PN-AAY-586

AGENCY FOR INTERNATIONAL DEVELOPMENT
WASHINGTON, D.C. 20523

DATE: 10/16/87

MEMORANDUM

TO: AID/PPC/CDIE/DI, room 209 SA-18

FROM: AID/SCI, Victoria Ose

SUBJECT: Transmittal of AID/SCI Progress Report(s)

Attached for permanent retention/proper disposition is the following:

AID/SCI Progress Report No. 4-586

Final rec'd 10/16/87

Attachment

2 cys

4.506

THE USE OF AGROBACTERIUM PLASMID VECTORS WHICH CONTAIN SYNTHETIC DNA FRAGMENTS TO MODIFY NUTRITIVE VALUE OF POTATOES

FINAL REPORT

August 1987

AID Project 936-5542
Obligation No. 3641294

Dr. J. H. Dodds
International Potato Center
P. O. Box 5969
Lima, Peru

Dr. J. Jaynes
Department of Biochemistry
Louisiana State University
Baton Rouge, LA 70803
U. S. A.

(1) PROJECT OBJECTIVE.

Short DNA fragments have been synthesized *in vitro* which code for proteins shown to be high in essential amino acids. These DNAs (spDNA), when fused to appropriate gene promoters, are expressed in *Escherichia coli* (1). We constructed infectious Agrobacterium (Ri) plasmid cloning vectors that contain, within the boundary of their "T" DNA, the chloramphenicol acetyl transferase gene (CAT gene). The spDNA has been inserted within the confines of the CAT gene with the correct reading frame maintained and the resultant gene fragment fussed to the Nopaline synthase promoter (unpublished results). We infected potato plants with Agrobacterium cells that harbor the above mentioned plasmid in order to obtain plant cells which possess the Nopaline synthase - CAT - spDNA complex within their genome. The primary thrust of this work has been to determine the level of transcription and translation of this gene complex towards the goal of improving the essential amino acid content of the potato.

(2) IMPORTANCE OF POTATO AS A BASIC FOOD.

Of the eight major food crops which may be regarded as staple, in the broadest sense of the word, seven are grain - millet, oats, rye, maize, rice, barley, and wheat. The other, produced in greater quantity, though on a smaller area than any one of the grain crops, is a tuber crop, the potato. It originated in the Andean region where it represents still today a major portion of the diet. Meanwhile potato is cultivated throughout the world which makes then of more than ordinary interest.

Potato is very efficient in producing calories per area and time and outranks the cereals in this respect. For the lesser developed countries it is important that it is not only a source of energy but also of proteins (2% in relation to fresh weight). The ratio for calories and protein is adequate for human requirements. The quality of the protein with respect to most of the essential amino acids is very good, only the sulfur containing amino acids methionine and cysteine are relatively low.

Therefore, it would be possible to increase the methionine content in the tuber proteins the nutritional value of the potato could be improved tremendously.

Hoff et al found little variation in the amino acid composition of the proteins of seven varieties of Solanum tuberosum and in tuber proteins analyzed from 45 wild species none was detected with a significantly higher methionine value. Therefore, breeding for a higher methionine content will be difficult because of lack of genetic variability. Any method which would allow to add the trait of increased methionine content in tuber proteins to a variety or breeding clone with many other valuable features without the necessity to go through the sexual breeding cycle which breaks up the combination of valuable genes could be of great advantage.

An important aspect for the proposed project is that, in contrast to cereals, the potato is capable of being infected by Agrobacterium species; in addition, it is quite amenable to tissue culture techniques. Regeneration of potato plants from single cells, protoplasts and callus has been described for potatoes of different origin and ploidy level (8), (9), (10). Also a genotype dependence of the response in tissue culture, "tissue culturability", was found.

Thus, the potato is very appropriate to be altered by gene transfer, utilizing recombinant DNA technology; if successful it would mean to have improved a major food crop through this method.

(3) AGROBACTERIUM AS NATURE'S GENETIC ENGINEER.

Agrobacterium rhizogenes causes a proliferation of roots on the plants it infects, and this disease has been termed "hairy root". The etiology of hairy root is quite distinct from that of crown gall, which is caused by Agrobacterium tumefaciens. The factor in A. tumefaciens responsible for causing plant neoplasms has been shown, without equivocation, to lie on a large plasmid (pTi). Furthermore, a discrete portion of the plasmid DNA (T-DNA) is found in the DNA of the infected plant tissue. This T-DNA determines two fundamental characteristics of crown gall tumor cells: i) the cells grow in the absence of phytohormones that are required normally for cell growth in callus culture, and ii) the cells synthesize unusual tumore-specific compounds, called opines. The Ti plasmid also confers on the bacterium the ability to catabolize the particular opine that is produced in the tumor. This represents a classic example of bacterial transformation of a eukaryotic organism. It has been shown recently the hairy root disease, caused by A. rhizogenes, is also due

to the transfer of plasmid DNA to the plant cell. Also, the hairy root tumors produced opines as well.

One strategy for introduction of a foreign gene into a plant by means of a Ti plasmid and A. tumefaciens is to utilize a small recombinant plasmid suitable for cloning in Escherichia coli, into which is known fragment of T-DNA has been spliced. This recombinant plasmid is cleaved open at a site within the T-DNA. A piece of "passenger" DNA is spliced into this opening. The passenger DNA consists of the foreign gene one intends to incorporate into the plant DNA as well as a selectable marker, say a bacterial gene for resistance to an antibiotic. This plasmid is then recloned into a larger plasmid and then introduced into an Agrobacterium strain carrying an unmodified Ti plasmid. During growth of the bacteria a rare double-recombination will sometimes take place resulting in bacteria whose T-DNA harbors an insert: the passenger DNA. Such bacteria can be identified and selected by their survival on media containing the antibiotic. These bacteria can then be used to insert their T-DNA (modified with passenger DNA) into a plant genome.

It is clear that his protocol can give rise to modified plant cells growing in culture. However, it has proven difficult to obtain healthy plants from these transformed plant cells. It should be noted that the same experimental strategy can be utilized employing the A. rhizogenes plasmid and inciting hairy tumors and culturing the infected root tissue. This would appear to be the method of choice as the Ri plasmids give rise to transformed plant cells that can regenerate into intact healthy, fertile plants.

(4) TECHNICAL WORK PLAN FOLLOWED.

Introduction of spDNA into A. rhizogenes Plasmid

Figure 1 illustrates the overall strategy of how to introduce the spDNA into the plant. The plasmid pGA414 (Figure 2) has been constructed by F. White and possesses the chloramphenicol acetyl transferase (CAT) gene under the control of the nopaline synthase promoter. The plasmid also contains resistance genes for the antiobiotics ampicillin and kanamycin. The restriction endonuclease EcoR1 has been utilized to cut pGA414 in one position within the CAT gene, spDNA has been inserted in this position and the modified plasmid closed with the aid of the enzyme T4 DNA ligase. It

should be noted that spDNA, when inserted into the CAT gene, is in the correct reading frame. The resultant plasmid pGA415.10 (Figure 3) has been purified and cut with the enzyme Bam HI. This plasmid has been ligated to the plasmid pFW105 (Figure 4) which has also been cut with Bam HI. This step accomplished the task of insertion of the passenger DNA within the T-DNA as pFW105 contains the Hind III17 fragment of the T-DNA found on the A. rhizogenes hairy root plasmid. The plasmid pFW105-2 (Figure 5) has been isolated from the previous ligation step. The plasmid can then be transformed into a wild-type A. rhizogenes strain and several kanamycin resistant clones can be selected for the homogeneous product. To select for the double recombination event a plasmid (pPH1JI; Figure 6), incompatible with the plasmid pFW105-2, will be introduced by transformation. The putative "double recombinants" were then selected by their resistance to kanamycin and gentamycin. These Agrobacterium strains were further characterized by Southern Blotting techniques and restriction enzyme mapping to ensure that the spDNA has been inserted properly within the T-DNA region. DNA sequence analysis will also be utilized to ascertain that the spDNA is within the CAT gene and that it is in the correct reading frame and that all is adjacent to the nopaline synthase promoter.

Infection of Plant Tissue

Preliminary experiments were carried out to develop optimal conditions for transformation of potato cells by A.r. Besides stem pieces cultured on agar medium section of tuber tissue and stems of in vitro plantlets will be utilized. Section of potato tuber tissues have shown strong morphogenetic response when incubated on a basic MS medium supplemented with cytokinins. These tissues were, therefore, also used for inoculation and incubation on the induction medium for adventitious buds.

The resulting outgrowths will be subcultured on hormone-free medium in the presence of carbenicillin to eliminate the bacteria and select for transformed auxinautotroph cells. Pieces will then be transferred to media with different hormone combinations. Their capacity to induce morphogenesis has been evaluated.

In view of the necessary exchange of the resulting transformed and control plant material between Peru and USA, the most adequate tissue defined in the preliminary studies was taken from pathogen-tested in vitro material and inoculation and all following steps will be carried out under aseptic conditions.

One part of the transformed tissue will be permanently kept on auxin-free medium and transferred to the same medium regularly. Samples of this were sent to LSU after an importation permit has been obtained from the US Quarantine. Similar tests were carried out by Jesse M. Jaynes as mentioned above to determine if the gene is present and if it is being translated and described.

The other part will be propagated and incubated on the optimum regeneration medium which was identified in the preliminary experiments.

Plant regeneration *in vitro* were propagated and transferred to soil. Their tubers were planted in quarantine growth room together with material propagated *in vitro* of the original clone. Dry matter, and methionine content of the tubers, as well as of leave tissue were compared.

(5) RESULTS.

Results are presented in the form of published papers (copies attached).

Increasing Bacterial Disease Resistance in Plants Utilizing Antibacterial Genes from Insects

Jesse M. Jaynes, Kleanthis G. Xanthopoulos, Luis Destéfano-Beltrán, and John H. Dodds

Summary

The introduction of genes into plants encoding potent antibacterial proteins, derived from insects, may significantly augment the level of their resistance to bacterial disease. Using modern techniques, genes of choice can be introduced into plant tissue and this tissue can be manipulated to produce viable plants. The potato has been chosen as the model system, not only because of its plasticity of development, which allows for the relatively easy regeneration of whole plants from transformed tissue, but also because the potato ranks among the top four plants in the world in terms of economic importance.

Introduction

The welfare of humanity is inextricably bound up with the efficient cultivation of plants. Plant disease is a disruptive and, at times, catastrophic occurrence. For instance, late blight, a disease resulting from an infection by a fungal pathogen, caused the starvation of one million people and forced the immigration of another two million to North America owing to its decimation of the potato crop (the infamous Irish Potato Famine of 1845–60). Clearly, the economic and social impact of plant disease can be substantial. It is estimated that as much as one-third of the total crops lost to man can be directly attributed to plant disease.[1] The loss in just one crop, the potato, associated with bacterial disease, can be as high as 25% (above 4 billion US$ annually) of the total worldwide production.[2] While plant breeding, the movement of desirable traits between plants by the use of the traditional Mendelian techniques, has profoundly improved the important inheritable characters (particularly yield) of the world's major crop plants, there are inherent weaknesses with these methods in relation to the production of disease-resistant plants. For example, it takes a great amount of time, perhaps as long as ten years, to introduce, select, and establish a particular trait into a plant cultivar and, often, some traits are impossible to incorporate by these traditional techniques.[3]

When confronted with a disease problem, the plant breeder has relied on the fact that somewhere, among plants of the same or closely related species, at least one individual can be found which retains resistance to that particular disease pathogen. This single plant can then be incorporated into the breeding program by attempting to introduce its genes into traditional varieties that are already well accepted, through the use of sexual crosses. It is assumed that this will eventually produce a hybrid plant which retains all of the desirable traits, including the new one, resistance to the disease. This has been the hallmark of breeding plants for disease resistance.

Disease pathogens, like all other organisms of the world, are constantly forged on the 'anvil' of natural selection and are very efficient in eventually overcoming a plant's defenses. So, a continual war of attrition has developed between the pathogen and the plant breeder, with no apparent winner having been declared.

One of the main weaknesses of plant breeding is its dependency upon sexual crosses that are limited to the genes that exist in only one relatively small group of organisms – the species. Recombinant DNA, the manipulation of genes in the test tube, allows the transcendence of inter-species barriers and makes novel genetic combinations possible.[4]

The direct intervention into the evolution of domesticated plants on the molecular level is possible because of advances on several fronts: (1) the ability to regenerate whole plants from plant parts and (2) the use of *Agrobacterium* to transfer genes of choice into the plant genome.[5] These techniques allow us to make gene combinations previously impossible to achieve by conventional means – to go beyond species barriers and endow plants with new genes of potential significance.

Humoral Immunity in Insects

Humoral immunity can be induced in insects by an injection of either live, non-pathogenic bacteria or heat-killed pathogens. This phenomenon has been studied by many investigators in a number of different insects. However, diapausing pupae of the giant silk moth *Hyalophora cecropia*, have proven to be particularly useful experimental animals (mainly because of their large size) to study the humoral defense mechanisms of insects. When *H. cecropia* pupae are immunized they produce a set of proteins which are normally not present in the hemolymph of the animals.[6] Immunized pupae of *H. cecropia* are therefore an ideal biological system for the enrichment of the RNA and proteins that are synthesized from the inductive genes for immunity. Boman and his co-workers have taken advantage of this fact, both in the purification of 15 different immune proteins and in the isolation of immune RNA, later to be used for the preparation of a cDNA bank. After a short period of RNA synthesis, the insects respond to the exposure of live bacteria by the production of a potent antibacterial activity which is due to the synthesis of at least three novel classes of bactericidal proteins: lysozyme, cecropins, and attacins.[7] The lysozyme protein is very similar to that found in chicken egg white. The three principal cecropins, A, B, and D, are small basic proteins with a comparatively long hydrophobic region. The attacins are larger and there are two main forms: basic and acidic.

Lysozyme

H. cecropia lysozyme was purified from immune hemolymph and was identified as a chicken-type lysozyme. The primary structure of this enzyme was elucidated by determination of the complete amino acid sequence.[8] A synthetic oligonucleotide probe was

SEQUENCE OF MATURE LYSOZYME GENE AND PROTEIN

```
TGC CGT TCG CAG TTC GCT TTG CAT TGC GAT GCG AAA CGT TTC ACG AGA TGC GGG
Cys Arg Ser Gln Phe Ala Leu His Cys Asp Ala Lys Arg Phe Thr Arg Cys Gly
```

Partial leader peptide — Mature lysozyme

```
TTA GTG CAG GAG CTT AGG AGA CGA GGC TTC GAT GAA ACT TTG ATG AGT AAC TGG
Leu Val Gln Glu Leu Arg Arg Arg Gly Phe Asp Glu Thr Leu Met Ser Asn Trp

GTC TGC CTT GTC GAG AAC GAA AGC GGA CGG TTT ACC GAT AAA ATC GGT AAA GTT
Val Cys Leu Val Glu Asn Glu Ser Gly Arg Phe Thr Asp Lys Ile Gly Lys Val

AAC AAG AAC GGA TCT CGA GAC TAC GGC CTC TTC CAG ATC AAT GAC AAA TAC TGG
Asn Lys Asn Gly Ser Arg Asp Tyr Gly Leu Phe Gln Ile Asn Asp Lys Tyr Trp

TGC AGT AAG GGA TCC ACT CCT GGA AAG GAT TGC AAC GTG ACT TGT AAT CAG CTA
Cys Ser Lys Gly Ser Thr Pro Gly Lys Asp Cys Asn Val Thr Cys Asn Gln Leu

CTG ACT GAC GAC ATT AGC GTG GCA GCT ACG TGC GCG AAG AAG ATT TAC AAA CGC
Leu Thr Asp Asp Ile Ser Val Ala Ala Thr Cys Ala Lys Lys Ile Tyr Lys Arg

CAC AAG TTT GAC GCT TGG TAC GGA TGG AAA AAT CAC TGT CAA CAT GGA CTG CCA
His Lys Phe Asp Ala Trp Tyr Gly Trp Lys Asn His Cys Gln His Gly Leu Pro

GAT ATT AGC GAC TGT TAG
Asp Ile Ser Asp Cys Stop
```

Fig. 1. *Nucleotide and deduced amino acid sequences of the Cecropia lysozyme. Part of the leader peptide is indicated by a line under the respective sequence.*

constructed, based on the protein sequence, and used to isolate cDNA clones. One clone, pCP701, with the largest insert size of about 420 base pairs (bp), was sequenced and found to include the full structural information for the mature enzyme (Fig. 1). The primary sequence of the mRNA that corresponds to clone pCP701 and the amino acid sequence obtained were in agreement except for one residue. More detailed studies, which included repeated amino acid sequence determinations from different lysozyme preparations, identified three variants of enzyme. It is surmised that these are allelic variants and that all could have originated from point mutations in their respective codons.

Attacins

Attacins are the largest antibacterial molecules found in the hemolymph of immunized *H. cecropia* pupae, with a molecular weight of about 20,000 daltons (20 kDa). They are comprised of six different forms, which can be fractionated according to their isoelectric point.[9] The results from the amino acid sequence of the N-terminus of five of the attacins indicates the presence of three basic and two acidic forms which are slightly different from each other. It has been suggested that they are the products of two related genes. Two cDNA clones, pCP517 and pCP521, were isolated that correspond to these two genes. Comparison of the two clones has revealed 76% homology in the coding region (Fig. 2) and it has become evident that the genes originated from a common ancestral gene. The six attacin forms found in the hemolymph are probably products of secondary modification of the two precursor molecules.

Cecropins

The cecropins are the most potent group of the antibacterial factors, with a broad spectrum of antibacterial activity against both Gram-negative and Gram-positive bacteria. They are small, around 4 kDa, and basic. They are comprised of three major forms (A, B, and D). Comparison of the amino acid sequences of the different forms has revealed a high degree of homology. They all have a basic N-terminal region and a hydrophobic stretch in the C-terminal part of the molecule. It seems that the cecropins are products of three related genes that, as in the case of the attacin genes, have originated by gene duplication. Recombinant cDNA clones corresponding to the cecropin-B form have been isolated which, when analyzed together, include information for the entire coding region of the cecropin-B molecule[10] (Fig. 3).

Agrobacterium: Nature's Genetic Engineer

The bacteria of the genus *Agrobacterium* have been of interest to plant biologists for many decades. Long before the developments in genetic engineering, it was found that some strains of these bacteria are the causal agents of cancer-like tumorous growths called 'crown-gall' in a wide range of plants.[11] If a dicotyledonous plant is wounded with a sterile needle and the wound is then infected with an inoculum of *Agrobacterium tumefaciens*, the area around the wound begins to swell within a few days. After three to four weeks the wound will have developed into a large cancerous growth. It was of great interest for scientists to elucidate the causal mechanisms of this cancer-forming reaction and it was not long until analysis was made of *Agrobacterium* to determine if they contained plasmids which may be responsible for causing the aberrant growth. Results were rapidly obtained and it became clear that the tumor-inducing principles of *Agrobacterium* are genes normally found on a large plasmid (pTi). Since this time the determination of the factor(s) for the plant disease called 'hairy root', a disease caused by *A. rhizogenes*, have also been shown, without equivocation, to lie on a large plasmid (pRi). These plasmids have been characterized in exquisite biochemical detail, utilizing the currently available techniques of recombinant DNA, and the molecular mechanisms involved in the plant transformation process have begun to be understood.

A turning-point in plant genetic engineering was the discovery that the bacterium elicits its response by actually inserting a small fragment of the bacterial plasmid (T-DNA, or transfer DNA) into the plant nucleus where it becomes incorporated into the plant

genome and functions as a gene.[12] This discovery has allowed for the utilization of *Agrobacterium* and their plasmids as vehicles to carry foreign DNA into the plant nucleus. There are, however, limitations to the application of these techniques. They are as follows: (1) the plant must be susceptible to infection with the *Agrobacterium* plasmid and (2) the tissue-culture technology must be available for regeneration of transformed plants. These limitations have meant that to date there are no successful reports on genetic engineering of cereals, as these plants are resistant to *Agrobacterium* infection and it has proven to be less than routine to regenerate whole plants from single cells.

The potato, an important basic food crop in many developed and developing countries, is however, amenable to these techniques. We have successfully developed routine methods for the insertion of synthetic nucleic acids into the potato genome.[13]

One strategy for the introduction of a foreign gene into a potato plant by means of *A. rhizogenes* is to utilize a small recombinant plasmid suitable for cloning in *E. coli* into which a known fragment of T-DNA has been inserted. This recombinant plasmid is then cleaved open at a site within the T-DNA using a restriction endonuclease. A piece of 'passenger' DNA is spliced into this opening. The passenger DNA consists of the foreign gene (for example, the genes which code for proteins containing antibacterial activity) one intends to incorporate into the plant genome and a selectable marker, e.g. a bacterial gene coding for resistance to an antibiotic. This plasmid is then recloned into a larger plasmid and introduced into an *Agrobacterium* strain carrying an unmodified Ri plasmid. During growth of the bacteria, a rare double-recombination will sometimes take place between the homologous T-DNA regions resulting in bacteria whose T-DNA harbors an insert: the passenger DNA. Such bacteria can be identified and selected by their survival on media containing the antibiotic. These bacteria can then be used to insert their chimeric T-DNA into a plant genome[11] (Fig. 4).

At the outset, most of our studies have concentrated on the insertion of a synthetic gene coding for a protein high in its content of essential amino acids as a means of increasng the overall nutritional value of potato protein. Our attention is now shifting, however, towards the possibility of using both

SEQUENCE OF CECROPIN GENE AND PROTEIN

ATG AAT TTC TCA AGG ATA TTT TTC TTC GTG TTC GCT TTG GTT CTG GCT
Met Asn Phe Ser Arg Ile Phe Phe Phe Val Phe Ala Leu Val Leu Ala

Leader peptide

TCA ACA GTT TCG GCT GCA CCG GAG CCG AAA TGG AAA GTC TTC AAG AAA
Ser Thr Val Ser Ala Ala Pro Glu Pro, Lys Trp Lys Val Phe Lys Lys

Mature cecropin

ATT GAA AAA ATG GGT CGC AAC ATT CGA AAC CGT ATT GTC AAG GCT GGA
Ile Glu Lys Met Gly Arg Asn Ile Arg Asn Gly Ile Val Lys Ala Gly

CCA GCG ATC GCG GTT TTA GGC GAA GCC AAA GCG CTA GGA TAA
Pro Ala Ile Ala Val Leu Gly Glu Ala Lys Ala Leu Gly, Stop

Fig. 2. *Nucleotide and deduced amino acid sequences of the clone encoding for the precursor of cecropin B. The structure of the leader peptide and the mature cecropin B molecule is indicated by a line under the respective parts of the amino acid sequence.*

SEQUENCE OF THE MATURE ATTACIN GENE AND PROTEIN

GAC GCG CAC GGA GCC CTT ACG CTC AAC TCC GAT GGT ACC TCT GGT GCT GTG GTT
Asp Ala His Gly Ala Leu Thr Leu Asn Ser Asp Gly Thr Ser Gly Ala Val Val

AAA GTA CCC TTT GCT GGT AAC GAC AAG AAT ATA GTA AGC GCT ATC GGT TCC GTA
Lys Val Pro Phe Ala Gly Asn Asp Lys Asn Ile Val Ser Ala Ile Gly Ser Val

GAC TTA ACT GAT AGG CAG AAA CTA GGC GCT GCA ACC GCT GGA GTG GCA CTG GAT
Asp Leu Thr Asp Arg Gln Lys Leu Gly Ala Ala Thr Ala Gly Val Ala Leu Asp

AAT ATA AAC GGT CAC GGA CTA AGT CTC ACG GAT ACA CAC ATC CCC GGG TTC GGA
Asn Ile Asn Gly His Gly Leu Ser Leu Thr Asp Thr His Ile Pro Gly Phe Gly

GAC AAG ATG ACA GCA GCC GGC AAA GTG AAT GTC TTC CAC AAT GAT AAC CAC GAC
Asp Lys Met Thr Ala Ala Gly Lys Val Asn Val Phe His Asn Asp Asn His Asp

ATC ACA GCG AAG GCT TTC GCC ACC AGA AAC ATG CCG GAT ATT GCT AAT GTA CCT
Ile Thr Ala Lys Ala Phe Ala Thr Arg Asn Met Pro Asp Ile Ala Asn Val Pro

AAT TTC AAC ACT GTC GGT GGC GGA ATA GAC TAT ATG TTC AAA GAT AAG ATT GGT
Asn Phe Asn Thr Val Gly Gly Gly Ile Asp Tyr Met Phe Lys Asp Lys Ile Gly

GCA TCT GCG AGC GCC GCT CAC ACG GAC TTT ATC AAT CGC AAC GAC TAC TCT CTT
Ala Ser Ala Ser Ala Ala His Thr Asp Phe Ile Asn Arg Asn Asp Tyr Ser Leu

GAC GGG AAA CTG AAC CTC TTC AAG ACT CCT GAT ACC TCG ATT GAT TTC AAC GCC
Asp Gly Lys Leu Asn Leu Phe Lys Thr Pro Asp Thr Ser Ile Asp Phe Asn Ala

GGT TTC AAC AAG TTC GAT ACA CCT TTC ATG AAG TCC TCT TGG GAG CCT AAC TTC
Gly Phe Lys Lys Phe Asp Thr Pro Phe Met Lys Ser Ser Trp Glu Pro Asn Phe

GGA TTC TCA CTT TCT AAA TAT TTC TGA TTA
Gly Phe Ser Leu Ser Lys Tyr Phe Stop Stop

Fig. 3. *Nucleotide and deduced sequences of the clone encoding the mature form of the basic attacin*

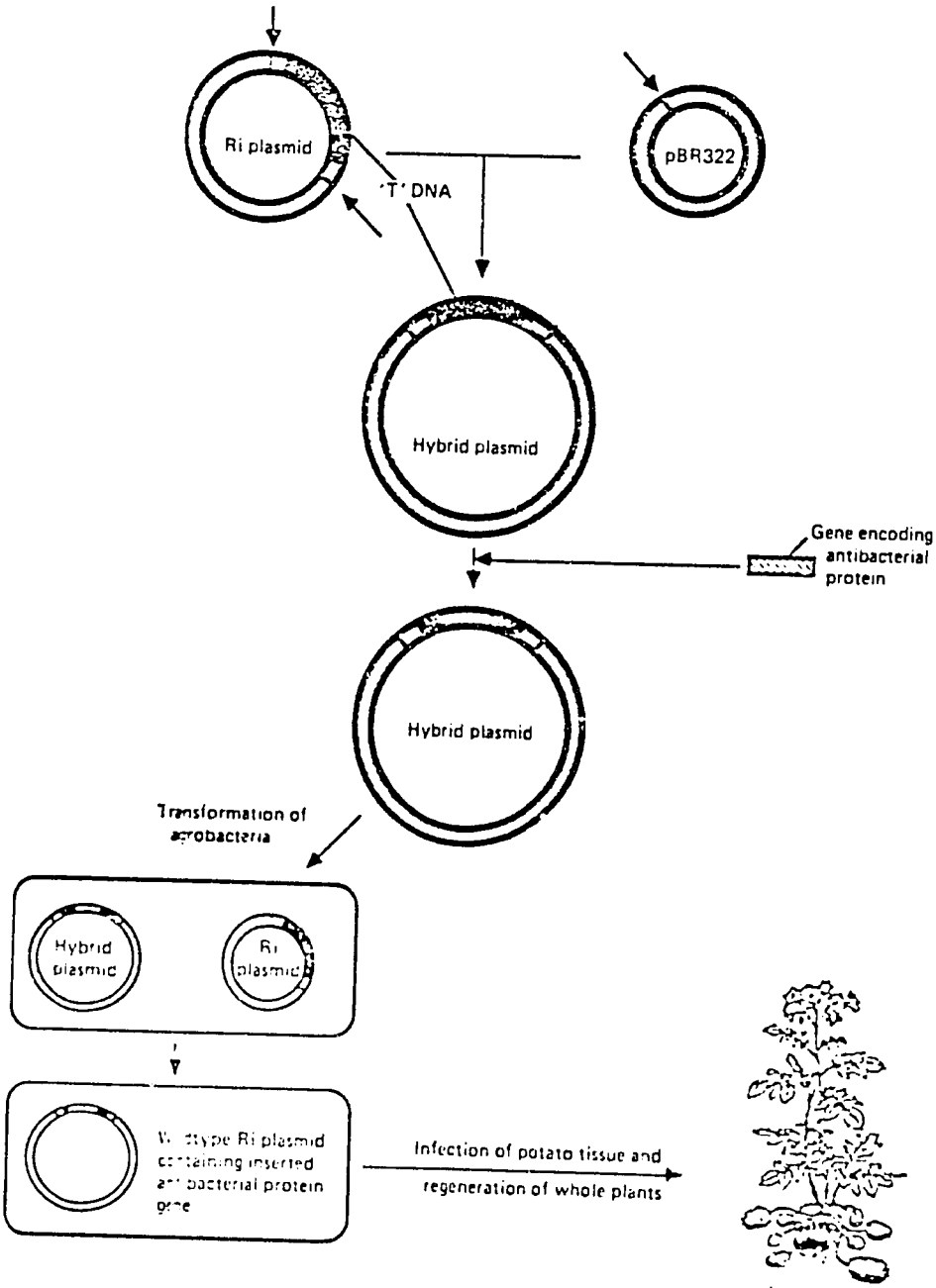


Fig. 4. *Through the use of restriction enzymes, modified Ri plasmids can be constructed which contain foreign genes (in this case, the genes encoding the antibacterial proteins). These new plasmids can be introduced into Agrobacteria and these strains can then be used to infect potato plants. The resultant transformed tissue can be regenerated into whole, reproductively viable plants and their level of increased bacterial disease resistance can be ascertained.*

synthetic and purified genes as a way of conferring novel forms of resistance to potato diseases and pests.

The potato is a model plant for tissue culture and its plasticity of development allows plants to be regenerated from almost any plant part. It is possible therefore to infect potato plants with *A. rhizogenes* and induce the formation of hairy roots; these roots can then be cultivated and transformed plants regenerated as shown (Fig. 5). In an analogous manner, it is also possible to use *A. tumefaciens* containing disarmed (non-tumor-forming) plasmids and regenerate transformed plants from leaf discs.[15] In this case a drug marker can be used as an additional selection permitting only transformed cells to survive.

Another advantage in using potato is that it is normally propagated asexually and therefore there are no problems of segregation and loss of the inserted information through the processes of meiotic recombination. However, it is also of interest to see how this newly inserted genetic information would segregate and if it is transmitted sexually to the progeny.

Bacterial Diseases of Potato

Although there are a number of bacterial diseases of potato, a majority of the economic losses can be attributed to just two of them. A short description of the etiology of these diseases follows.

Bacterial Soft Rot (Blackleg). The causal agent of this disease is *Erwinia carotovora*. Symptoms occur at any stage of plant development and the stems of infected plants typically exhibit an inky black decay which usually begins at the decaying seed piece (portion of the seed tuber) and may extend up the stem only a few millimeters for its entire length. Stem pith (inner part of the stem) is often decayed above the black discoloration, and vascular tissues in the stem are generally discolored. Infected plants are commonly stunted and have a stiff, erect growth habit, particularly early in the season. Foliage becomes chlorotic (loss of chlorophyll), and leaflets tend to roll upwards at the margins. Leaflets and, later, entire plants may wilt, slowly decline, and eventually die. Young shoots may be invaded and killed before emergence[16]. Tubers can be affected with soft rot while in storage or in the soil before harvest, and seed tubers decay after planting. Infection occurs through lenticels and wounds or through the stolon end of the tuber in the infected mother plant (Fig. 6).

Brown Rot (Bacterial Wilt). The causal agent is *Pseudomonas solanacearum*, which is infectious at any stage of the plant's growth. Wilting of leaves and collapse of stems may be severe in young, succulent plants of highly susceptible varieties. Initially, only one branch in a hill may show wilting. If disease development is rapid, all leaves on the plants in a hill may wilt quickly without much change in color. Tubers are also affected by a general decay and soft rot[17] (Fig. 7).

Total losses from bacterial infections in developing countries can amount to hundreds of millions of dollars (US$) annually and, in particularly bad years, it can approach several billion dollars worldwide. The extent of loss varies greatly from country to country and is influenced by climate and conditions of growth and storage. Bacterial soft rot is a major factor limiting the storage of potato tubers under tropical conditions.

Losses can be between 30- and 100% during the 2- to 6-months storage period, where temperatures can be between 27 and 32 °C.

As new heat-adapted clones are produced, allowing the potato to be grown in lowland tropical conditions, control of bacterial disease becomes a high priority. The cultivation of genetically resistant material has so far been the only effective method for the control of bacterial wilt. However, experience has shown that the available genetic resistance has a tendency to break down if the material is moved into climatic zones for which it is ill-adapted. Moreover, the genetic basis of the source of resistance, traditionally utilized in breeding, is extremely small – two Colombian clones of *Solanum phureja*. The reliance on this small gene pool has been a concern to breeders and plant pathologists for a very long time.

Use of Genes Encoding for Bactericidal Proteins to Confer Resistance to Bacterial Disease

As mentioned above, the hemolymph of an immunized *H. Cecropia* pupa contains at least three groups of antibacterial proteins: lysozyme, the antibacterial protein found in egg white and human tears; and two other classes of antibacterial peptides: cecropins and attacins. Thus these insects have evolved a rather successful and novel means to fight bacterial infections with a system a traditional immunologist would think lacked specificity.

Their antibacterial proteins have a rather broad spectrum activity and are very effective in killing many different types of plant pathogenic bacteria (Table I). With this array of antibacterial proteins, a genetically engineered plant would possess a rather potent arsenal composed of three different antibacterial proteins which appear to work in different ways to actively destroy the bacterial pathogen. This multi-level defense system would present a formidable challenge to the invading bacteria, one which would be very difficult to evolve a means to circumvent. The probability that a pathogen would become naturally resistant to all three toxins at once is rather remote (about 1 in 10^{18}). Also, it has been found that, when used together, there is a synergistic effect exerted which will make it even more difficult for the bacterial pathogen to compete.[18] In essence, it was demonstrated that attacin enhanced the activity of cecropin and lysozyme when tested together on *E. coli*. Primary results obtained in our laboratory (J.M.J.) seem to indicate that, at least for the plant pathogens listed in Table

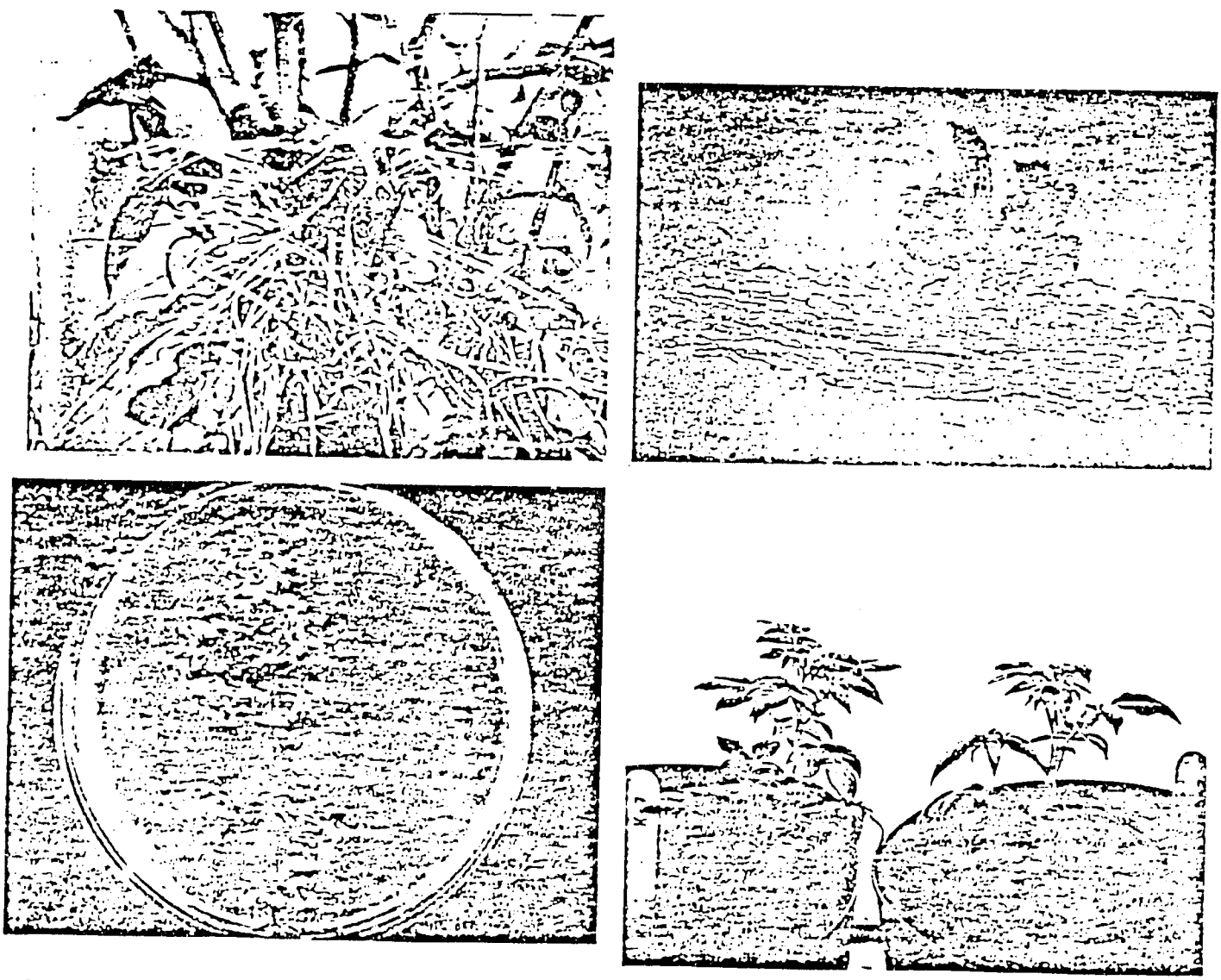


Fig. 5. Agrobacterium rhizogenes incites hairy roots on potato plants propagated in vitro. Bacterial-free individual roots from these plants were placed on nutrient media to induce the formation of plantlets. A light-microscope section of an infected root shows that small plantlets begin to differentiate from a few cells of the root. Several weeks later, individual plantlets can be seen growing from regions of the root. Within a few months, large and healthy plants can be grown in pots.

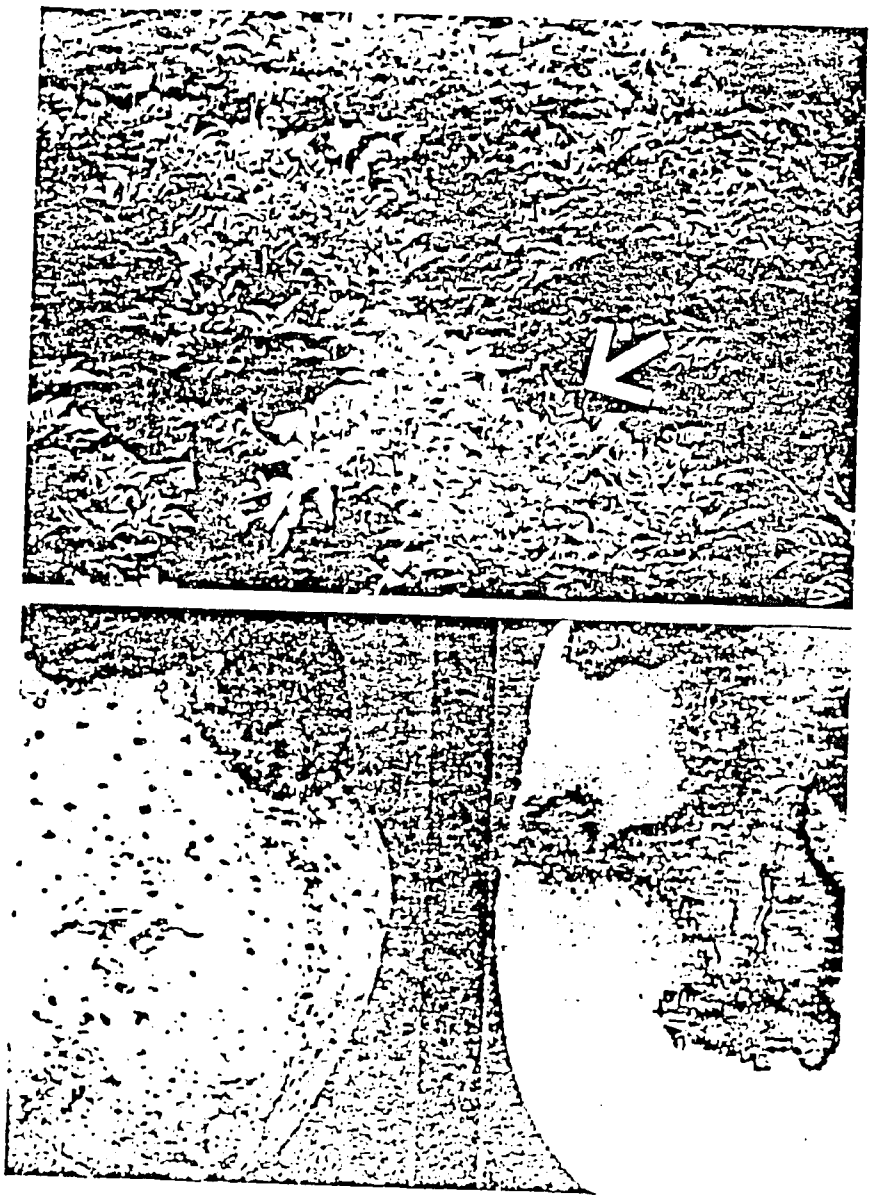


Fig. 6. *A plant infected by* Erwinia (*blackleg*) *can be seen in the top photograph and is characterized by the chlorotic appearance of the foliage. Leaflets and, later, entire plants will eventually wilt and die. The bottom photograph shows the general symptoms of the disease on the tuber characterized by an extensive soft rot.*

I and for some animal pathogens (*Brucella abortus*), there is a measurable synergism between cecropin and chicken egg-white lysozyme (unpublished results). While the exact modes of action of these antibacterial proteins have not been conclusively determined, it is clear that their actions are specific to prokaryotic organisms and are benign to Chang liver cells[18] and a number of other eukaryotic cells *in vitro* (unpublished results, J.M.J.)

The recent incorporation of a bacterial-encoded gene for herbicide resistance[20] and the firefly luciferase gene[21] into tobacco plants illustrates the fact that gene transfer, at least in dicotyledonous plants, is now a routine process. Furthermore, it has recently been shown that a gene (chloramphenicol acetyl transferase, CAT), under the control of a Drosophila promotor, was efficiently expressed in transformed plants[22]. It is interesting to note that the level of expression of the CAT protein, from this promoter, was as high as the level of expression obtained from the 35 S promoter of cauliflower mosaic virus (a very efficient plant gene promoter). These data support our belief that we can achieve incorporation and expression of the antibacterial genes in plants. It should be pointed out that, at this time, we have not obtained transformed plant material which contain the insect antibacterial genes, but experiments are currently under way which should yield plants, in a reasonably short period of time. These plants would then be available for assessment of the level of expression of these proteins and it will then be possible to determine the efficacy of this novel approach to supplement a plant's natural resistance to bacterial infection. Therefore the genes encoding proteins derived from the humoral immune response in *H. cecropia* are an attractive genetic system to incorporate into the genome of plants to protect them from diseases induced by bacteria.

Conclusions

Genetic engineering offers the possibility of introducing a single trait, without altering other agronomically important characters, into a well-accepted or traditional plant variety, providing for a method of 'fine-tuning' specific cultivars. This technology will allow the incorporation of desirable and inheritable traits into plants, with some ease, rapidity, and with a high probability of success when allied with modern plant tissue culture techniques. The use of these new methods, however, is not a goal in themselves and should be coupled to conventional breeding in order to achieve plant modification.

TABLE I. Antibacterial activity on representative plant pathogenic bacteria

Organism	Lethal concentration for Cecropin A* (μM)
Escherichia species†	0·28–0·36
Pseudomonas species‡	0·98–1·27
Erwinia species§	1·17–5·28
Clavibacter michiganensis‖	5·27
Xanthomonas species¶	1·27–5·77

* The lethal concentration was calculated from the inhibition zones around 2 mm (2 μl total volume) wells in thin agar plates according to Hultmark *et al.*[23] Synthetic cecropin A has been shown to be as potent as the natural cecropin B.[24] The data represents the average of two separate determinations. The bacteria were also tested against the basic form of the attacin. All species were found to be sensitive, however, only one concentration was used (0·75 μg/μl), owing to lack of sufficient attacin to conduct a lethal concentration assay.

† *Escherichia* species were used as controls because of their extreme sensitivity to the cecropins.

‡ Four different *Pseudomonas* species were assayed for their sensitivity to the toxin.

§ Three different *Erwinia* species were tested, including the pathogen which causes soft rot.

‖ *Clavibacter michiganensis* was included as it causes a ring-rot disease in potato tubers.

¶ *Xanthomonas* species were included as they are the causative agents of many plant diseases (citrus canker, for example), although none of major significance in potato.

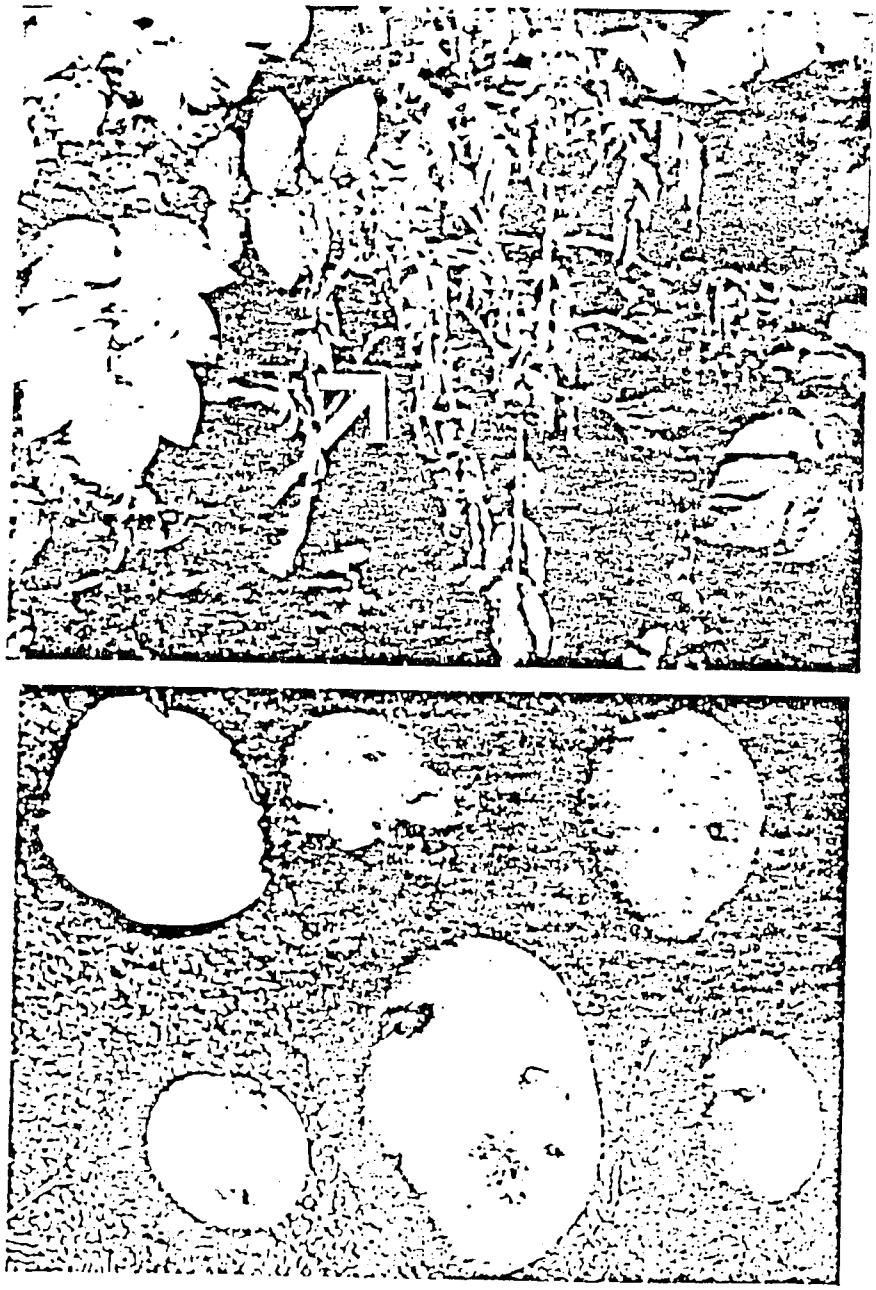


Fig. 7. *A plant infected by* Pseudomonas *(bacterial wilt) can be seen in the top photograph and is characterized by a very rapid and lethal wilting. The bottom photograph illustrates the symptoms of the disease on the tubers. As with* Erwinia, *there can also be a very extensive soft rot of the tuber.*

When used together, the scope is greatly enlarged for the possibility of plant improvement.

Natural resistance to disease and pest organisms, in plants, is a very complicated process. At present it is an almost impossible task to identify, isolate, and characterize the genes responsible for pathogen resistance. We believe that, with the introduction of the genes encoding antibacterial proteins into the plant genome, a crop plant's natural resistance to bacterial pathogens can be significantly augmented.

The world is a vast repository of potentially valuable genetic material locked within organisms by the natural barriers which isolate species. Recombinant DNA technology allows the Earth's genetic resources to be exploited by providing a means of overcoming these barriers. Indeed, the potential for a new horizon in plant breeding is here and it may be possible, sometime in the near future, to endow plants with a permanent resistance to a variety of pathogens.

REFERENCES

1 Agrios, G. N. (1978). *Plant Pathology*. Academic Press, New York.

2 Sawyer, R. L. (1984). *Potatoes for the Developing World*. International Potato Center, Lima, Peru.

3 Smith, O. & Plaisted, R. L. (1977). Potato breeding and improvement. In *Potatoes: Production and Storage*, pp. 725–754. AVI, Westport, Connecticut.

4 Schliel, R. (1985). *Genetics and Molecular Biology*. Addison-Wesley, Reading, Massachusetts.

5 Chilton, M. D. (1983). A vector for introducing new genes into plants. *Sci. Amer.* 248, 50–59.

6 Boman, H. G. & Steiner, H. (1981). Humoral immunity in Cecropia pupae. In *Curr. Top. Microbiol. Immunol.* 94/95, 75–91.

7 Hultmark, D., Steiner, H., Rasmuson, T. & Boman, H. G. (1980). Purification and properties of three inducible bacterial proteins from the hemolymph of immunized pupae of *Hyalophora cecropia*. *Eur. J. Biochem.* 106, 7–16.

8 Boman, H. G., Faye, I., v. Hofsten, P., Kockum, K., Lee, J.-Y., Xanthopoulos, K. G., Bennich, H., Engstrom, A., Merrified, R. B. & Andreu, D. (1985). On the primary structure of lysozyme, cecropins, and attacins from *Hyalophora cecropia*. *Dev. Com. Imm.* 9, 551–558.

9 Hultmark, D., Engstrom, A., Andersson, K., Steiner, H., Bennich, H. & Boman, H. G. (1983). Insect immunity: attacins, a family of antibacterial proteins from *Hyalophora cecropia*. *EMBO J.* 2, 571–576.

10 v. Hofsten, P., Faye, I., Kockum, K., Lee, J.-Y., Xanthopoulos, K. G., Boman, I. A., Boman, H. G., Engstrom, A., Andreu, D. & Merrifield, R. B. (1985). Molecular cloning, cDNA sequencing, and chemical synthesis of cecropin B from *Hyalophora cecropia*. *Proc. Natl. Acad. Sci. USA* 82, 2240–2243.

11 Albinger, G. & Bliderbeck, R. (1977). Übertragung der Fähigkeit zur Wurzelinduktion von *Agrobacterium rhizogenes* auf *A. tumefaciens*. *Phytopath. Z.* 90, 306–310.

12 Matthysse, A. G. (1977). Variations in plasmid DNA sequences present in crown gall tumour lines. *J. Gen. Micro.* 102, 427–430.

13 Espinoza, N. O., Yang, M. S., Jaynes, J. M. & Dodds, J. H. (1986). Genetic engineering of potato. II. Insertion and detection of a synthetic gene for improved protein quality. Submitted for publication in *Plant Cell Reports*.

14 Yang, M. S., Jaynes, J. M., Espinoza, N. O. & Dodds, J. H. (1986). Genetic engineering of potato. I. Construction of a synthetic gene for improved protein quality. Submitted for publication in *Plant Cell Reports*.

15 Horsch, R. B., Fraley, R. T., Rogers, S. G., Sanders, P. R., Lloyd, A. & Hoffman, N. (1984). Inheritance of functional foreign genes in plants. *Science* 223, 496–498.

16 Molina, J. J. & Harrison, M. K. (1980). The role of *Erwinia carotovora* in the epidemiology of potato blackleg. *Am. Potato J.* 23, 41–57.

17 Buddenhagen, I. & Kelman, A. (1964). Biological and Physiological aspects of bacterial wilt caused by *Pseudomonas solanacearum*. *Ann. Rev. Phytopathol.* 2, 203–230.

18 Boman, H. G., Faye, I., v. Hofsten, P., Kockum, K., Lee, J.-Y., Xanthopoulos, K. G., Bennich, H., Engstrom, A., Merri-

[illegible] (1985). On the primary structure of lysozyme, cecropins, and attacins from *Hyalophora cecropia*. *Dev. Comp. Immunol.* 9, 551–558.
19 Steiner, H., Hultmark, D., Engstrom, A., Bennich, H. & Boman, H. G. (1981). Sequence and specificity of two antibacterial proteins involved in insect immunity. *Nature* 292, 246–248.
20 Shah, D. M., Horsch, R. B., Klee, H. J., Kishore, G. M., Winter, J. A., Tumer, N. E., Hironaka, C. M., Sanders, P. R., Gasser, C. S., Aykent, S., Siegel, N. R., Rogers, S. G. & Fraley, R. T. (1986). Engineering herbicide resistance in transgenic plants. *Science* 233, 478–481.
21 Ow, D. W., Wood, K. V., DeLuca, M., De Wet, J. R., Helinski, D. R. & Howell, S. H. (1986). Transient and stable expression of the firefly luciferase gene in plant cells and transgenic plants. *Science* 234, 856–859.
22 Ou-Lee, T-M., Turgeon, R. & Wu, R. (1986). Expression of a foreign gene linked to either a plant-virus or a Drosophila promoter, after electroporation of protoplasts of rice, wheat, and sorghum. *Proc. Natl. Acad. Sci. USA* 83, 6815–6819.
23 Hultmark, D., Engstrom, A., Bennich, H., Kapur, R. & Boman, H. G. (1982). Insect immunity: isolation and structure of cecropin D and four minor antibacterial components from Cecropia pupae. *Eur. J. Biochem.* 127, 207–217.
24 Andreu, D., Merrifield, R. B., Steiner, H. & Boman, H. G. (1985). N-terminal analogues of cecropin A: synthesis, antibacterial activity, and conformational properties. *Biochem.* 24, 1683–1688.

JESSE M. JAYNES AND LUIS DESTÉFANO-BELTRÁN *are at the Department of Biochemistry, 322 Choppin Hall, Louisiana State University, Baton Rouge, LA 70803, USA.* KLEANTHIS G. XANTHOPOULOS *is at the Department of Microbiology, University of Stockholm, S-106 91 Stockholm, Sweden, and* JOHN H. DODDS *is at the International Potato Center, P.O. Box 5969, Lima, Peru.*

Mitogen-Specific Nonresponsive Mutants and the Initiation of Cellular Proliferation

Harvey R. Herschman

Summary

A large number of molecular, physiological, and biochemical events accompany the mitogen-induced transition from a quiescent, nonproliferating state to a cycling state. However, it is difficult to distinguish causal steps in the commitment to cell division from correlative events. To approach this problem we have isolated mitogen-specific nonproliferative variants of 3T3 cells, namely cell lines that are unable to mount a mitogenic response to specific mitogens.

Introduction: Regulation of the Cell Cycle in a Multicellular Organism

With respect to cell division, individual cells in a multicellular organism exist in three states. Some cells – such neurons, fused muscle cells or red cells – are terminally differentiated and will never again divide. Others are constantly cycling. Finally, many cells, at any given time, are in a quiescent, nonproliferating state but retain the potential to re-enter – in response to cell-type specific mitogenic signals – the proliferative cycle. Thus, for instance, resting, antigen-responsive cells will undergo antigen-driven clonal expansion. Mammary-gland cells provide a second example; under the influence of pregnancy-induced mitogenic stimuli these cells undergo a hyperplastic as well as a hypertrophic response. A third example is that of hepatocytes; these cells are stimulated to grow in response to hepatectomy. An altered or inappropriate response to signals regulating controlled entry into and exit from the cell cycle forms the basis for a wide range of pathologies.

It is difficult to study events related to a mitogenic response in intact animals. Tissues are heterogeneous cell populations, only a subset of which will respond to a particular proliferation signal. Existing proliferating cells will be randomly distributed about the cell cycle. In an intact animal one cannot distinguish a direct effect of the administered agent from effects in response to an intermediary system. Finally, the large quantities of mitogenic peptides necessary for *in vivo* experiments are frequently prohibitive.

Model Systems for Studying Mitogenesis

The ideal model system to study the mitogenic response would be a clonal cell culture population that could reversibly and synchronously exit from and enter into the cell cycle in response to biologically relevant factors. The clonal murine 3T3 cell line[1] has many of the desired properties. 3T3 cells grown in medium with differing serum concentrations exit from the cell cycle at densities proportional to the serum level and enter a nonproliferating, 'G_0' state. When subsequently exposed to an elevated serum concentration, the cells re-enter G_1 and, 8–12 h after serum addition, begin DNA synthesis. A variety of protein growth factors such as epidermal growth factor (EGF), fibroblast growth factor (FGF) or platelet-derived growth factor (PDGF) can also stimulate DNA synthesis and subsequent cell division in quiescent 3T3 cells. Fatty-acid derivatives such as prostaglandin F2α and macrocyclic tumor promoters such as tetradecanoyl phorbol acetate (TPA) are also potent mitogens for 3T3 cells.

A larger number of events, collectively termed the 'pleiotypic response',[2] occur following addition of mitogens to quiescent 3T3 cells. These include (but are not limited to) changes in transport of ions and small molecules, phosphorylation of many proteins, in-

Plant Science, 41 (1985) 121—124
Elsevier Scientific Publishers Ireland Ltd.

ADVENTITIOUS SHOOT FORMATION ON CULTURED POTATO ROOTS

N.O. ESPINOZA and J.H. DODDS*

International Potato Center, P.O. Box 5969, Lima (Perú)

(Received December 7th, 1984)
(Revision received June 28th, 1985)
(Accepted August 7th, 1985)

Isolated roots of five different potato genotypes were cultured on media for the regeneration of plants. Plants were regenerated from four of the genotypes and in two cases the regeneration took place, apparently without an intermediate callus phase. Sections were made of this directly regenerating material which appears to form from the epidermal layer of the cultured root.

Key words: potato; adventitious buds; root culture; direct differentiation

Introduction

The potato is one of the world's most important crop plants, and is often used as a model plant species in tissue culture because of its outstanding plasticity of development. The regeneration of plants has been demonstrated from a wide selection of tissue culture material including meristems [1], leaf discs [2], stem segments [3], callus from protoplasts [4], and isolated cells [5].

The regeneration of intact plants from cultivated roots of other Solanaceous plants has been reported both from the root pericycle [6,7] and after intermediate callus formation [8] However, there are no reports of the regeneration of plants from cultured roots of potato. In experiments in vivo Howard [9] and Miedema [10] damaged roots of potato and removed the growing apex of the shoot, under these conditions small numbers of plants could be regenerated from roots.

This series of experiments was designed to study the regeneration of plants from potato roots cultured in vitro. This paper also describes the cellular origins of these regenerated plantlets to determine whether an intermediate callus phase is formed during differentiation.

Materials and methods

The starting material was obtained from plantlets maintained in vitro in the potato germplasm collection of the International Potato Center. The selected clones were K-2 (AH 78.5262); K-7 (AH 79.5503); BR-63.65 (800222); BR-63.5 (800212) and P-2 (374080.1). The plants were maintained on Murashige and Skoog (MS) [11] salts medium with the addition of calcium pantothenate (2 mg/l), sucrose (3% w/v) and agar (0.8% w/v). Plantlets were removed from the culture tubes and the roots were cut into 3 cm long segments to be inoculated into petri dishes containing a number of different experimental media. All manipulations were carried out under aseptic conditions.

The experimental culture media contained the organic and inorganic constituents of MS [11] supplemented with 0.8% (w/v) agar, 6 benzylamino purine (1 mg/l), indoleacetic acid (1 mg/l) and gibberellic acid (10 mg/l). Sucrose was added at six differ-

*Editorial Comments
Abbreviation: MS, Murashige and Skoog.

0168-9452/85/$03.30 © 1985 Elsevier Scientific Publishers Ireland Ltd.
Printed and Published in Ireland

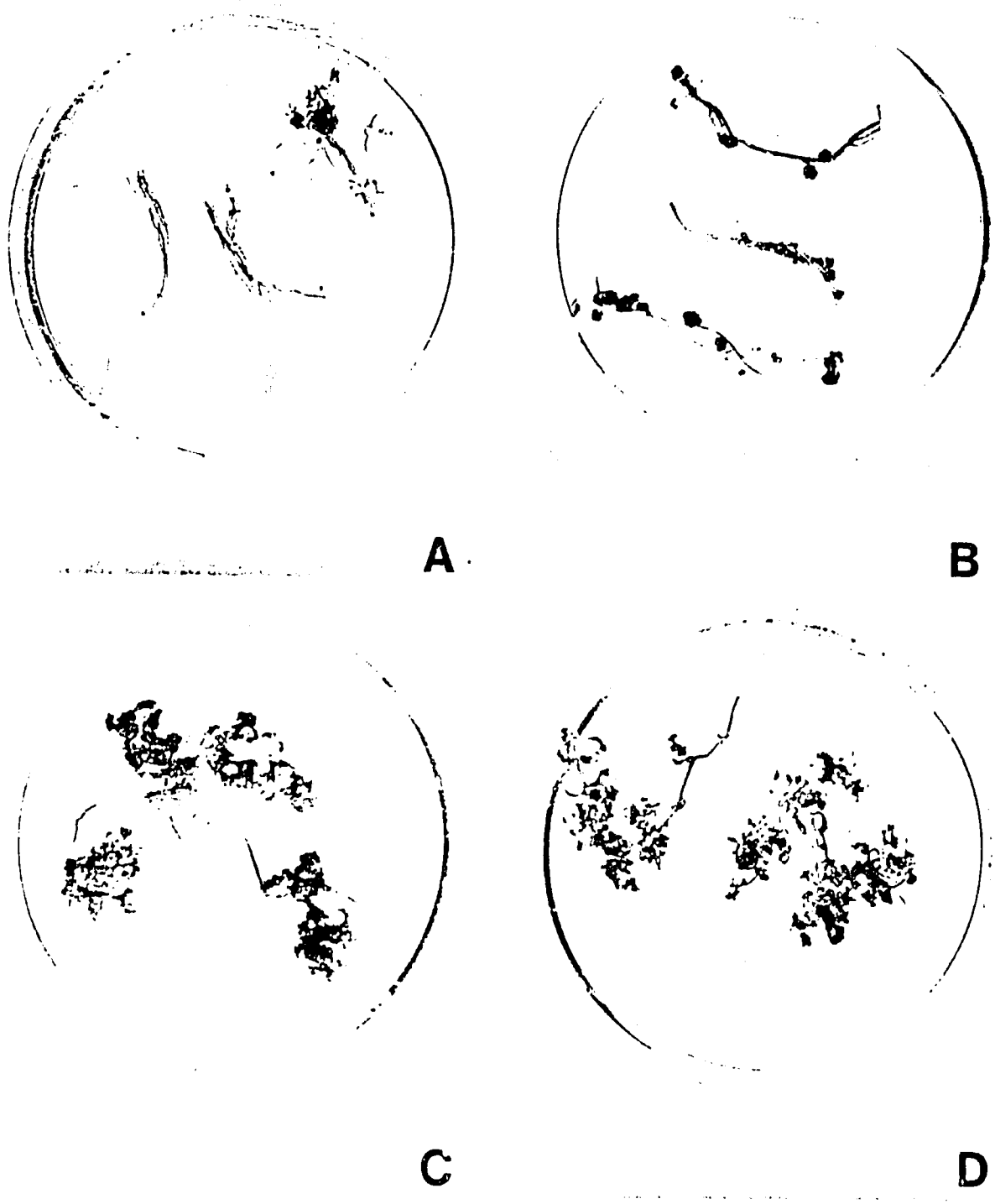


Fig. 1. Three centimeter long root segments from various potato clones. (a) Clone BR-63.5 grown on MS medium with benzylamino purine, without sucrose, indoleacetic acid induces little or no growth; (b) growth of clone BR-63.65 with MS medium with benzylamino purine, indoleacetic acid, gibberellic acid and 2.5% sucrose induces small compact nodules of callus; (c) clone BR-63.5 on MS medium with benzylamino purine, indoleacetic acid and gibberellic acid + 1% sucrose induces large friable callus which will regenerate plantlets; (d) growth of clone K-2 on MS medium with benzylamino purine, indoleacetic acid and gibberellic acid with 0.5% sucrose induces formation of large numbers of plantlets directly from the root epidermis.

Table I. Formation of callus and adventitious shoots on cultured potato roots. Measurements were made after 42 days in culture. Sucrose concentration as indicated in an otherwise complete MS medium containing benzylamino purine, indoleacetic acid and gibberellic acid. Results in Table I are from three experiments, each employing 60 root segments per treatment.

Clone	Sucrose concentration (%)					
	0	0.25	0.5	1.0	2.5	5.0
K-2	–[a]	+[b]	++	+++	+++	C[c]
K-7	–	++	+++	++++	+	–
BR-63.5	–	C	C	C++	C	–
BR-63.65	–	C	C	C+	C	–
P2	–	C	C	C	C	–

[a]– = no callus or adventitious shoots.
[b]+ = adventitious shoots.
[c]C = callus.

ent concentrations from 0 to 5% (w/v) (Table I). The media were adjusted to pH 5.6 and autoclaved at 121°C for 15 min.

Once inoculated the roots were cultured at 18–22°C; a light intensity of 1000 lux was provided during a 16-h day by Gro-Lux fluorescent tubes. After 42 days the cultures were scored and histological sections were cut of the regenerating material.

Tissues were fixed in a chromic acid, acetic acid and formalin mixture, dehydrated in alcohol and embedded in paraffin wax. Serial sections were cut (11 μm thick) and stained with safranin/crystal violet/fast green mixture [12]. Stained material was observed and photographed with an Olympus BH-2 microscope.

Results and discussion

Of the five clones tested, adventitious shoots were induced in four of them (Table I and Fig. 1). In about 30% of the plantlets regenerated (which numbered several hundred), a small callus formed and plants then regenerated from the callus (Fig. 1). In about 80% of the cases (a total of more than 1200 plantlets) the shoots regenerated with no apparent callus phase (Fig. 2). In the absence of sucrose there was no induction of either callus or adventitious shoots. As seen in Table I concentrations of sucrose between 0.5 and 1.0% are optimal for adventitious shoot formation. These small adventitious shoots are easily excised and transferred to propagation medium to obtain intact rooted plants.

In most cases there appears to be no callus phase involved in the induction process and this may have significant implications for the genetic fidelity of the regeneration system [13]. Experiments are now being carried out to see how many clones can be regenerated by this method.

Preliminary experiments indicate that it is also possible to regenerate intact plants from

Fig. 2. Section cut through a plantlet forming directly from root epidermis in clone K-7 on MS medium containing benzylamino purine, indoleacetic acid, gibberellic acid and 0.5% (w/v) sucrose. Wax embedded material was serially cut in 11 μm sections and stained with safranin/crystal violet/fast green. (Orig. mag. ×1000).

'hairy roots' on potato plants induced by *Agrobacterium rhizogenes* using the same media. These results may have important implications for on-going experiments involving genetic engineering of potato using *A. rhizogenes* as a gene vector.

Acknowledgement

The authors wish to thank Mr. Fidel Torres for help with preparation of material for microscopy.

References

1 W.M. Roca, N.O. Espinoza, M.R. Roca and J.E. Bryan, Am. Potato J., 59 (1978) 691.
2 K.J. Webb, E.O. Osifo and G.G. Henshaw, Plant Sci. Lett., 30 (1983) 1.
3 E. Thomas, S.W.J. Bright, J. Franklin, V. Lancaster, B.J. Miflin and R. Gibson, Theor. Appl. Genet., 62 (1982) 65.
4 J.F. Shepard and R.E. Totten, Plant Physiol., 60 (1977) 313.
5 A.C. Cassells, E.M. Goetz and S. Austin, Potato Res., 26 (1983) 367.
6 J.G. Torrey, Plant Physiol., 33 (1958) 258.
7 A. Zeleer, O. Soferman and S. Izhar, Plant Cell Rep., 2 (1983) 252.
8 E. Thomas and H.E. Street, Ann. Bot.. 36 (1972) 239.
9 H.W. Howard, Nature, 203 (1964) 1303.
10 P. Miedema, Euphytica, 16 (1967) 163.
11 T. Murashige and F. Skoog, Physiol. Plant., 15 (1962) 473.
12 G.P. Berlyn and J.P. Miksche, in: The Iowa State University Press, Botanical Microtechnique and Cytochemistry, Ames, Iowa, 1976.
13 W.R. Scowcroft and P.J. Larkin, in: Pitman Press, CIBA Foundation Symposium 97, Better Crops For Food, London, 1983, p. 177.

GENETIC ENGINEERING OF THE POTATO

Dr. Jesse M. Jaynes
Department of Biochemistry
322 Chopin Hall
Louisiana State University
Baton Rouge, Louisiana 70803

Mr. Nelson Espinoza
International Potato Center
P. O. Box 5969
Lima, Peru

Dr. John H. Dodds
International Potato Center
P. O. Box 5969
Lima, Peru

ABSTRACT Application of recombinant DNA technology and plant transformation techniques with the modern science of plant tissue culture for potato improvement.

BIBLIOGRAPHY

Genetic Engineering of the Potato

"Construction and Expression of Synthetic DNA Fragments Coding for Polypeptides with Elevated Levels of Essential Amino Acids." Jesse M. Jaynes et al. in Applied Microbiology and Biotechnology Vol. 21, pages 200-205; January, 1985.

"A Vector for Introducing New Genes into Plants." Mary-Dell Chilton in Scientific American, pages 36-45; June, 1983.

"A Simple and General Method for Transferring Genes Into Plants." Robert B. Horsch et al. in Science Vol. 227, pages 1229-1231; March 8, 1985.

"Potatoes for the Developing World." 150 p. International Potato Center, 1984.

THE AUTHORS

Jesse M. Jaynes, Nelson O. Espinoza, and John E. Dodds ("Genetic Engineering of the Potato") began their collaboration in 1983. Jaynes is an assistant professor of biochemistry at Louisiana State University and also holds an appointment with the Louisiana State Agricutural Experiment Station. He attended the public schools of Tooele, Utah, and obtained an A.S. degree from the College of Eastern Utah. He earned a B.S. degree from Southern Utah State College and a Ph.D. degree in biochemistry from Brigham Young University where he studied with Dr. Leo P. Vernon. After graduate school, he was a post-doctoral fellow with Dr. Ray Valentine at the University of California, Davis. He then moved to Montana State University, first as a post-doctoral fellow and subsequently became a research assistant profesor in the plant pathology department. His current research interests are focused on the application of recombinant DNA technology to solve problems confronting the peoples of the developing world. Espinoza obtained a degree in biological science in 1976 from the San Marcos National University of Lima, Peru. He joined the staff of the International Potato Center in 1977 working in potato micropropagation, in vitro conservation, plant regeneration, and protoplast culture. Since 1978, he has been the national correspondent for the Peruvian section of the International Association of Plant Tissue Culture. Dodds obtained B.S. and Ph.D. degrees from University College, London, in the field of plant biology. He then spent two years of post-doctoral study on plant hormone biochemistry with Dr. M. A. Hall at University College of Wales, Aberystwyth. Short post-doctoral periods were spent with

professors Raghavan and Roberts at Ohio State University and Idaho State University, respectively. He co-authored a text book entitled "Experiments in Plant Tissue Culture" with professor Roberts. He has worked as a lecturer at the University of Birmingham, England, and as a consultant in Mexico. He currently holds the position of Tissue Culture Specialist at the International Potato Center in Lima, Peru.

Introduction

The potato originated in the Andean region of South American and has proven to be extremely important as a staple food (see illustration one). It was taken to Europe by the Spaniards and went through a process of genetic adaptation to the temperate climate there. The crop is now grown in more countries than any other food crop except corn. It has a role in haute cuisine but more importantly it is utilized as a basic calorie and protein source by millions who live in the ~~torrid zone of~~ tropics the world (see illustration two). To serve these latter consumers, the International Potato Center was formed in 1972. CIP collaborates with research and development agencies throughout the world to achieve two basic objectives:

- Increase the potato's yielding ability, stability, and efficiency of production in deveoping areas where it is now grown, and,
- Improve the potato's adaptability to grow more extensively and efficiently in cold, high regions, as well as in warm, low tropical regions.

The nutritional value of the potato when used in haute cuisine is of little concern to the consumers of these types of foods. However, when the potato is used as a basic food, often comprising more than 80% of the diet, its nutritional value is of primary importance.

Protein malnutrition can usually be ascribed to a diet which is deficient in one or more of the essential amino acids. There are twenty common amino acids which are utilized in the synthesis of

proteins. Essential amino acids are those ten protein building blocks which most animals, including humans, cannot synthesize de novo. Thus, a nutritionally adequate diet must include a minimum daily consumption of these amino acids. Essential amino acid content, or protein quality, is as important a feature of the diet as total protein quantity or total calorie intake.

When diets are high in carbohydrates and low in protein over a protracted period, essential amino acid deficiencies result. The name given to this condition is "Kwashiorkor", which is an African word meaning "deposed child" (deposed from the mother's breast by a newborn sibling). This debilitating malnourished state, characterized by a bloated stomach and reddish-orange discolored hair, is more often found in children than adults because of their great need for essential amino acids during growth and development. In order for normal physical and mental maturation to occur, the above mentioned daily requirement of amino acids is a requisite (see illustration three).

The potato, although one of the best sources of plant proteins, is deficient in certain essential amino acids. For a number of reasons, it is difficult to envisage that a plant breeding program would be capable of producing significant increases in the essential amino acid content of the potato. Firstly, the genetics of potato breeding is complex which means that increased essential amino acid content may be offset by a loss in other agronomically important characters; secondly, it is probable that the storage proteins (the main protein

components in tubers) are highly conserved in their structure and would be little modified by these conventional techniques.

The innovative method of genetic engineering, however, offers a novel approach to modifying the essential amino acid composition of potato, thus increasing its nutritive value. Our approach is not to try and modify the existing proteins of the potato but rather to supplement the existing proteins with new synthetic proteins, which have a high content of essential amino acids. In order to produce a synthetic protein of known amino acid composition, we constructed a synthetic DNA fragment with the appropriate codon sequence (the three base linear array of the genetic code).

Construction of Synthetic DNA Sequence

Synthetic DNA, of a desired sequence, can now be constructed using modern chemical techniques. This DNA can then be inserted into various microorganisms via recombinant DNA technology (the recombination of DNA in the test tube). The cloning (replication) and expression of synthetic DNAs, that contain repeated codons for a single amino acid, can be used as a mean to supplement the essential amino acids, of plant protein. As stated previously, however, plant proteins are deficient in more than a single essential amino acid; therefore, in the design of a useful synthetic protein, the total amino acid composition of plant protein sources must be taken into consideration.

We have constructed (using a DNA synthesis machine), cloned, and

obtained in bacteria the expression of synthetic genes that code for proteins with a high content of most deficient essential amino acids of plant-derived proteins. The sequences for several of these synthetic gene fragments were designed and the resulting protein sequence was obtained by inspection of the genetic code (see illustration four). The designation of proteins A and B represents sequences of the proteins derived from both possible reading directions of gene fragment 1. That is, gene fragment 1 was constructed symmetrically so that a protein containing a high content of essential amino acids would be produced no matter which strand of the synthetic DNA was ultimately read by the plant. The composition of proteins A and B for the five most deficient essential amino acids of plant-derived proteins was compared with those found in proteins from several foods (see illustration five). Proper suplementation of plant-derived foods with proteins encoded within these synthetic gene fragments would markedly improve the essential amino acid balance.

These methods of gene synthesis are flexible enough to produce proteins possessing any particular amino acid composition, therefore, genes could be specifically designed to supplement any desired animal feed or human food. It should be pointed out that the insertion of lysine at frequent intervals in these synthetic proteins provides numerous sites for proteolytic attack by trypsin (one of the main protein-degrading enzymes found in the digestive tract). This feature is important, as it increases the bioavailability of the supplemental protein. Bioavailability

refers to the amount of amino acids actually taken up from a particular dietary protein and used by the animal to make its own protein.

How to Get the Gene Fragment into Plants

Plant breeding, the movement of desirable traits between plants by the use of the traditional Mendelian techniques, has, without question, improved the yields of the world's major crop plants such as the potato. There are, however, certain limitations to these techniques. For example, it takes a great amount of time, perhaps as long as ten years, to introduce, select, and establish a particular trait into a plant cultivar and ofter some traits are impossible to incorporate by these traditional techniques.

Genetic engineering offers the possibility of introducing a single trait, without altering other agronomically important characters of the plant, into a well accepted or traditional variety, thus allowing for a method of "fine-tuning" these specific genotypes. The use of these new methods, however, is not a goal in itself and should be coupled to conventional breeding methods to achieve plant improvement.

Agrobacteria, often regarded as Nature's first "genetic engineer", allows the exploitation of recombinant DNA technology for plant improvement. Two species of the genus Agrobacterium have been shown to affect plant growth and development.

Arrobacterium rhizogenes causes a proliferation of roots at the point of infection on the host plant; this response has been termed "hairy root". The etiology of hairy root is quite distinct from that of crown gall (a type of plant tumor), which is caused by Arrobacterium tumefaciens. The factors, in both A. rhizogenes and A. tumefaciens, causing changes in growth characteristics, have been shown, without equivocation, to be located on large plasmids (circular, self-replicating DNAs found in bacteria). Most importantly, a discrete portion of this plasmid DNA (called T-DNA) is found to be incorporated into the genome of the infected plant, which is known as the process of plant transformation. This T-DNA determines several fundamental characteristics of transformed plant tissue. For example, these plant cells will grow without adding phytohormones to the culture medium, which are normally necessary for cell growth in culture. These transformed cells will also synthesize unusual compounds called "opines" (see illustration six), which prove to be useful markers for selection of transformed plants.

One strategy, for introducing foreign DNA into a plant, by means of A. tumefaciens plasmids, is to use a small recombinant plasmid into which a known fragment of T-DNA has been inserted (see Mary-Dell Chilton's article in Scientific American, June 1983 issue). Using this method, we opened the recombinant plasmid by means of a restriction enzyme, which recognizes a specific short sequence of DNA and causes a double strand break at a site within the T-DNA. At this breakage point, the synthetic essential amino acid encoding (EAAE) DNA was inserted. Along with the EAAE-DNA, a

selectable marker, i.e., the bacterial gene encoding for resistance to a specific antibiotic, was included. This new plasmid was then introduced into a A. tumefaciens strain carrying the unmodified agrobacterium plasmid. Since these different plasmids contain homologous (similar) sequences, a rare double-recombination between the two plasmids will sometimes occur and result in a new plasmid in which the T-DNA harbors the insert of EAAE-DNA. The agrobacteria containing the new plasmid can be identified and selected by their survival on media containing the selecting antibiotic. These selected bacteria are then used to transfer the modified T-DNA (containing the EAAE-DNA) into the plant genome.

This protocol allows production of modified plant cells growing in culture; however, we found difficulty in obtaining healthy plants from these transformed cells, since they still contained tumerous genes. It should be noted that we used the same experimental strategy as Chilton, but employing an A. rhizogenes plasmid vector system to introduce the EAAE-DNA into potato plants. We are also using a modification of Chilton's method to insert the EAAE-DNA into potato plants by using "disarmed" A. tumerciens plasmid vectors. These disarmed vectors have had the tumor-forming genes removed and plants can be regenerated directly from sections of the transformed leaves.

It is clear that the technology exists to insert, on a routine basis, foreign DNA into the potato plant. To ensure the expression of a

particular desirable gene or DNA fragment in the plant, one must insert it next to the proper control regions of another already existing gene, which has had its protein encoding part removed. These control regions or promoters as they are commonly called, are usually found at the beginning of the gene and are vital switches attuned to the many influences that regulate and temper the expression of the gene to its final protein product. It is highly desirable to use promoters that allow abundant expression of the EAAE-protein and also control its tissue-specific expression within the potato tuber.

Tissue Culture of Transformed Plants

From the previous sections, it is clear that recombinant DNA techniques exit for the insertion of synthetic genes into *Agrobacterium* plasmids; however, the controlled transformation of plants by the use of modified bacteria is dependent on two important features. First, the bacterium must be capable of infecting the host plant. The usefulness of this infection process is limited at the present time, to a few major crop plants such as the potato. Second, tissue culture techniques must be available for the regeneration of transformed plants. The potato is a model plant for tissue culture and its plasticity of development allows intact plants to be regenerated from almost any plant part. It is possible, therefore, to infect potato plants with *A. rhizogenes* (containing the EAAE-DNA) and induce the formation of hairy roots; these roots can then be cultivated and transformed plants regenerated as shown (see illustration seven). We can also regenerate transformed plants from leaf

discs using disarmed plasmids. In the potato plant, which is normally asexually propagated, there are no problems of segregation and loss of the inserted information through the processes of meiotic recombination, although, it would be interesting to see how this newly inserted information (EAAE-DNA) segregates and if it is transmitted sexually to the progeny. In the cereals, such as rice, corn, and wheat, tissue culture technics do not exist to regenerate whole plants from single cells, a process that is extremely simple in the case of the potato. Thus, the idea of genetically engineering cereals must await the development of different technologies.

Distribution of Transformed Potato Germplasm

When transformed plants have been regenerated they can be propagated rapidly *in vitro* by using conventional tissue culture techniques. These micropropagation methods offer a number of advantages, since the material is pathogen free and can be readily exported to national agricultural programs in the developing world. Illustration nine shows the growth of potato plants *in vitro* to the stage of having small seedling-like plantlets ready for planting. Since a stock of sterile, pathogen-free, *in vitro* plantlets can be maintained and new planting material produced each year, it is no longer necessary to save part of the crop for the next season's planting. All the tubers harvested then may be used for consumption.

Methods have been developed at the International Potato Center

that allow for the worldwide distribution, at no cost to the recipient, of new germplasm to national potato programs (see illustration ten). A few in vitro plantlets exported to a national program through CIP's regional distribution network (see illustration twelve), can be propagated rapidly to produce large numbers of plants for farmers' fields. A good example of this is the case of the potato-growing areas around Dalat in the central highlands of Vietnam. In vitro potato plantlets, sent to Vietnam in 1980 by CIP, have been rapidly micropropagated by small farmers using simple home-constructed equipment. Most of the tissue culture operations were carried out in the kitchen or bedroom (see illustration eleven). These few plants have now been propagated to such an extent that almost 500 hectares (equivalent to about six million plants) are now grown annually in the Dalat area with material derived from these plants.

Some Possible Future Applications

Through our collaborative research we have demonstrated that it is now possible to incorporate desirable genes into the potato via the methods of recombinant DNA technology. The major limitation to the large-scale application of these genetic engineering techniques, for the improvement of the potato and other crop plants, is the general lack of knowledge about plant genes and how to identify, isolate, and purify them. This article has highlighted an innovative approach--that is, if you cannot locate and purify desirable genes then they must be constructed synthetically.

We are in the process of producing synthetic DNA sequences that may interfere with potato virus or viroid replication. When these sequences are incorporated into the potato, the plant may become permanently "immunized" against infection (see illustration thirteen).

It may also be possible to use a non-infectious viroid as a novel vector for the insertion of desirable traits into plants, possibly even in the cereals. We are also producing plants containing the genes that code for enzymes that degrade bacterial cells walls; this may protect the plant against bacterial infection.

The preliminary results obtained from these experiments are contributing to the basic knowledge of plant biochemistry and gene structure and may, in the future, lead to the development of improved potato varieties. It takes about ten growing seasons to make a new potato variety by traditional breeding methods. Only further time will tell if genetic engineering technology will enhance our capabilities to introduce new characteristics into potato plants. If so, we will have attained a new horizon in the production of improved plant varieties for the ultimate benefit of all peoples.

The Potato: A Model Crop Plant for Tissue Culture

N. O. Espinoza, R. Estrada. D. Silva-Rodriguez, P. Tovar, R. Lizarraga, and J. H. Dodds

International Potato Center, P.O. Box 5969, Lima, Peru

It is a commonly held misconception that plant tissue culture is a new technology. In reality, the concept that the individual cells of an organism are totipotent is implicit in the statement of the cell theory [1]. T. Schwann in 1839 expressed the view that each living cell of a multicellular organism should be capable of independent development if provided with the appropriate external conditions. During the latter part of the 19th century several plant scientists performed experiments on fragments of tissues isolated from higher plants. Wound callus formation on isolated stem and root slice fragments were described, callus being the disorganized proliferation of actively dividing cells.

G. Haberlandt in 1902 [2] began the concept of cell culture, and was the first person to attempt to cultivate isolated plant cells *in vitro* on an artificial medium. A tribute to Haberlandt's genius, with a translation of his paper 'Experiments on the culture of isolated plants cells' was published in 1969 (3). He suggested the utilization of embryo sac fluids as a type of culture medium and the possibilty of culturing artficial embryos from vegetative cells.

Early in the 20th century interest shifted to the culture of meristematic tissues in the form of isolated root tips: these were the first sterile organ cultures to be performed. W. J. Robbins [4] and W. Kotte [5] both published results on culture of isolated roots. It was P. R. White [6], however, experimenting with tomato roots in 1934, who succeeded for the first time in demonstrating the potentially indefinite culture of isolated roots.

During the 1960s it was shown that cultured anthers (containing haploid pollen grains or microspores) have the potential to produce haploid embryos and plantlets [7, 8, 9, 10]. Later, with the aid of chemical treatments, it became possible to culture microspores, double the chromosone number, and collect seed from homozygous diploid plants within a very short space of time.

Another important development in the 1960s was the enzymic isolation and culture of protoplasts [11]. This method involves removing the cell wall with purified preparations of cellulase and pectinase, while regulating protoplast expansion with an external osmoticum. The cultured protoplasts regenerate new cell walls, form cell colonies, and ultimately form plantlets.

By the late 1970s and early 1980s techniques of cell, tissue, and organ culture like some of those destined above were beginning to make a significant contribution to agriculture and industry; however, many of the techniques described above are not successful with all plants. For example, in some grasses it may be possible to culture isolated meristems [12] but it is not possible to regenerate plants from protoplasts of the same species [13].

The potato is the one of the few basic food crops that reacts in a 'textbook' manner to the whole array of tissue culture techniques. In this article we would like to describe the array of techniques being applied to the potato to improve both quality and quantity of potato production.

Pathogen elimination

Plant material destined for culture *in vitro* must be surface sterilized, because fungi and bacteria will grow on the culture medium far faster than the plant material and will quickly overgrow and kill the plants. In certain exceptional circumstances the growth of bacteria and fungi can be controlled with antibiotics [13].

Viruses are much more difficult to eradicate and represent a major problem in potato tissue culture [14]. In simple terms, if the plants are not freed from virus then any rapid asexual propagation system also rapidly propagates the disease. The method normally used for eradication of virus is thermotherapy treatment followed by meristem isolation and culture (figure 1) [15, 16]. Infected plants are subjected to thermotherapy treatment (36°C 16hr/30°C 8hr) for four weeks in continuous light. The principle of thermotherapy is to inhibit the replication of the virus while still allowing growth of the meristem.

The dissection of a potato meristem is shown in figure 2. Once placed into culture on an appropriate medium it takes several weeks to regenerate a plant. This plant can then be rapidly propagated *in vitro* (see later) and enough material produced for virus testing. The testing of material prior to issuing a 'certificate of health' (phytosanitary) should be rigorous [17]. The International Potato Centre phytosanitary statement reads as follows: The vegetative plant parts were indexed and found to be negative for viruses: A,M,S,T,X,Y, potato leaf roll, Andean potato latent, Andean potato mottle, tomato black ring and tobacco ring spot viruses by symptoms on inoculated indicator hosts, by latex or ELISA serology and electron microscopy. Potato spindle tuber viroid (PSTV) was negative by the nucleic acid spot hybridization test (NASH).

This pathogen tested (this term is preferred to pathogen free) material forms a nuclear stock from which certified seed tubers can be produced [18].

In vitro micropropagation

Although it is possible to propagate large numbers of potato plants by regeneration of plants from single cells or callus these methods are not normally used, as they can affect the clonal stability of the material (see later). The standard micropropagation methods depend, therefore, on the release of dormancy of axillary buds, these methods are shown diagrammatically in figure 3.

At the International Potato Centre two micropropagation methods are used [19, 20].

Single node cuttings. In vitro plantlets from the pathogen-tested collection are removed from the tubes and cut into single nodes. Each node has a small

Outlook on Agriculture, Volume 15, No. 1, 1986.
0030-7270/86 $0.00 + .50
© 1986 Pergamon Press Printed in Great Britain.

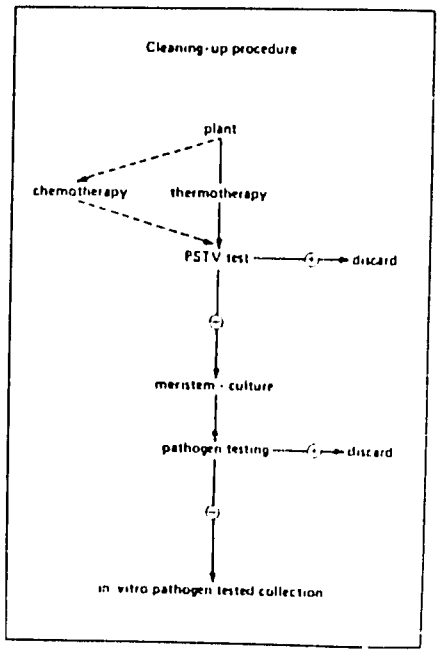


Figure 1 Diagramatic scheme to show the passage of material to obtain pathogen-tested status.

leaf and an axillary bud. The small leaf is carefully excised and the remaining nodal cutting is transferred to a tube of Murashige and Skoog propagation medium (Table 1). Figure 4 shows inoculation and growth of the nodal cutting after 2, 4 and 6 weeks. After 5–8 weeks the *in vitro* plantlets are ready for sub-culture. The process can be repeated as many times as needed to produce desired number of plantlets. Propagation rate can lead to the production of several million plants in a year.

Shaken liquid cultures. An alternative and more rapid and cost-effective method of propagation is to excise whole stems of *in vitro* plants and layer them in flasks with liquid propagation medium (Table 1). After 2–3 weeks the axilary buds along the whole length of the stem will have grown out and plantlets are ready to be subcultured (figure 5).

Production of single rooted plantlets. The Potato Center uses methods (a) and/or (b) to produce large enough quantities of material for single node cuttings, which are inoculated into sterile plastic boxes with 16 to 25 nodes/box. After 3–4 weeks these nodal cuttings have grown and rooted (figure 6) and are ready for transfer to potting mix as shown in figure 7. These plantlets can be used as mother plants for conventional rapid propagation or can be transferred to seed beds or fields for production of certified basic seed tubers.

Conservation of germplasm

In the last decade increasing attention has been given to the conservation of plant genetic resources, especially in economically important crop plants where the genetic base is being eroded [21, 22]. In the case of potato, collection of germplasm has been steadily carried out for many years and the International Potato Centre now

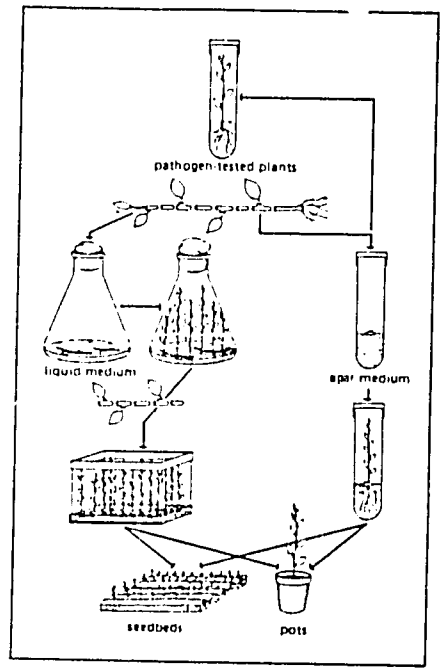


Figure 3 Diagram of standard micropropagation methods used at the International Potato Center.

holds a collection of some 6000 accessions [23]. Within this collection there are many sterile clones; the rest are highly heterozygous, meaning that seeds from this material are very distinct from the parent clones. The germplasm collection is currently maintained as a vegetatively propagated field collection which requires large quantities of space and labour and is also at a high risk from natural disasters – for example, frost,

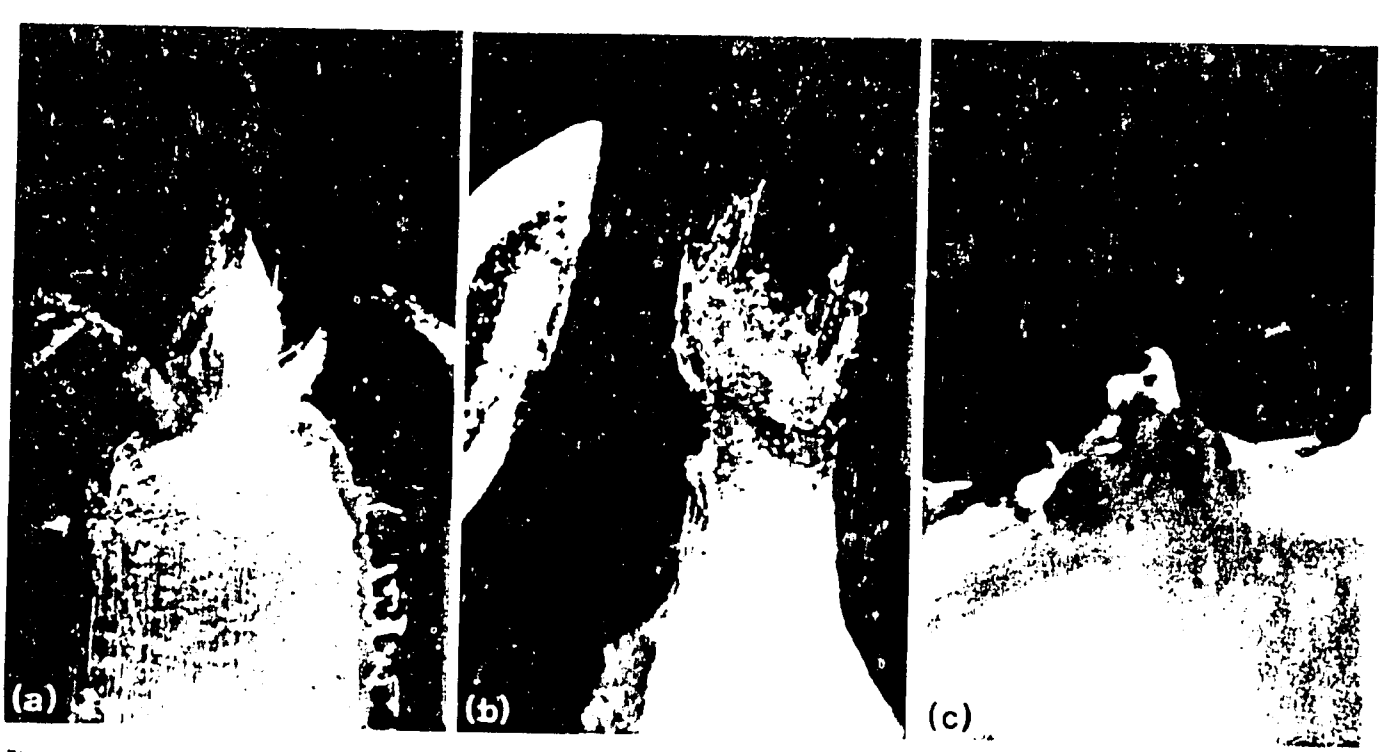


Figure 2 Sequence of dissection of meristem of potato. a to c. In (c) the two leaf primordial can be seen, indicating apical dome.

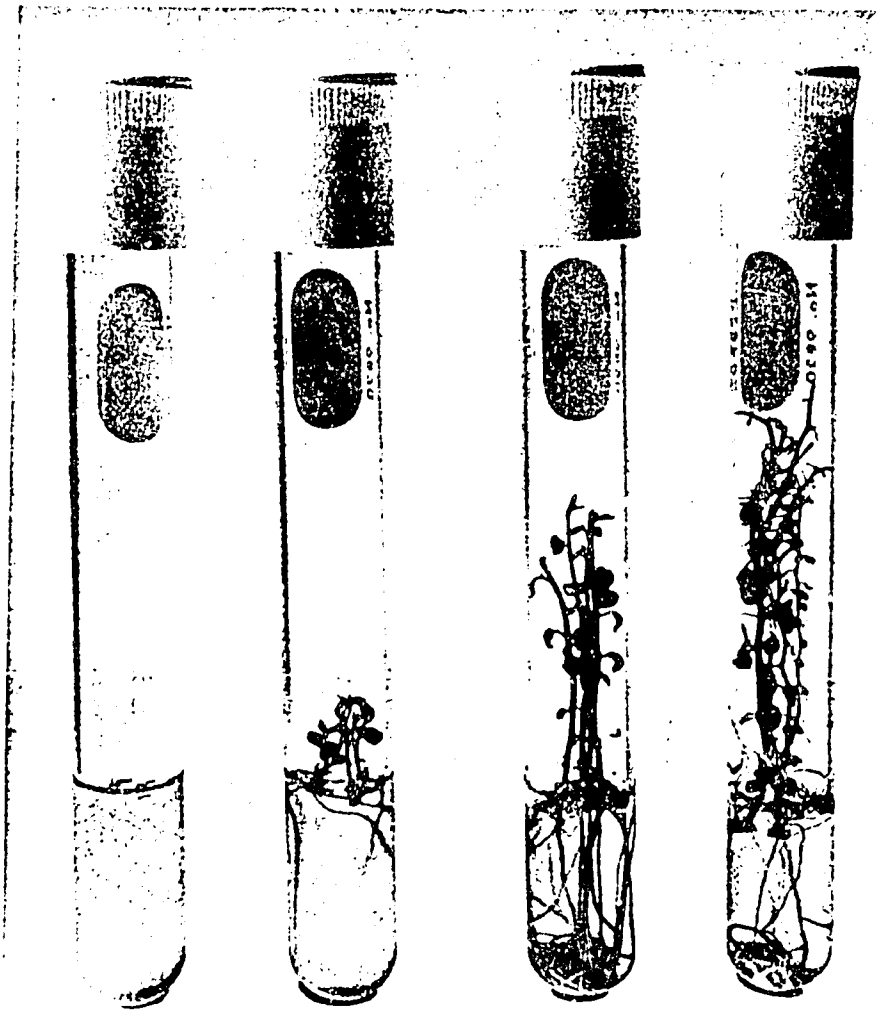


Figure 4 Growth and development of nodal explants, 0, 2, 4, and 6 weeks after inoculation on standard propagation medium.

Figure 8 *In vitro* conserved germplasm. This plant has been in the culture tube for nearly 12 months.

hail, and pathogen attack. The collection is currently being transferred to a system of *in vitro* storage. This is a long-term project and will probably take about 8-10 years to complete. The methodology being applied is to transfer the material to sterile (though not free of viruses) *in vitro* culture. The young shoots are then transferred to a storage medium (Table 1) and incubated at 8°C, 1000 lux, 16-hour day. Under these conditions the growth

Figure 7 *In vitro* plantlet after transfer to jiffy pot.

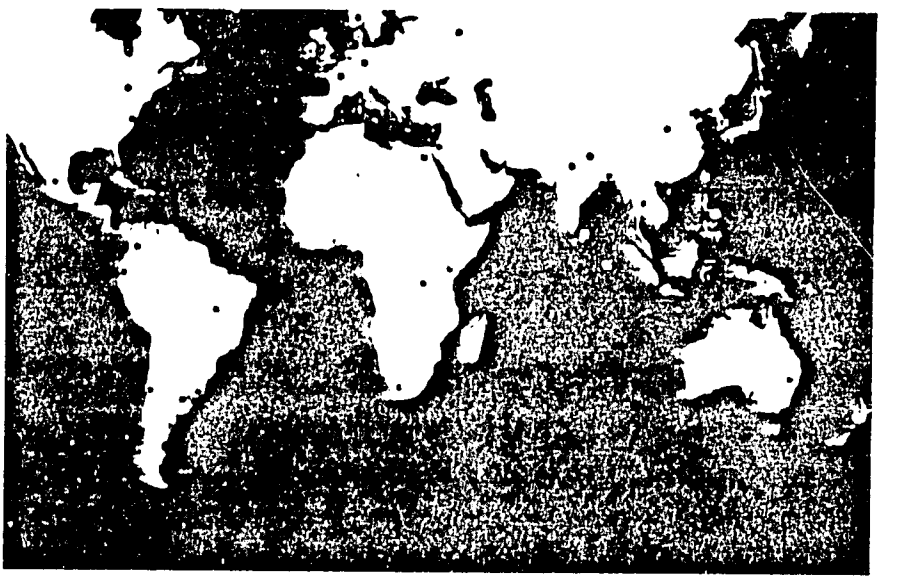


Figure 9 World map showing distribution of *in vitro* potato germplasm by the International Potato Center.

TABLE 1

Compound (mg/l)	Single node propagation	Shaken liquid propagation	*In vitro* conservation	*In vitro* tuber induction
NH_4NO_3	1650	1650	1650	1650
KNO_3	1900	1900	1900	1900
$CaC1_2$ $2H_2O$	440	440	440	440
$MgSO_4$	370	370	370	370
KH_2PO_4	170	170	170	170
H_3BO_3	6.2	6.2	6.2	6.2
$MnSO_4$ $4H_2O$	16.9	16.9	16.9	16.9
$ZnSO_47H_2O$	8.6	8.6	8.6	8.6
KI	0.83	0.83	0.83	0.83
$Na_2MoO_42H_2O$	0.25	0.25	0.25	0.25
$CuSO_45H_2O$	0.025	0.025	0.025	0.025
$CaCl_2$ $6H_2O$	0.025	0.025	0.025	0.025
Na_2EDTA	37.3	37.3	37.3	37.3
$FeSO_4$ $7H_2O$	27.8	27.8	27.8	27.8
Thiamine HC1	0.4	0.4	0.4	0.4
Ca Pantothenic acid	2.0	2.0		
Gibberellic acid	0.25	0.4		
Benzylamino purine		0.5		5.0
Napthalene acetic acid		0.01		
Chlorochlorine chloride				500
Inositol	100	100	100	100
Sucrose	3%	2%	3%	8%
Mannitol			4%	
Agar	0.8%		0.8%	

rate of the cultures is significantly reduced; a culture that normally needs to be transferred every 6–8 weeks needs to be transferred only once every two years (figure 8) [24].

International export of germplasm

The International Potato Center has a worldwide mandate to develop and disseminate knowledge for greater use of the potato as a basic food. As part of the overall strategy of achieving that aim the centre exports improved pathogen-tested germplasm to many developing countries. A growing proportion of these exports is now made as *in vitro* plantlets. Figure 9 shows the extent to which this distribution has now extended worldwide.

Using the micropropagation methods previously described, small tissue culture plantlets are produced on individual rooted plantlets in small glass test tubes. The tubes are packaged in a special polystyrene package to which is attached a phytosanitary certificate (figure 10); this is then sent air mail to the recipient potato programme. In 1984, the International Potato Center exported over 2500 plants using this method. Upon receipt of these plantlets *in vitro* propagation can be continued or the plantlets can be transfered to potting mix for conventional rapid propagation methods.

Figure 5 Growth of *in vitro* plants in a shaken liquid culture. Gibberellic acid in the medium releases dormancy of axillary buds and promotes rapid growth.

Organogenesis and somaclonal variation

The potato plant shows an extraordinary plasticity of development [25, 26]. It is possible to regenerate whole plants from isolated protoplasts (27); single cells (28); callus cultures (29); and explants of almost any part of the plant [30, 31]. Figure 11 shows regeneration of plants from cultured roots.

When plants are regenerated from any of these tissues by the formation of callus, followed by *de novo* production of buds, there is evidence that some of the plants regenerated are not genetically identical to the starting material [32, 33].

It is suggested by some workers that the variation induced by these methods, termed somaclonal variation, may be useful as a method for improving existing potato clones [34]. The hypothesis is that minor variations can be induced, then the material is screened and those plants improved in

Figure 6 Production of single-rooted plantlets by inoculating single nodes in sterile boxes.

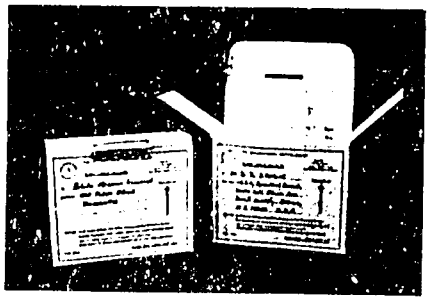


Figure 10 Export package. The polystyrene box contains tubes with *in vitro* plants. The package is sent air freight.

a specific trait are selected and cloned, using the standard micropropagation methods outlined previously. Several research institutions are testing these methods, and some have reported production of improved varieties.

Anther culture

The culture of potato anthers as a method for producing diploid (dihaploid) plants from tetraploids has been described [35]. There is a clonal dependence, however, in response to this technique; some clones easily produce 'pollen derived' plantlets, while other clones do not yield any plantlets. Preliminary evidence suggests that the differences in clonal responsiveness may be under genetic control [36].

The technique of anther culture is a relatively simple one. Anthers at an appropriate stage of development are excised from a surface-sterilized bud and cultured on an appropriate medium. In responsive clones, after a few days of culture a small percentage of the grains change their normal developmental pathway (to germinate and grow a pollen tube) and instead begin to undergo cell division. The mitotic divisions take place in a highly organised way and a pollen-derived embryo forms. This pollen embryonic pathway follows the same development stages as the formation of a seed embryo [37]. The pollen embryos can eventually be grown to intact plants, and form a useful source of material for potato breeders.

In vitro tuber production

The conventional method for propagation of potatoes is by planting tubers. In recent years techniques have been developed which allow tubers to be produced *in vitro* [38, 39, 40]. These *in vitro* tubers have the advantages of being small, light, and aseptic (sterile); also, when plantlets derived from them are grown in the field they appear to be perfectly normal. These *in vitro* tubers may represent a novel method to export potato germplasm and possibly commercial seed tubers.

The starting material for *in vitro* tuber induction are flasks of shaken liquid cultures used in micropropagation (figure 5). The liquid propagation media can be carefully removed and substituted with tuber-induction media that contains 500 ppm of CCC (Chloro Choline Chloride) (see Table 1). Once this tuberization stimulus has been applied, it takes only five weeks until physiologically mature tubers are ready to be harvested (figure 12). This technique has been applied at the International Potato Center to over 50 potato genotypes, all of which have responded well.

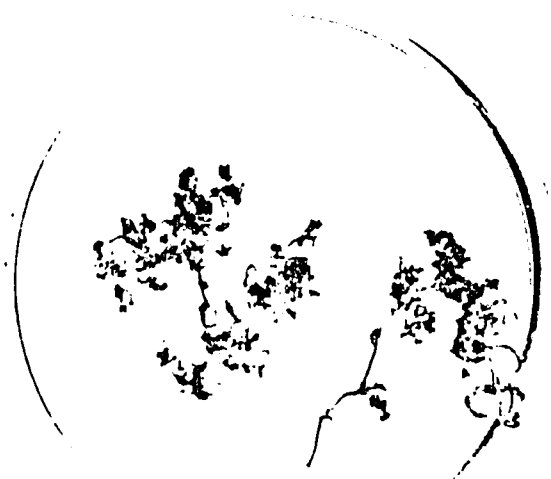
The tubers produced *in vitro* have similar characteristics to field-produced tubers in terms of colour and shape (figure 13) and the electrophoretic patterns of the tuber storage proteins are identical. It may be expected that in the next few years *in vitro* tubers will find their role in the various integrated schemes of potato seed tuber production.

Genetic engineering

The idea of genetically engineering crop plants is one that is currently receiving much attention [41, 42]. The methods of gene insertion are based on the principle of using *Agrobacterium* plasmids as vectors for the insertion of genes into a receptor crop species [43]. For successful results to be achieved in this area a number of criteria must be met.

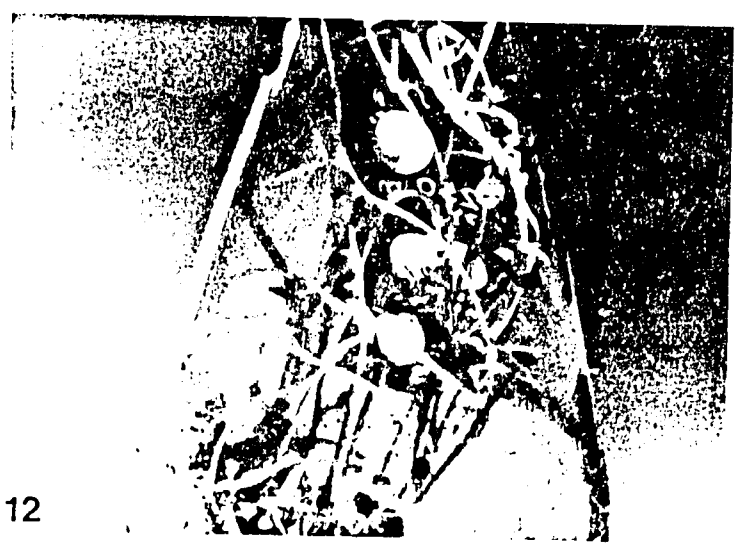
1. The plant must be succeptible to *Agrobacterium* infection.
2. It must be possible to regenerate plants from the transformed cells.
3. The inheritance characteristics of the inserted factor must be determined, or the plant must be asexually propagated.

The potato is one of the few important crop plants that can meet all three of these criteria. Potato plants *in vitro* can be wounded and infected with



11

Figure 11 Small plantlets regenerating from roots of potato inoculated on to the surface of agar solidifed regeneration medium.



12

Figure 12 Induction of *in vitro* tubers on rapidly propagated plants.

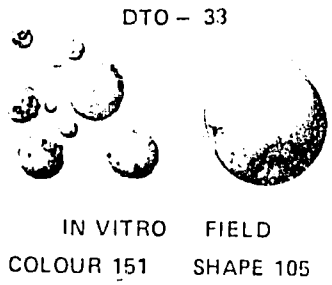


Figure 13 Comparison of *in vitro* with field produced tubers. Shape and color characteristics are identical.

Agrobacterium. After about two weeks a wound reaction takes place.

It is possible to regenerate transformed plants from these tissues (figure 14). A transformed plant is one in which part of the *Agrobacterium* plasmid has been integrated into the host-plant genome. The plasmid may be carrying a synthetic DNA sequence or desirable gene that has been inserted [44]. The technology for genetic engineering now exists in potato; the problem is one of deciding what genes should be inserted to improve the plant. We are currently attempting to insert genes for improved storage protein quality and improved virus resistance.

Figure 14 Regeneration of transformed plants after infection with *Agrobacterium*.

Conclusions

It is clear that the potato is an exceptional plant for tissue culture studies. Many of the techniques can already be routinely applied to the potato to give a direct impact on production; these would include pathogen elimination, multiplication, conservation, and export. For other more experimental techniques, such as genetic engineering, the potato offers a system to study that will have direct agricultural importance.

References

[1] Schwann, T. Mikroskopische Untersuchungen über die Ubereinstunmung in der Struktur und der Watchstum der Thiere and Pflanzen. Berlin, 1839. Reprinted as Ostwalds Klassiker der exakten Wissenschaften. No 176, 1910

[2] Haberlandt, G. *Sber. Akad. Wiss. Wein*, **111**, 69, 1902.

[3] Krikorian, A. D. and Berquam, D. L. *Bot. Rev.*, **35**, 59, 1969.

[4] Robbins, W. J. *Bot. Gaz.* **73**, 376, 1922.

[5] Kotte, W. *Ber. disch. Bot. Ges.*, **40**, 269, 1922.

[6] White, P. R. *Plant Physiol.*, **9**, 585, 1934.

[7] Guha, S. Maheshwari, S. C. *Nature*, **204**, 497, 1964.

[8] Nitsch, J. P. *Z. Pflanzenzucht*, **67**, 3, 1972.

[9] Sunderland, N. In 'Plant Tissue and Cell Culture' (Ed H. E. Street). p. 161, 1973.

[10] Nitsch, J. P., and Nitsch, C. *Science*, **163**, 85, 1969.

[11] Cocking, E. C. *Nature*, **187**, 927, 1960.

[12] Dale, P. J. *Plant Sci Lett.*, **19**, 303, 1980.

[13] Jones, M. G. K. and Dale, P. J. *Z. Pflanzenphysiol.*, **105**, 267, 1982.

[14] Mellor, F. C. and Stace-Smith, R. *Phytopath*, **57**, 674, 1967.

[15] Pennazio, S. *Riv. dell'Ortoflorofrutticoltura Ital.*, **5**, 446, 1971.

[16] Ouak, F. In 'Applied and Fundamental Aspects of Plant Cell, Tissue and Organ Culture' (Eds. Reinet & Bajaj) 1977.

[17] Roca, W. M., Rodriguez, J., Beltran, J., Roa, J. and Mafla, G. 5th Intl. Tissue Culture Congress Tokyo 1982.

[18] Roca, W. M., Espinoza, N. O., Roca, M. R. and Bryan, J. E. *Amer Pot. J.*, **55**, 691, 1978

[19] Espinoza, N. O., Estrada, R., Tovar, P., Rodriguez, D., Bryan, J. E. and Dodds, J. H. Specialised Technology Document CIP, Lima. 1984.

[20] Dodds, J. H. Planning Conference: Innovative Methods for Potato Propagation. CIP, Lima. 1984.

[21] Jackson M. and Ford Lloyd B. 'Crop Genetic Resources: Conservation and Utilization'. Edward Arnold, London 1984.

[22] Henshaw, G. G. In 'Crop Genetic Resources for Today and Tomorrow'. Eds. Frankel and Hawkes. Cambridge University Press p 349 1975.

[23] Huaman, Z. In 'Crop Genetic Resources'. IBPGR. 1984.

[24] Estrada, R. Schilde-Rentschler, L., and Espinoza, N. O. In *Proc. Intl Conf Research for the potato in the year 2000*. Intl. Potato Center Lima Peru 1982.

[25] Wenzel, G., Uhrig, H. and Burgermeister, W. In 'Genetic Manipulation. Impact on Man and Society'. ICSU Press 1983.

[26] Wenzel, G., Bapat, V. A. and Uhrig, H. In 'Plant Cell Culture in Plant Improvement'. (Ed K. L. Cortes) Plenum, New York. p. 337. 1982.

[27] Shepard, J. F. and Totten, R. E. *Plant Physiol.*, **60**, 313, 1977.

[28] Butenko, R. G., Kuchko, A., Vitenko, V. A. and Avetisov, V. A. Sov. *Plant Physiol.*, **24**, 660, 1977.

[29] Shepard, J. F., Bidney, D. and Shahin, E. *Science*, **208**, 17, 1980.

[30] Roest, S. and Bakelmann, G. S. *Potato Res.*, **23**, 167, 1980.

[31] Espinoza, N. O. and Dodds, J. H. *Plant Sci. Lett.* (in press) 1985.

[32] Scowcroft, W. R. and Larkin, P. J. In 'Better Crops for Food'. Pitman, London. p. 177. 1983.

[33] Larkin, P. J. and Scowcroft, W. R. *Theor. Appl. Gen.*, **60**, 197, 1981.

[34] Jones, M. G. K. In *Proc Planning Conference Innovative Techniques for Potato Propagation*. CIP, Lima, Peru. 1984.

[35] Sopory, S. K., Jacobsen, E. and Wenzel, E. *Plant Sci. Lett.* **12**, 47, 1982.

[36] Wenzel, G. and Foroughi-Wehr, B. In 'Cell Culture and Somatic Cell Genetics of Plants. Vol 1.' (Ed. I Vasil).

[37] Dodds, J. H. and Reynolds, T. L. *Z. Pflanzenphysiol.*, **97**, 271, 1980.

[38] Wang, P. J. and Hu, C. *Amer Pot. J.*, **59**, 33, 1982.

[39] Koda, Y. and Okasawa, Y. *Jap J. Crop Sci.*, **59**, 33, 1982.

[40] Hussey, G. and Stacey, N. J. *Ann. Bot.*, **48**, 787, 1981.

[41] Dodds, J. H. (Ed) 'Plant Genetic Engineering'. Cambridge University Press, 1985.

[42] Bengochea, T. and Dodds, J. H. *Outlook on Agriculture*, **12**, 16, 1983.

[43] Jaynes, J., Espinoza, N. O. and Dodds, J. H. In *Proc. 1st Intl Conf Plant Tissue Culture*, Brazilia, Brazil (in press) 1985.

[44] Jaynes, J., Espinoza, N. O. and Dodds, J. H. *Sci. American* (in press) 1986.

Reprinted from Trends in Biotechnology

elsevier
publications
CAMBRIDGE

REVIEWS

Plant protein improvement by genetic engineering: use of synthetic genes

J. M. Jaynes, M. S. Yang, N. Espinoza and J. H. Dodds

Methods now exist to construct genes coding for synthetic proteins enriched in essential amino acid content. The production of these synthetic proteins in potato tubers can improve the nutritive value of the potato and increase its importance as a basic food crop.

Protein nutrition

The biosynthesis of amino acids from simpler precursors is a process vital to all forms of life as these amino acids are the building blocks of proteins[1]. Organisms differ markedly with respect to their ability to synthesize amino acids. In fact, virtually all members of the animal kingdom are incapable of manufacturing some amino acids. There are twenty common amino acids which are utilized in the fabrication of proteins and essential amino acids are those protein building blocks which cannot be synthesized by the animal. It is generally agreed that humans require eight of the twenty common amino acids in their diet[2]. A nutritionally adequate diet must include a minimum daily consumption of these amino acids (Fig. 1).

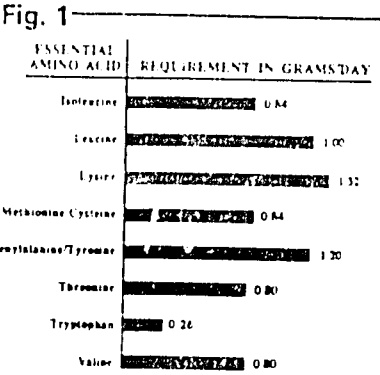
When diets are high in carbohydrates and low in protein over a protracted period, this results in deficiencies of essential amino acids and leads to a condition called 'Kwashiorkor' which is an African word meaning 'deposed child' (deposed from the mother's breast by a newborn sibling). This debilitating and malnourished state, characterized by a bloated stomach and reddish-orange discolored hair, is more often found in children than adults because of their great need for essential amino acids during growth and development. For normal physical and mental maturation, a balanced daily source of essential amino acids is a requisite. This is as important a feature of the diet as total protein quantity or total calorie intake.

Some foods, such as milk, eggs and meat, have very high nutritional values because they contain a high level of essential amino acids[3]. On the other hand, most foodstuffs obtained from plants possess a poor nutritional value because of their relatively low content of some or, in a few cases, all of the essential amino acids. Generally, the essential amino acids which are found to be most limiting in plants are isoleucine, lysine, methionine, threonine, and tryptophan (Fig. 2).

In the Western world few amino acid deficiency problems arise, primarily because the diet is composed of a mixture of a wide range of animal and plant proteins. In many developing countries, however, a single crop can be perhaps 80–90% of the total food intake. Rice, for example, is the major staple in Asia while potatoes are the staple in the Andean region of South America. With heavy dependence on plant protein from a single source, its essential amino acid composition becomes of critical importance. In these situations it would be highly beneficial to 'engineer' the plant to produce proteins with a balanced essential amino acid content. Several potential methods exist to achieve this objective. Firstly, utilization of conventional plant breeding techniques to improve protein quality; secondly, manipulation of the existing storage protein genes to increase levels of the essential amino acids; and thirdly, construction of synthetic genes which encode proteins enriched in essential amino acids for overall enhancement of the quality of total plant protein.

Although the first method has been applied with encouraging results in the case of maize[4] (i.e. the Opaque 2 mutant, so-called high lysine mutant) the screening techniques and effort involved make this an ineffective method. Besides, plant proteins are deficient in more than one of the essential amino acids which compounds the level of difficulty to achieve a nutritionally complete

Fig. 1

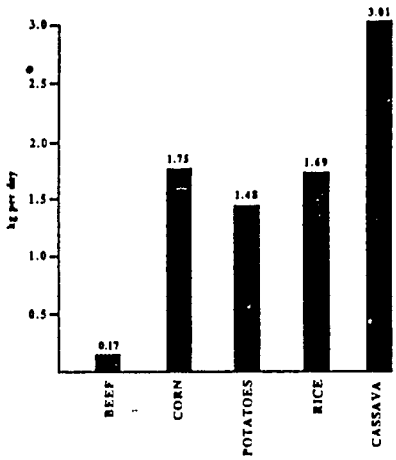


The daily essential amino acid requirements for a 20 kg child as listed are those necessary to maintain good health and normal growth and development as established by FAO. (A 90 kg adult male's daily essential amino acid requirement, to maintain good health, would be approximately the same – emphasizing the extreme importance of an adequate diet during early life.) The amino acid histidine has not been shown as it is generally regarded as being essential in humans for only the first three months of life. Also, the intake of cysteine and tyrosine can 'spare' (reduce) the requirements for methionine and phenylalanine, respectively.

J. M. Jaynes and M. S. Yang are at the Department of Biochemistry, 322 Choppin Hall, Louisiana State University, Baton Rouge, Louisiana 70803, USA. N. Espinoza and J. H. Dodds are at the International Potato Center, P.O. Box 5969, Lima, Peru.

© 1986, Elsevier Science Publishers B.V., Amsterdam 0166 – 9430/86/$02.00

Fig. 2



The lower quality of plant-derived proteins is apparent when one looks at the total amount of consumption necessary for a 20 kg child to receive 100% of his essential amino acid requirement and compare this value with the amount required from beef protein. The calculations assumed that the proteins were 100% bioavailable, so these values should be viewed as lower limits.

maize utilizing this method. The second method has been discussed previously in *TIBTECH*[5].

The innovative methods of genetic engineering offer a novel approach to modifying the essential amino acid composition of plant proteins and can thus increase their nutritive value. Our approach is not to try and modify the existing plant proteins, but rather to supplement them with new synthetic proteins which have a high content of essential amino acids. To produce a synthetic protein of known amino acid composition, a DNA fragment with an appropriate codon sequence can be produced using modern chemical techniques and introduced into various micro-organisms using recombinant DNA technology[6]. The cloning and expression of synthetic DNAs, containing repeated codons for a single amino acid, could be used as a means to supplement the essential amino acid content of plant protein[7]. However, because plant proteins are deficient in more than a single essential amino acid, the design of a useful synthetic protein must take into consideration the total amino acid composition of plant proteins.

Gene design and synthesis

It is now possible, utilizing machines, to synthesize DNA fragments with an individual length up to about 100 bases[8]. Using such a machine we have constructed, cloned and obtained expression of genes in bacteria which code for proteins with a high content of the essential amino acids found to be most deficient in plant-derived proteins[9]. This was done in bacteria first, in order to facilitate the analysis of the new genes and their protein products. The sequences for several of these synthetic gene fragments – the 'high essential amino acid encoding' gene (HEAAE-gene) – have been deduced and the particular encoded protein sequences were obtained by inspection of the genetic code (Fig. 3).

Fig. 3

Gene Fragment 1

Reading direction of top strand (A)->->->

```
AATTCGGGGATCGTAAGAAATGGATGGATCGTCATCCATTTCTTCATCCATTTCTTAC
    GCCCCTAGCATTCTTTACCTACCTAGCAGTAGGTAAAGAAGTAGGTAAAGAATG

GATCCATCCATTTCTTAAGAAATGGATGAAGAAATGGATGACGATCCATCCATTTCTT
CTAGGTAGGTAAAGAATTCTTTACCTACTTCTTTACCTACTGCTAGGTAGGTAAAGAA

CATCCATTTCTTCATCCATTTCTTACGATCAAGAAATGGATGAAGAAATGGATGAAGA
GTAGGTAAAGAAGTAGGTAAAGAATGCTAGTTCTTTACCTACTTCTTTACCTACTTCT

AATGGATGAAGAAATGGATGCATCCATTTCTTAAGAAATGGATGAAGAAATGGATGAA
TTACCTACTTCTTTACCTACGTAGGTAAAGAATTCTTTACCTACTTCTTTACCTACTT

GAAATGGATGACGATCGATCGTAAGAAATGGATGACGATCCATCCATTTCTTACGATC
CTTTACCTACTGCTAGCTAGCATTCTTTACCTACTGCTAGGTAGGTAAAGAATGCTAG

CCCG
GGGCTTAA
```

<-<-<- *Reading direction of bottom strand* (B)

Sequence of protein (A)->->->

```
  GlyAspArgLysLysTrpMetAspArgHisProPheLeuHisProPheLeuThrIleHisProPheLeu-
- LysLysTrpMetLysLysTrpMetThrIleHisProPheLeuHisProPheLeuHisProPheLeuThr-
- IleLysLysTrpMetLysLysTrpMetLysLysTrpMetLysLysTrpMetHisProPheLeuLysLys-
- TrpMetLysLysTrpMetLysLysTrpMetThrIleAspArgLysLysTrpMetThrIleHisProPhe-
- LeuThrIlePro
```

Sequence of protein (B)->->->

```
  GlyAspArgLysLysTrpMetAspArgHisProPheLeuThrIleAspArgHisProPheLeuHisPro-
- PheLeuHisProPheLeuLysLysTrpMetHisProPheLeuHisProPheLeuHisProPheLeuHis-
- ProPheLeuAspArgLysLysTrpMetLysLysTrpMetLysLysTrpMetAspArgHisProPheLeu-
- HisProPheLeuLysLysTrpMetAspArgLysLysTrpMetLysLysTrpMetThrIleHisProPhe-
- LeuThrIlePro
```

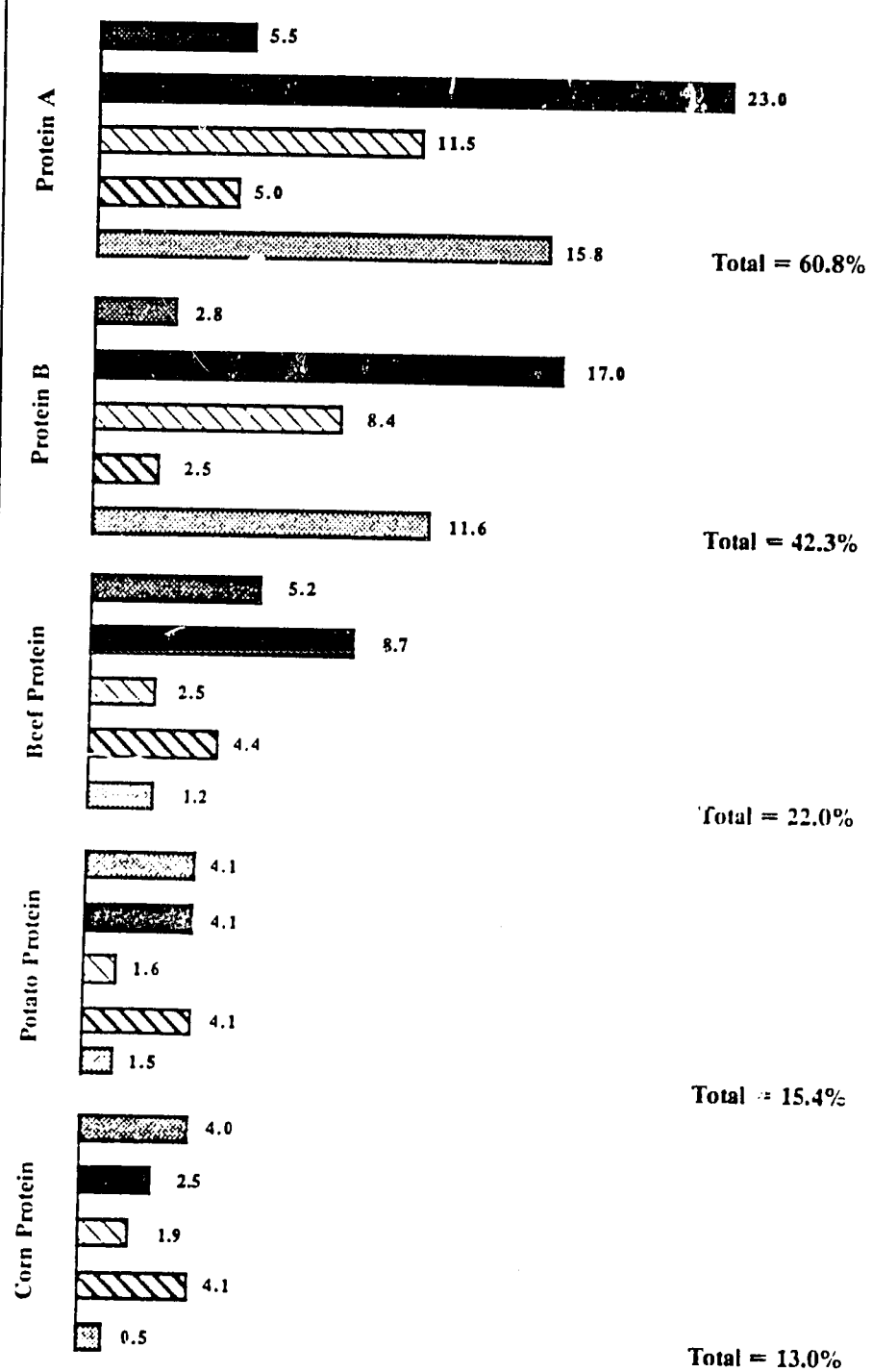
The nucleotide sequence of gene fragment 1 and the derived protein sequences. Gene fragment 1 yields two proteins designated A and B and one or the other will be produced depending upon the orientation of the gene when it is fused to the plant-gene promoter. In other words, the gene was constructed symmetrically so that a protein of high essential amino acid content would be produced no matter which DNA strand was read by the cell's protein synthesis machinery. Those amino acids highlighted are essential to the human and need to be consumed every day for good health. Protein A, the best so far analysed, is composed of about 80% essential amino acids.

Proteins A and B represent sequences derived from both possible reading directions of gene fragment 1. That is, gene fragment 1 was constructed symmetrically so that a protein, containing a high content of essential amino acids, would be produced no matter which strand of the synthetic DNA was ultimately read by the cell's protein synthesis machinery.

The composition of proteins A and B, for the five most deficient essential amino acids of plant-derived proteins, is compared with those found in proteins from several foods (Fig. 4). Beef protein has been shown for comparison purposes as it is composed of an unusually high content of these essential amino acids. Proper supplementation of plant-derived foods with proteins encoded by these synthetic gene fragments would markedly improve their essential amino acid balance.

This method of gene synthesis is flexible enough to produce proteins possessing any particular amino acid composition. Therefore, proteins could be specifically designed to supplement any desired animal feed or human food. It should be pointed out that the insertion of lysine at frequent intervals in these synthetic proteins provides numerous sites for proteolytic attack by trypsin (one of the main protein-degrading enzymes found in the digestive tract). This feature is important as it increases the bioavailability of the supplemental protein – the amount of amino acids absorbed from a particular dietary protein and used by the organism to make its own protein. The next major step has been the insertion of the synthetic genes into an important crop plant, the potato, using an *Agrobacterium*-based gene vector system.

Fig. 4



The percent composition found in the synthetic proteins A and B, for the five most deficient essential amino acids of plant-derived proteins, is compared with the content of proteins from some common foods. Synthetic protein A is composed of almost three times the content of these most important essential amino acids than is beef protein. ▒ Isoleucine: ■ Lysine: ▨ Methionine: ▨ Threonine: ▒ Tryptophan.

How the synthetic genes were introduced into potato plants

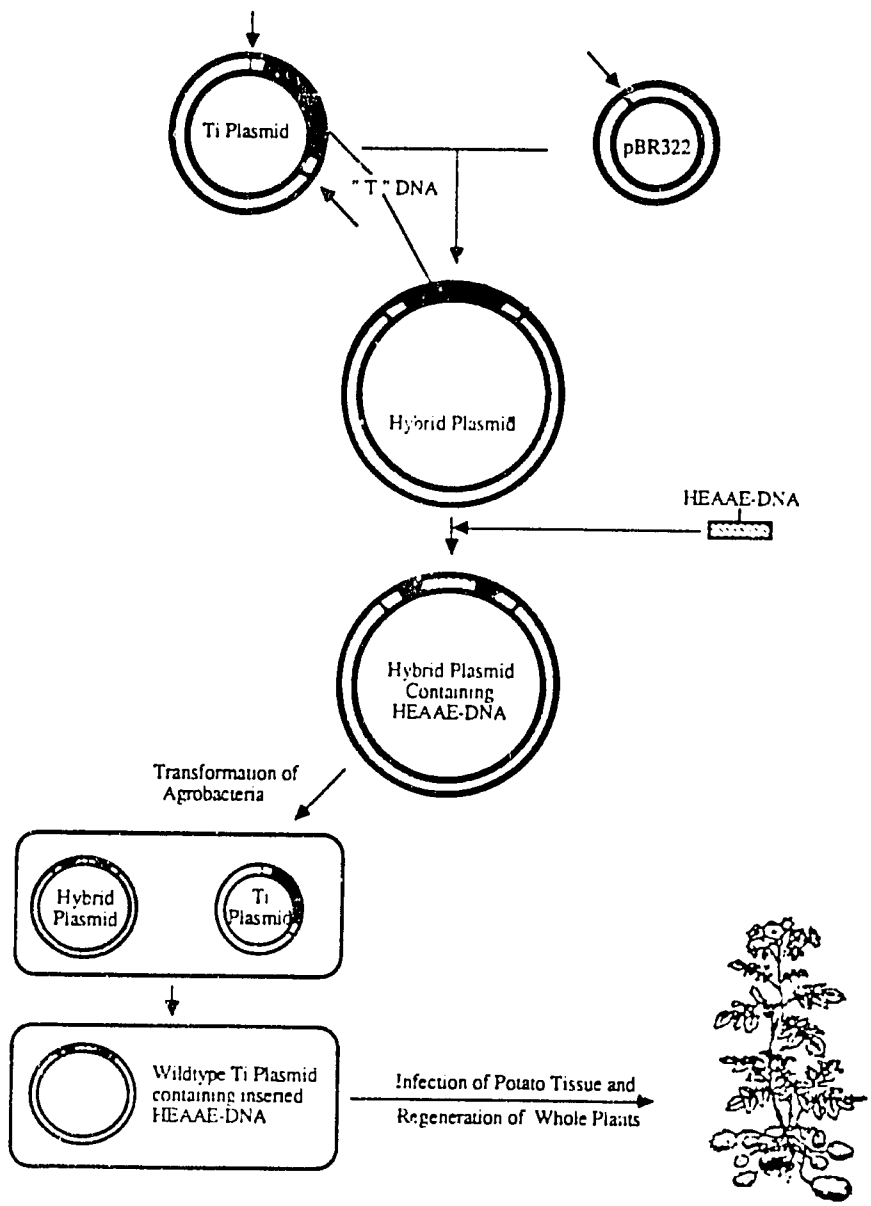
Plant breeding, the movement of desirable traits between plants by the use of the traditional techniques, has without question profoundly improved the important inheritable characters (yield, resistance to disease, etc.) of the world's major crop plants such as the potato. There are, however, certain limitations to these techniques. For example, it can take as long as ten years to introduce, select and establish a particular trait into a plant cultivar, and some traits are impossible to incorporate by these traditional techniques[10].

Genetic engineering offers the possibility of introducing a single trait, without altering other agronomically important characters of the cultivar

into a well accepted or traditional variety, allowing for a method of 'fine-tuning' specific genotypes. *Agrobacterium* spp., often regarded as Nature's first 'genetic engineers', allow the exploitation of recombinant DNA technology for plant improvement[11]. Several species have been shown to affect plant growth and development. *Agrobacterium rhizogenes* causes a proliferation of roots at the point of infection on the host plant and this response has been termed 'hairy root'. The etiology (expression of symptoms) of hairy root is quite distinct from that of crown gall (a type of plant tumor), which is caused by *Agrobacterium tumefaciens*. The genes causing changes in the growth characteristics of infected plants have been shown to be located on large plasmids in both *A. rhizogenes* and *A. tumefaciens*[12]. Most importantly, a discrete portion of this plasmid DNA (called T-DNA) is found to be incorporated into the genome of the infected plant, a process known as 'plant transformation'. This T-DNA determines several fundamental characteristics of transformed plant tissue[13]. For example, these plant cells will grow without adding phytohormones to the culture medium, which are normally necessary for cell growth in culture. Also, the transformed cells will synthesize unusual compounds called 'opines'. In addition, the plasmids confer to the bacterium the ability to metabolize the particular opine produced by the transformed plant tissue. The evolutionary significance of transformation is that *Agrobacterium* causes the plant to produce special substances (the opines) which only the bacteria can utilize as a food source; thus the bacteria have built a secure niche to promote their perpetuation at the expense of the plant.

One strategy for introducing foreign DNA into a plant, by means of *Agrobacterium* plasmids, is to use a small recombinant plasmid into which a known fragment of T-DNA has been inserted[14] (Fig. 5). This protocol can give rise to modified plant cells growing in culture. However, it is difficult to obtain healthy plants from these transformed cells. We have used a similar experimental strategy but employing an *A. rhizogenes* plasmid vector system in order to introduce the HEAAE-DNA into potato plant (Yang, M. S., Jaynes, J. M., Dodds, J. H. and White, F. F., unpublished). This procedure incites hairy or 'rooty' tumors and the infected root tissue can then be propagated. This appears to be a good method as the roots transformed with the modified T-DNA gave rise to plant cells which could be regenerated into intact plants (Fig. 6). The presence of HEAAE-DNA, its transcription into mRNA and translation into HEAAE-protein has been demonstrated in the potato by Southern transfer, 'northern blotting' and 'western' blotting, respectively. This method has proven to be very successful for the introduction and expression of HEAAE-DNA in transformed, regenerated potato plants (Espinoza, N., Yang, M. S., Jaynes, E. M., Dodds, J. H. and Schnorr, K. unpublished). A newer technique has emerged recently where *A. tumefaciens* plasmid

Fig. 5



Through the use of restriction enzymes, modified Ti plasmids can be constructed which contain foreign genes (in this case, the HEAAE-gene). These new plasmids can be introduced into Agrobacteria *and these strains can then be used to infect potato plants. The resultant transformed tissue can be regenerated into whole, reproductively viable plants.*

vectors have been constructed with the tumor-forming genes removed[15]. These so-called 'disarmed' vectors have allowed for the regeneration of plants from sections of the transformed leaf tissue. Transformed potato tissue employing this newer method has also been obtained.

Tissue culture of transformed potato plants

It is clear that techniques exist to insert the HEAAE-DNA into *Agrobacterium* plasmids; however, the controlled transformation of plants by the use of modified *Agrobacterium* spp. is dependent on two important features. Firstly, the bacterium must be capable of infecting the host plant. The usefulness of this infection process is limited, at the present time, to a few major crop plants such as the potato. Secondly, tissue culture techniques must be available for the regeneration of transformed plants. Cereals, such as rice, corn and wheat, are also quite low in essential amino acid content and it would be a significant advance to insert and obtain expression of HEAAE-DNA in these plants. However, cereals remain refractory to transformation by *Agrobacterium* and techniques do not exist to routinely regenerate whole cereal plants from single cells, a process which is extremely simple in the case of the potato[16]. Therefore, in order to genetically engineer cereals we must exploit new and different technologies.

The potato is a model plant for tissue culture and its plasticity of development allows intact plants to be regenerated from almost any plant part. It is possible to infect potato plants with *A. rhizogenes* (containing the HEAAE-DNA on a modified plasmid) and induce the formation of hairy roots; these roots can then be propagated and transformed plants regenerated. We have also regenerated transformed plants from leaf tissue using *A. tumefaciens* containing disarmed plasmid vectors.

In the potato plant, which is normally asexually propagated, there are no problems of segregation and loss of the inserted genetic information through meiotic recombination, although it is of interest to determine how this newly introduced gene (HEAAE-DNA) segregates and if it is transmitted sexually to the progeny.

Foreign DNA can be inserted at will into plants such as the potato. However, to ensure expression of a particular desirable gene, it must be inserted next to the proper control region of another already existing gene which has had its protein encoding part removed. These control regions, or promoters, usually found at the beginning of the gene, are vital switches attuned to the many influences which regulate the expression of the gene to its final protein product[17].

The potato, like many important plants, contains its food deposits in a specialized organ, in this case, a tuber. It would be of little import to improve the nutritional value of potato leaves as these are not consumed. Thus the ultimate goal of this research is to obtain high level expression of HEAAE-DNA only within the tuber (Fig. 7). This will increase the gene's value and importance as an inheritable genetic trait if this goal can be accomplished. It is important to have a method which can quickly analyse for the presence of the HEAAE-protein in the potato tuber. Techniques have now been developed which allow for the rapid (four weeks) induction of tubers *in vitro*[18]. These tubers are small, normally 3–5 mm in diameter but are morphologically and biochemically identical to field produced tubers (Fig. 8). This method will allow for speedy and efficient screening of the transformed regenerated plants for any increase in essential amino acid content of the tubers.

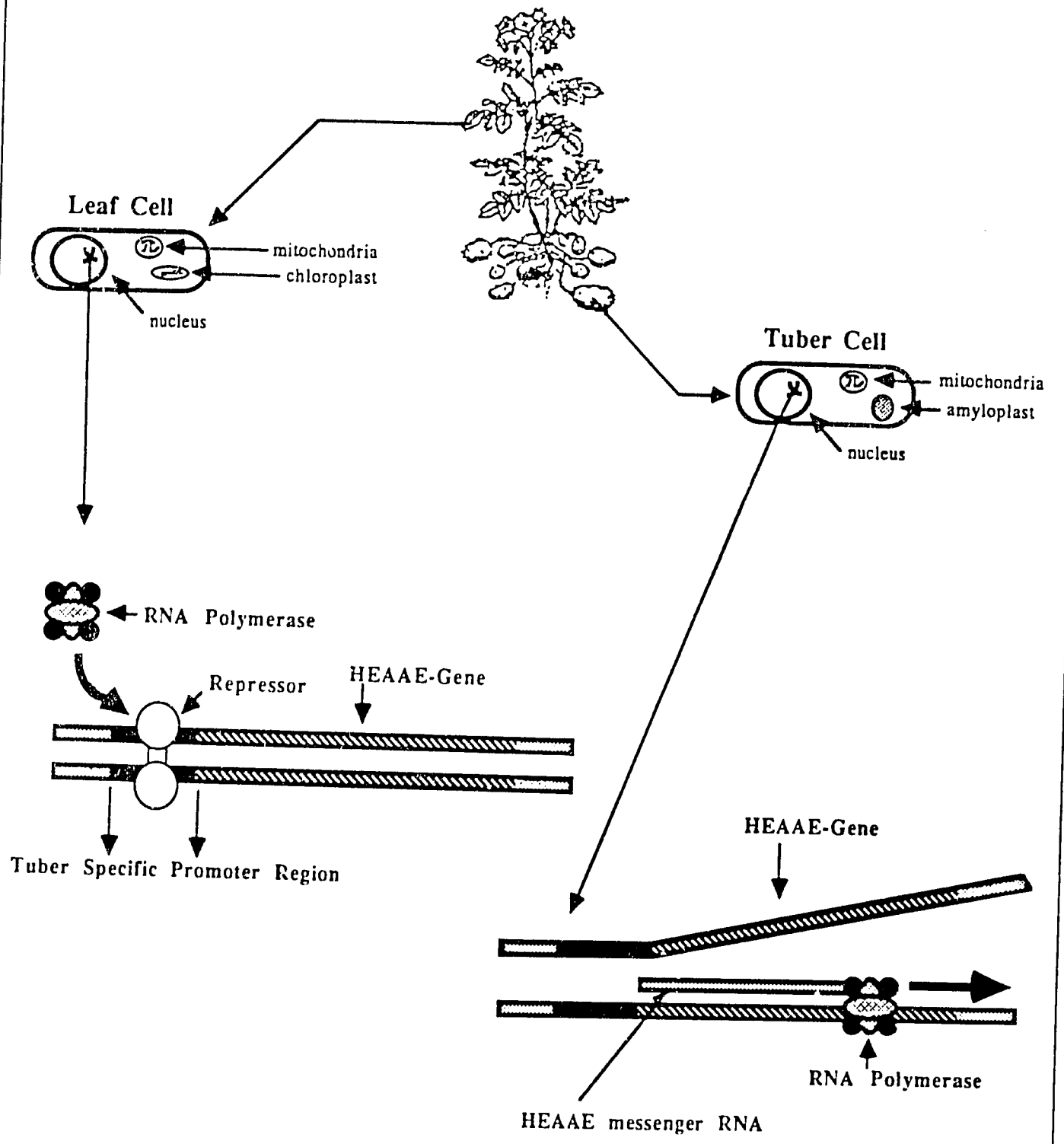
Fig. 6

Agrobacterium rhizogenes *incites hairy roots on* in vitro *propagated potato plants. Bacterial-free individual roots from these plants were placed on nutrient media to induce the formation of plantlets.*

Conclusions

The technology now exists to synthesize 'custom made' genes for proteins with an enriched essential amino acid content. We have been able to insert these genes into the potato and detect expression of the gene. An analysis of amino acid profiles of transformed and non-transformed plants is currently under study. Just how much HEAAE-protein will need to be produced within the tuber to make a significant impact on the essential amino acid content of the potato has yet to be ascertained.

Fig. 7



Modified Ti plasmids which contain foreign genes (e.g., the HEAAE-gene) can be constructed. The T-DNA region of the Ti plasmid was inserted into the E. coli *plasmid, pBR322. The pBR322 portion of this hybrid plasmid (i) permitted propagation of the hybrid in* E. coli *and (ii) bore an antibiotic resistance marker permitting subsequent selection of plasmid-containing clones. The HEAAE-DNA was then inserted into the T-DNA region of the hybrid plasmid. This new plasmid was then introduced into an* A. tumefaciens *strain carrying the unmodified* Agrobacterium *plasmid. Since these different plasmids contain homologous (similar) sequences, a rare double-recombination between the two plasmids will sometimes occur and result in a new plasmid in which the T-DNA harbors the insert of HEAAE-DNA. Strains of* Agrobacterium *containing the new plasmid can be identified and selected by their survival on media containing the antibiotic. These selected bacteria were then used to transfer the modified T-DNA (containing the HEAAE-DNA) into the plant genome.*

Fig. 8

In vitro tubers can be grown from a number of different cultivars and are remarkably similar in shape and color to those produced under field conditions. On average they are 5 mm in diameter. Plants grown in the field, derived from both sources of tubers, yield about the same amount of consumable tubers.

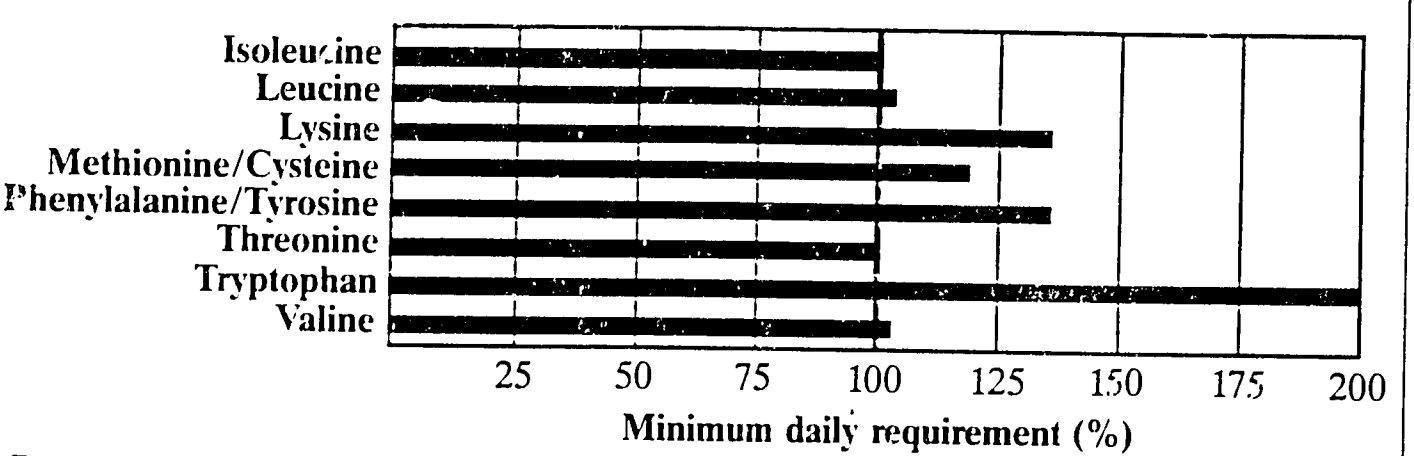
According to our calculations, if enough of the modified potato was fed to a 20 kg child to satisfy 20% of his calorific needs (600 g), then about 28% of the protein in the potato must be the genetically engineered HEAAE-protein (Fig. 9). Given the appropriate promoter sequence, we believe it should be possible to achieve this level of production. Certainly, any detectable increase would be significant. However, if we can achieve this level of expression of the protein then it is possible that the child would not need any additional protein and could exist solely on the modified potato with growth and development proceeding normally.

We have demonstrated that it is now possible to incorporate desirable genes into the potato via the methods of recombinant DNA technology, plant molecular biology and plant cell tissue culture. The major limitation to the large-scale application of these techniques for the improvement of the potato and other crop plants, is the general lack of knowledge about useful plant genes and how to identify, isolate, and purify them. This article has highlighted an innovative approach – if desirable genes cannot be located and purified, then they can be constructed synthetically. When applied to the genetic engineering of an economically important basic food crop with an agronomically significant synthetic gene we believe this will eventually culminate in a modified potato with superior nutritional qualities.

References

1 Doolittle, D. A. (1985) *Sci. Amer.* 253, 88–99
2 Arroyave, G. (1975) in *Protein-Calorie Malnutrition* (Olson, R., ed.), pp. 1–17, Academic Press
3 Hansen, R. G., Wyse, B. W. and Sorenson, A. W. (1979) *Nutritional Quality Index of Foods*, AVI Publishing
4 Mertz, E. T., Bates, L. S. and Nelson, O. E. (1964) *Science* 145, 279–280
5 Rao, A. S. and Singh, R. (1986) *Trends Biotechnol.* 4, 108–109
6 Gilbert, W. and Villa-Komaroff, L. (1980) *Sci. Amer.* 242, 74–94
7 Kangas, T. T., Cooney, C. L. and Gomez, R. F. (1982) *Appl. Environ. Microbiol.* 43, 629–636
8 Brown, E. L., Belagaje, R., Ryan, M. J. and Khorana, H. G. (1979) *Methods Enzymol.* 68, 109–151
9 Jaynes, J. M., Langridge, P., Anderson, K., Bond, C., Newman, C. W. and Newman, R. (1985) *Appl. Micro. Biotechnol.* 21, 200–205
10 Harlan, J. R. (1976) *Sci. Amer.* 235, 88–97
11 Albinger, G. and Beiderbeck, R. (1977) *Phytopathol. Z.* 90, 306–310
12 Kado, C. I. (1976) *Annu. Rev. Phytopathol.* 14, 265–308
13 Chilton, M. D., Saiki, R. K., Yadar, N., Gordon, M. P. and Quentier, F. (1980) *Proc. Natl Acad. Sci. USA* 77, 4060–4064
14 Chilton, M. D. (1983) *Sci. Amer.* 248, 50–59
15 Horsch, R. B., Fraley, R. T., Rogers, S. G., Sanders, P. R., Lloyd, A. and Hoffman, N. (1984) *Science* 223, 496–498
16 Espinoza, N. O., Estrada, R., Silva-Rodriguez, D., Tovar, P., Lizarraga, R. and Dodds, J. H. (1986) *Outlook Agric.* 15, 21–26
17 Schleif, R. (1985) *Genetics and Molecular Biology*, Addison-Wesley Publishing
18 Hussey, G. and Stacey, N. J. (1981) *Ann. Bot.* 48, 787–796

Fig. 9



The essential amino acid profile of the putative nutritionally complete modified potato tuber is shown above. The data presented assumes the consumption of 20% of the caloric requirement (600 g) of the modified potato tuber for a 20 kg child. The black rectangles are those essential amino acids provided by the HEAAE-protein showing that with the correct level of expression the essential amino acid deficiencies of the potato can be overcome. The potato would thus become a complete food.

CROP PLANT GENETIC ENGINEERING. SCIENCE FICTION TO SCIENCE FACT

By

John H. Dodds*
International Potato Center
P. O. Box 5969
Lima, Peru

and

Jesse M. Jaynes
Louisiana State University
Dept. of Biochemistry
Baton Rouge, Louisiana 70803-1806
U. S. A.

* To whom editorial comments should be sent.

INTRODUCTION

Recombinant DNA technology is a term that covers a wide range of biochemical techniques used to cut, splice and move DNA from one organism to another. The manipulation of genes in the test tube, more popularly known as genetic engineering began as a basic scientific study to learn more about gene expression and gene structure in bacteria. In the last 10 years the techniques of recombinant DNA technology have moved from the university research laboratory to the industrial production level. The techniques are applicable to all organisms and studies have been performed by genetic engineering on the genomes of viruses, bacteria, yeasts, animals and plants.

It is the latter, genetic engineering of plants which is to be covered in this article.

Let us look first at the major crop plants presently under study and later see the type of gene inserts that may lead to improvement of these crops.

THE WORLDS MAJOR CROP PLANTS

The importance of principal food crops can be defined in a number of ways. Table 1 shows the value of production of principal food crops in developing countries. Despite the enormous economic value of these crops many research scientists have directed their efforts to genetic engineering of model plant species such as petunia or tobacco. (1,2,3) The reason for choosing these model species is that they offer many advantages many crop plant species for basic scientific studies. In the case of tobacco and petunia the following criteria are all easily met.

(1) Conventional genetics and cytology well studied.

(2) Easy to manipulate and regenerate in vitro.

(3) Susceptible to infection by Agrobacterium.

(4) Short generation time (seed to flower).

(5) Many diploids available.

Let us now look at the major crop plant species and see the limitations that exist.

(A) Cereals.

The major cereal crops such as rice, maize and wheat are able to meet some of the criteria needed to facilitate genetic engineering studies, for example, well studied cytology and a relatively short generation time. However, many limitations exist with all the cereals.

1) Regeneration of whole plants from single cells and protoplasts has been reported recently in a few cases with certain limited genotypes(4) it is fair to say however that no routine regeneration system exists for cereals.

2) Cereals are not susceptible to infection by Agrobacterium, this means that the standard A. tumefaciens or A. rhizogenes vector cannot be used. To overcome this recent experiments have shown that electroporation (electrical treatment of protoplasts) will allow the direct uptake of plasmid constructs. However point 1)

then comes back into play and it is impossible to regenerate whole plants containing these inserts. Transformed cereal callus cultures are of little direct agricultival importance!

Experiments are in progress in many laboratories to:

(a) Increase frequency of protoplast regeneration or (b) attempt direct uptake of plasmids not into protoplasts but into more organized structures such as zygotes or developing somatic embryos. Care must be exercised however that chimeras can result by this methods.

Cereals are normally propagated by seed, this implies that gene inserts made by genetic engineering into the nuclear genome will undergo segregation during seed production. This will add to the complexity of the genetics of the gene inserts of interest. This problem is normally not encountered with the major vegetatively propagated food crops.

(B) Potato

The potato has already been described as a model crop plant for tissue culture studies (5). It can be regenerated with relative ease from almost any type of explant. The potato is also susceptible to infection by the vector *Agrobacterium tumefaciens* or *A. rhizogenes*. A later section covers in detail the methodology of gene synthesis regeneration of plants and tests for gene insertion and expression.

(C) Sweet Potato

Of the different worldwide important crops, sweet potato ranks fifth in both economic importance and contribution to the diet in calories and proteins, in developing countries. In comparison with other basic food crops little basic research has been carried out on sweet potatoes. Results have been described in the literature showing that techniques exist for regeneration and propagation of sweet potatoes in vitro (6,7). Preliminary experiments carried out at the International Potato Center have shown that sweet potatoes are amenable to infection with Agrobacter species and we have been able to successfully transform sweet potatoes by insertion of a synthetic gene coding for a protein rich in essential acids (8).

THE POTATO AS A MODEL FOR CROP PLANT GENETIC ENGINEERING STUDIES.

Inoculation with Agrobacterium.

The potato is amenable to infection with Agrobacterium. If the stem of in vitro potato plantlets are wounded with a sterile needle and inoculated with Agrobacterium rhizogenes, after a few days hairy roots will form. These hairy roots are characteristic of the malformation caused by the bacterium and are formed as a result of hormonal changes at the cellular level (Figure 1). Likewise inoculation with A. tumefaciens will cause the formation of a tumor at the point of inoculation.

These hairy roots can be excised and put into culture, on an appropriate medium plantlets can be regenerated from the cultured transformed roots (Figure 2) (9). These plantlets can be propagated in vitro using standard single node propagation methods (10).

In the case of potato it is possible to induce the formation of storage tubers in vitro ii. This is almost the only case in which the food component of the plant can be induced under in vitro conditions (Figure 3). This means that evaluation of the full growth cycle can be carried out in vitro.

WHAT GENES SHOULD BE INSERTED? SYNTHETIC VERSUS NATURAL GENES.

Once it was established that for certain plant species Agrobacterium plasmids offered an excellent system for gene insertion the next problem was which genes to insert. The answers to that question came quickly, improved nutritional qualities, disease resistance, pest resistance, photosynthetic efficiency and many others. However, it also became clear that it is one thing to have a reproducible system for gene insertion but quite another to have genes to insert. Our knowledge of methods for gene identification and purification are still poor, so in some cases a different approach has been taken, that of using synthetic gene construction.

The laboratory synthesis of DNAs began approximately 25 years ago. Khorana and co-workers have been at the forefront of oligonucleotide synthesis for many years. Their work culminated in 1979, with the synthesis of the structural gene for tyrosine RNA, some 200 base pairs in length. This was followed a few years later by an English group who accomplished the chemical synthesis of the gene for human interferon. This was no small feat as the gene fragment was 517 base pairs in length.

Since this time, a number of advancements have been made, including mechanization of this rather tedious chemical coupling process. Most of the available machines utilize the method developed by Caruthers and co-workers, commonly called the phosphite method. Utilizing one of the commercially available machines, it is possible to make an oligonucleotide of 50 bases in length with good yield in about 18 hours of machine time.

A plant gene is like all other genes, simply a string of nucleic acid bases. It follows, therefore, that if a number of bases are added together chemically, in the proper order, then what one is doing is to synthesize a gene. What sorts of genes would be useful to synthesize and insert into plants?

This article will discuss several types of synthetic genes. In the first instance, our interest may be to obtain the product of the new gene, i.e. a synthetic protein. Later, synthetic genes might play a regulatory role in the cell, for example, disease resistance.

(A) Genes Encoding Nutritional Proteins

To overcome deficiency of essential amino acids in plant proteins, we have synthesized, utilizing a DNA synthesis machine, genes which code for proteins high in essential amino acids. These proteins were designed to be highly bioavailable (digestable by the animal gut) and have been shown to be biologically stable. Figure 4 illustrates how these synthetic genes were constructed and clones into bacteria. We have verified the structure of several of these genes and the sequence of one of them is shown in Figure 5 (12).

It is worth noting that if the gene is inserted "upside down" it would still produce a protein rich in essential amino acids, since the gene was designed to be relatively symmetrical. In figure 6, the amino acid profiles encode a by the synthetic gene fragment I are compared with those found in lactalbumin and zein. Lactalbumin is a high quality milk protein and zein is the poor quality major storage protein of maize kernals. It can be seen that proper supplementation of plant proteins with those derived from these synthetic genes would markedly improve the balance of their most limiting essential amino acids. This method of gene synthesis is flexible enough to produce proteins possessing a particular amino acid composition specifically designed to supplement any desired feed of foodstuff. This synthetic gene has been inserted into Agrobacterium plasmids using an intermediate plasmid as a carrier.

The synthetic gene was then inserted into potato using the Agrobacterium inoculation system previously described, and we have been successful in obtaining insertion and expression of this synthetic gene.

The above mentioned results are an excellent demonstration of how custom made genes can be synthesised and used to affect nutritional qualities of a crop plant.

Many naturally existing nutritionally rich plant proteins have been analyzed and cDNA clones isolated from these, some of these genes may be useful for specific needs, for example the storage protein of brazil nut has an exceedingly high content of methionine (more than 25%).

Bean protein has long been proposed as a highly rich source of proteins and recent experiments have shown it is possible to obtain expression of cDNA clones of phaseolin in other plants such as tobacco (13). Experiments are also in progress to modify existing proteins such as bean proteins to increase their nutritional value and then use these as gene inserts.

In this area we believe that both synthetic and natural genes have a useful role to play in crop plant improvement.

(B) Disease Resistance Genes

(i) Viruses and Viroids.

It has recently been found that bacteria regulate expression of some genes in a rather novel way. Under conditions where the cell would repress the biosynthesis of a particular protein an additional level of control is exerted. This new type of control is called "micRNA" control (mRNA-interfering complementary RNA). This micRNA is complementary to the 5′ end of the gene and when it is produced has the ultimate effect of reducing the amount of protein produced by messenger RNA by annealing to the mRNA, thus removing it from normal protein synthesis.

This mode of gene regulation may offer a revolutionary means to control the expression of viruses in animals and plants, and therefore, the diseases they cause. Animals and plants have evolved very precise and elegant mechanisms which allow the regulated expression of their genes. Many viruses have "co-evolved" in order to exploit the eukaryotic cell by mimicking the general structure of plant and animal genes. Of course, the end result of this is disease which, in the case of vertebrates, is combated by the vastly complex immune system. But, many viruses can eventually circumvent this response (by slightly altering a surface protein, for

example, thus making the virus "invisible" to the immune system) and cause persistent infections or totally overwhelm the system (e.g. the AIDS virus) and cause sickness and/or death of the animal. Plants apparently do not possess a sophisticated disease resistance mechanism like the immune system and may be viewed to be more at risk to infection. But, by the same reasoning, so are the viruses -- they are dependent on the animal and plant system of gene regulation and expression to synthesize and translate their own genes. This dependency, in our view, is the key to the control of virus disease.

We believe that the above micRNA system may be used to block viroid replication. Viroids are single-stranded, circular ribonucleic acids (RNAs) of a few hundred nucleotides. Viroids are the smallest self-replicable structures known and represent the lowest level of life. They are the causative agents of a number of plant disease and elicit mild to lethal symptoms depending upon the fine structure of the viroid and the susceptibility of the host plant.

A great deal of effort is now being expended to elucidate the general mechanisms of replication and etiology of disease symptoms of the viroids. Indeed, some information has been obtained which allows one to construct a possible mechanism of replication of the viroid within the plant cell.

Figure 7 illustrates a scheme for replication of the viroid which causes a disease in potatoes: Potato Spindle Tuber Viroid (PSTV). What is significant is the fact that in order for replication to occur, the host cell RNA polymerases must recognize and bind to the viroid in order for initiation and subsequent replication of the viroid to occur. Thus, if we endowed plants with the ability to produce regulatory RNAs complementary to specific regions of the viroid molecule this would block replication.

(ii) Bacteria.

Similarly genetic engineering may be a useful way of conferring bacterial disease resistance in potato. Bacterial diseases of potatoes such as soft rot (Erwinia carotovora) and bacterial wilt (Pseudomonas solanacearum), cause potato losses in developing countries amounting to hundreds of millions of dollars. As the potato is increasingly grown in warm tropical climates the problems of bacterial diseases become of even greater importance. Until recently there has been only one source of bacterial wilt resistance available to potato breeders. This, coupled to the enormous range of bacterial races that exist, makes conventional breeding for bacterial resistance difficult. We are therefore looking to genetic engineering as an alternative method to confer resistance. To do this we may be able to make use of a range of bactericidal proteins in other organisms.

One of these organism is the silk moth (Hyalophora), in which the pupae respond to bacterial infection by the synthesis of about 15-20 antibacterial proteins. From these newly synthesized proteins have been purified, lysozyme, the antibacterial protein found in egg white and human tears, and two other classes of antibacterial peptides called cecropins and attacins. Lysozyme is effective in limiting some growth in a broad spectrum of bacteria including Erwinia and Pseudomonas. While a great deal of work remains to determine the exact mode of action of these toxins, it is clear that they attack only bacteria and do not affect higher plant or animal cells. These antibacterial proteins isolated from the silk moth are thus an attractive genetic system to incorporate into the genome of potato plants to protect them from diseases such as Erwinia and Pseudomonas.

(C) Pest Resistance Genes

Insect attack of food crops can lead to tremendous losses either by direct destruction of the plants by the insects, or by causing insect damage that allows the infection and spread of other pathogens. Any possible means to increase a plant's tolerance to insect damage would thus be of significant value, especially to farmers in the developing world, who often lack the financial resources to protect their crops.

Several commercial laboratories are exploring the use of genetic engineering with a natural insecticide to protect plants from insect damage. This natural insecticide, isolated from Bacillus thuringensis, is a protein that forms a high molecular weight crystal. These crystals are toxic to the larvae of a number of lepidopteran insects. Work is currently in progress at the International Potato Center to insert the gene for thuringensis toxic protein into potato plants. In addition to this toxin, we are also looking into the possibility of using an enzyme called chitinase as a natural anti-insect compound. The chitinase enzyme will attack the skeleton and gut of the insect allowing it to contract bacterial infections leading to the eventual death.

Perhaps the use of both of these constructions in tandem would be a useful way of conferring insect resistance.

REFERENCES

1. Greenwood J.S., Chrispeels, M.J. 1985. Correct targeting of the bean storage protein phaseolin in the seeds of transformed tobacco. Plant Physiol. 79: 65-71
2. Sun, M. 1986. Engineering crops to resist weed killers. Science 231: 1360-1361.
3. Fraley, R.T., Rogers, S.G.; Horsch, R.B.; Sanders, P.R.; Flick,J.S; Adams, S.P.; Bittner, M.L.; Brand, L.A.; Fink, C.L.; Fry, J.S.; Galluppi, G.R.; Goldberg, S.B.; Hoffmann, N.L.; Woo, S.C. 1983. Expression of Bacterial Genes in Plant Cells. Proc. Natl. Acad. Sci. USA (80): 4803-4807.
4. Fujimora, T.; Sakurai, M.; Akagi, H., Negishi, T.; Hirose, A. 1986 Regeneration of Rice Plants from Protoplasts Proc. 6th International Congress of Plant Tissue and Cell Culture JAPTC Minnesota PP. 161.
5. Espinoza, N.O.; Estrada, R.; Silva-Rodríguez, D.; Tovar, P.; Lizarraga, R.; Dodds, J.H. 1986 Outlook on Agriculture is (1): 21-26.
6. Carswell, G.K.; Locy, R.D. 1984. Root and Shoot Initiation by leaf, stem and storage root explants of sweet potato. Plant cell tissue organ cult 3: 229-236.
7. Litz, R.E.; Conover, R.A. 1978. In vitro propagation of sweet potato. Hortscience 13 (6): 659-660.
8. Jaynes, J.M.; Yang, M.S.; Espinoza, N.; Dodds, J.H. 1986. Plant protein improvement by genetic engineering: Use of synthetic genes. Tibtech (4): 314-320.
9. Espinoza, N.O.; Dodds, J.H. 1985. Adventitious shoot formation on cultured potato roots. Plant science (41): 121-124.
10. Espinoza, N.; Estrada, R.; Tovar, P.; Bryan, J.; Dodds, J.H. 1984 Tissue Culture Micropropagation, Conservation and Export of potato germplasm. Specialized Technology Document 1. International Potato Center, Lima, Peru.

11. Estrada, R.; Tovar, P.; Dodds, J.H. 1986 Induction of in vitro tubers in a broad range of potato genotypes - plant cell tissue organ cult 7: 3-10.

12. Jaynes, J.M.; Langridge, P.; Anderson, K.; Bond, C.; Sands, D.; Newman, C.W.; Newman, R. 1985. Construction and expression of synthetic DNA fragments coding for polypeptides with elevated levels of essential amino acids. Eur. J. Microbiol. Biotechnol. (21): 200-205.

13. Goldberg, R.B. 1986. Expression of soybean seed protein genes in transformed tobacco plants. Proc. 6th International Congress of plant tissue and cell culture. IAPTC Minnesota pp 259.

14. Crawford, M. 1986. Test of tobacco containing bacterial gene approved. Science 233 (4769): 1147.

ACKNOWLEDGEMENT

We wish to thank the US-AID for funds to support research on genetic engineering of potato.

LEGENDS TO FIGURES

Table 1

Table showing production and farmgate value of principal basic food crops in developing countries.

Figure 1

'In vitro plantlet of potato, wounded and inoculated with Agrobacterium rhizogenes. After approx 10 days 'hairy roots' form at the site of inoculation.

Figure 2

'Hairy roots' are excised and placed into regeneration medium. After approximately the month of culture plantlets regenerate from the epidermis of the cultived roots.

Figure 3

In vitro tuber can be induced on potato plantlets propagated in vitro, plantlets of the clone desireé were treated with cycocel (CCC) to induce the formation of tubers. Mature tuber are approximately 5mm in diameter.

Figure 4

Schematic representation of the steps involved to insert synthetic genes into Agrobacterium rhizogenes.

Figure 5

Base sequence of a synthetic protein coding gene (SP47) made on a DNA synthesis machine (b) above, is translated.

Figure 6

Profiles of essential amino acids in the synthetic proteins, both reading directions, as compared with other know nutritional protein sources.

Figure 7

Schematic representation of the mechanism of viroid replication. Small synthetic fragments (mic RNA) may serve to block viroid replication.

CIP CIRCULAR

INTERNATIONAL POTATO CENTER ISSN 0256-8632 Vol. 15, No. 1, March 1987

CIP Collaborative Research on Tissue Culture and Genetic Engineering for Potato Improvement

John H. Dodds[1]

From its foundation, the International Potato Center (CIP) has had a policy of collaborative research with other institutions to use the comparative advantage of each institution to pursue research on potato improvement. These types of collaborative agreements have allowed CIP to reach research objectives more rapidly and at low cost. Over the past few years, several such collaborative arrangements have been adopted by CIP to develop new areas of research in plant tissue culture and genetic engineering.

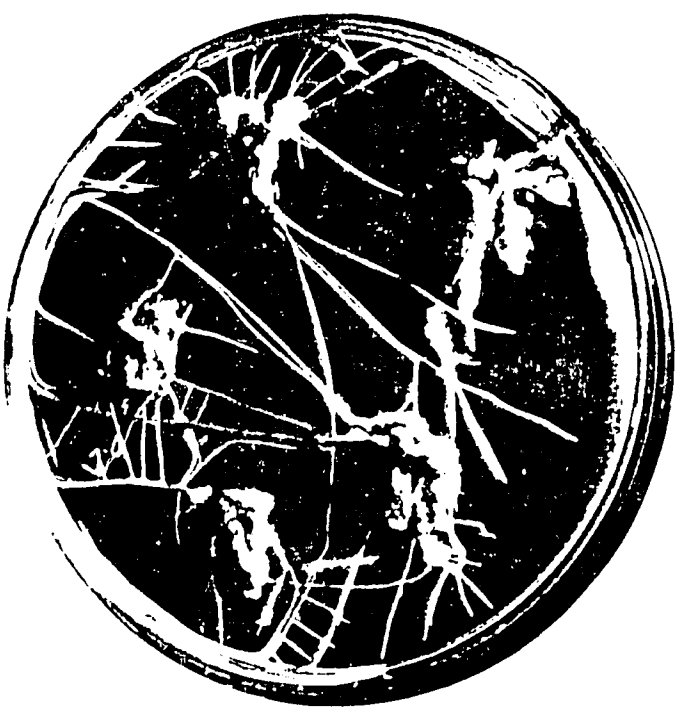
Plant *biotechnology* is an area where CIP has been active for many years, in fact many projects that would be considered biotechnology research in other institutions are already part of the routine work in CIP's tissue culture laboratory. This would include, for example, germplasm conservation and distribution in vitro of pathogen-tested materials (Schilde-Rentschler and Schmiediche, 1984; Tovar et al., 1985).

In recent years CIP has developed a number of new and exciting areas of research in tissue culture and genetic engineering in collaboration with many research institutions in different parts of the world. Some of these research activities are still in the initial phase and will need several more years of development until the resulting technologies can be transferred to practical use in farmers' fields. It is important, however, to explain how this type of research is progressing, what CIP's objectives are, and how this type of work will help CIP's current research programs and research within national potato programs. In some cases, even though the technologies themselves may be inappropriate for direct transfer to national programs, the improved germplasm resulting from these technologies may be of fundamental importance in breeding, leading to the release of new improved materials for national programs.

Training courses on different aspects of plant biotechnology, i.e., tissue culture, have been held over the last five years at CIP headquarters in Lima, in various countries such as Turkey, Vietnam, and Venezuela, and in collaboration with other agricultural research institutions.

Genetic Engineering

In collaboration with Dr. Jesse Jaynes of the Biochemistry Department at Louisiana State University, United States, CIP has developed a project to insert synthetic genes into potato plants. In this project we use *Agrobacterium* plasmid vectors



A petri dish containing roots formed at the site of inoculation of an in vitro plantlet (potato clone K-2). The roots were induced by inoculating the plantlet with *Agrobacterium*. Genetically engineered plants can be regenerated from these cultured roots.

[1] J. H. Dodds, Tissue Culture Specialist, International Potato Center, P.O. Box 5969, Lima, Peru.

CIP CIRCULAR

Published quarterly in English and Spanish by the International Potato Center (CIP)
P.O. Box 5969
Lima, Peru

Linda W. Peterson, Editor

CIP is a nonprofit, autonomous, scientific institution established by agreement with the Government of Peru for developing and disseminating knowledge for greater use of the potato as a basic food. CIP is supported by the Consultative Group on International Agricultural Research (CGIAR), which is an informal association of governments, private foundations, and international and regional organizations, dedicated to supporting a system of agricultural research centers and programs around the world. The information and conclusions reported in this publication do not necessarily reflect those of any of the aforementioned agencies, foundations, or governments.

CIP Regional Headquarters

Region I—Oscar S. Malamud
Andean Latin America
Apartado Aéreo 92654
Bogotá 8, D.E., Colombia
Tel: 2819468

Region II—Oscar Hidalgo
Non-Andean Latin America
c/o Centro Nacional de Pesquisa de Hortaliças
Caixa Postal D7-0213
70 359 Brasília, D.F., Brasil
Tel: (061) 556-2384

Region III—Sylvester Nganga
Tropical Africa
P.O. Box 25171, Nairobi, Kenya
Tel: 592-206

Region IV—Sidki Sadik
Near and Middle East
International Potato Center
P.O. Box 2416, Cairo, Egypt
Tel: 725-785

Region V—Roger Dorthaoui
North and West Africa (Francophone)
11 Rue des Orangers
2080 Ariana, Tunis, Tunisia
Tel: 715-047

Region VI—Mahesh D. Upadhya
South Asia, c/o NBPGR
Indian Agric. Res. Institute Campus
New Delhi, 110012, India
Tel: 588-055

Region VII—Peter Wander Zeng
Southeast Asia, c/o IRRI
P.O. Box 933, Manila, Philippines
Tel: 85-0015

Region VIII—Song Bo Fu
China, c/o The Chinese Academy of Agricultural Sciences
Bei Shi Diao Rd. No. 30
Beijing, People's Republic of China
Tel: 894-978

(Espinoza et al., 1986, 1987), a system of gene transfer now used by several laboratories worldwide, including laboratories in Europe, United States, Brazil, Mexico, and India. The primary objective of the project is to enhance the nutritional value of the potato by obtaining the supplementary production of a synthetic protein rich in essential amino acids. The synthetic protein is produced from a synthetic gene that can be synthesized by machine in the laboratory of Dr. Jaynes.

Through this research collaboration, CIP has been successful in inserting the synthetic gene into potato plants. We have also proved that the gene is transcribed and produces a corresponding messenger RNA (MRNA) molecule, which is then translated in the plant to produce the synthetic protein (Fig. 1 shows these translational steps). This synthetic gene project has proved for CIP that the technology now exists to genetically engineer genes into the potato (Fig. 2). Much more research, as well as the appropriate legislative measures, will be needed before these genetically engineered plants can be released to national potato programs.

On the basis of the success with the synthetic protein gene, CIP is directing attention to the possible use of genetic engineering to give the potato plant resistance to pests and diseases. Through collaborative links, CIP hopes to genetically engineer potato plants for resistance to viroid and virus infection by inserting gene sequences that will interfere with virus and viroid replication. This approach would allow potato plants to be grown for more generations in the field before degenerating with virus infection. CIP's primary interest is to give resistance to potato spindle tuber viroid (PSTV) and potato leafroll virus (PLRV).

CIP is also attempting to engineer resistance in the potato to diseases caused by bacteria such as *Pseudomonas* and *Erwinia*. For these experiments, we are using genes that have been purified and code for proteins with potent antibacterial activity. A genetic engineering approach to bacterial disease resistance may allow us to develop clones with resistance to both *Erwinia* and *Pseudomonas* in a given clone without major changes of the existing agronomically acceptable characters.

A

```
AATTCGGGGATCGTAAGAAATGGATGGATCGTCATCCATTTCTTCATCCATTTCTTAC
     GCCCCTAGCATTCTTTACCTACCTAGCAGTAGGTAAAGAAGTAGGTAAAGAATG

GATCCATCCATTTCTTAAGAAATGGATGAAGAAATGGATGACGATCCATCCATTTCTT
CTAGGTAGGTAAAGAATTCTTTACCTACTTCTTTACCTACTGCTAGGTAGGTAAAGAA

CATCCATTTCTTCATCCATTTCTTACGATCAAGAAATGGATGAAGAAATGGATGAAGA
GTAGGTAAAGAAGTAGGTAAAGAATGCTAGTTCTTTACCTACTTCTTTACCTACTTCT

AATGGATGAAGAAATGGATGCATCCATTTCTTAAGAAATGGATGAAGAAATGGATGAA
TTACCTACTTCTTTACCTACGTAGGTAAAGAATTCTTTACCTACTTCTTTACCTACTT

GAAATGGATGACGATCGATCGTAAGAAATGGATGACGATCCATCCATTTCTTACGATC
CTTTACCTACTGCTAGCTAGCATTCTTTACCTACTGCTAGGTAGGTAAAGAATGCTAG

CCCG
GGGCTTAA
```

B

```
GlyAspArgLysLysTrpMetAspArgHisProPheLeuHisProPheLeuThrIleHisProPhe
LeuLysLysTrpMetLysLysTrpMetThrIleHisProPheLeuHisProPheLeuHisProPheLeuThr
IleLysLysTrpMetLysLysTrpMetLysLysTrpMetLysLysTrpMetHisProPheLeuLysLysTrp
MetLysLysTrpMetLysLysTrpMetThrIleAspArgLysLysTrpMetThrIleHisProPheLeuThr
IlePro
```

Fig. 1. (A) A synthetic DNA sequence coding for a protein rich in essential amino acids that can be translated to give the protein molecule shown in (B).

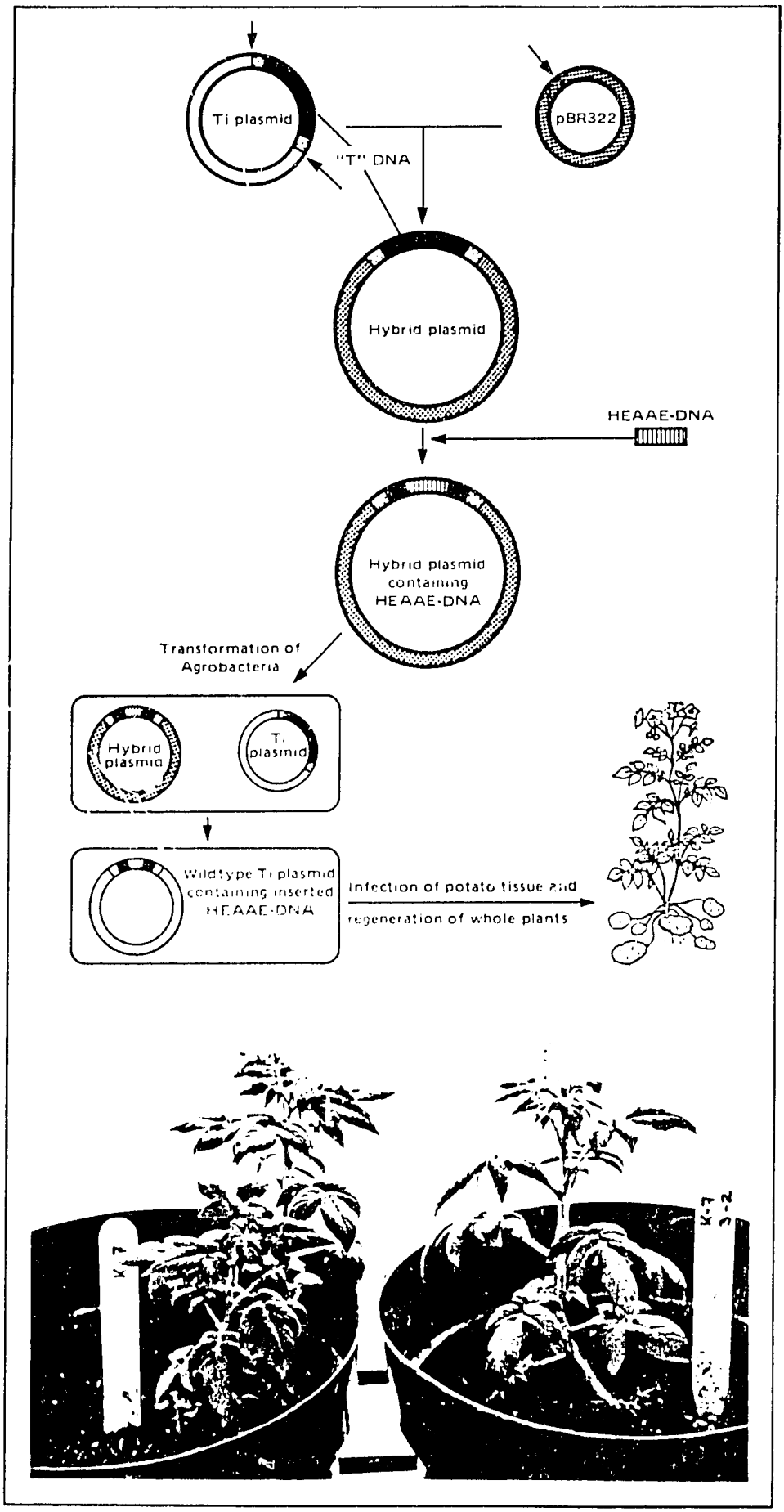


Fig. 2. *Above:* The diagram shows the steps followed in the genetic engineering of potato plants. A hybrid plasmid is constructed, the foreign gene is inserted, and the plasmid is introduced into *Agrobacterium*. The *Agrobacterium*, containing the foreign gene, will be used to inoculate the plant, in which the synthetic protein is to be produced. *Below:* two pots containing potato clone K-7: on the *right* is the genetically engineered plant containing the synthetic gene, and on the *left* is the control plant (non-engineered).

Protoplast Fusion

Protoplasts are "naked" plant cells in which the outer rigid cell wall has been removed by enzymic digestion. The absence of the cell wall makes it possible to fuse together protoplasts to produce hybrids that cannot be produced by conventional breeding. The potential of using this technology to produce hybrids between sexually incompatible species is an idea that has been discussed by tissue culture technologists for decades; however, to date, no agronomically useful fusion product has been produced.

In collaboration with Professor Esra Galun of the Weizmann Institute in Israel, CIP is developing a potato protoplast fusion program with a different objective and methodology. The idea is to transfer the nucleus of one genotype to the cytoplasm of another genotype (donor-recipient approach). This technique has proved useful in the transfer of cytoplasmic characters, particularly cytoplasmic male sterility in tobacco (Galun et al., 1981, 1983)—we hope to do the same with potato. The diagram in Figure 3 shows the principle of the donor-recipient method. The result of the fusion on the two protoplasts is that the nuclear genome of one clone is transplanted into the cytoplasmic genome of the second clone. This technology should allow us to produce female parent lines with male sterility; thus hybrid true potato seed can be produced without the need of emasculating female plants.

Anther Culture

The ability to regenerate haploid plants from pollen grains is one of the great success stories of plant tissue culture. In a collaborative research project between CIP and Dr. Andrea Sonnino of ENEA, the Italian Nuclear Research Agency, reasons are being investigated as to why in some potato genotypes it is easy to obtain dihaploids while in other genotypes it is impossible. This so-called *tissue culture ability* (TCA) (Sonnino, 1984; Wenzel and Uhrig, 1981) has been shown to be under genetic control, and it may now prove possible to move this characteristic from one genotype to another. This TCA also appears to control other aspects of tissue culture regeneration, including regeneration from somatic tissues such as leaves and stems.

Many experiments involve the regeneration of potato plantlets from cultured explants; for example, in genetic engineering experiments, plantlets are often

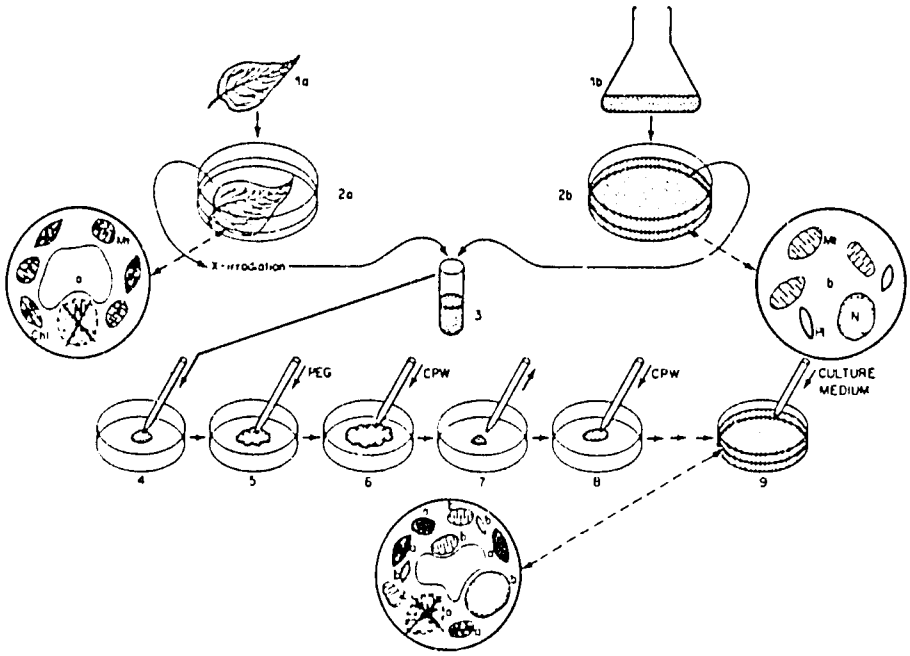


Fig. 3. The "donor-recipient" method of cytoplasmic hybridization: scheme of procedure. Legend for numbers: (1a) "donor" leaf as source of protoplasts; (1b) cell suspension culture as source of "recipient" protoplasts; (2a) maceration of "donor" leaf in enzyme solution; (2b) maceration of recipient cell suspension in enzyme solution; (3) mixing of X-irradiated "donor" and non-irradiated "recipient" protoplasts; (4) insertion of 0.25-ml-mixed protoplast suspension to the center of a petri dish; (5) addition of 0.35 ml polyethylene glycol (PEG) solution; (6) addition of 0.5 ml CPW; (7) removal of solution, leaving agglutinated protoplasts on the dish; (8) addition of 0.5 ml CPW; (9) final dilution of fused protoplasts with liquid medium. Protoplasts of "donor" (a) and "recipient" (b), containing nuclei (N), mitochondria (Mt), and chloroplasts (Chl) or plastids (Pl), as well as fusion product protoplasts with only one functional nucleus, are presented in a highly schematized manner (Galun and Aviv, 1983, p. 377).

generated from cultured leaf discs. In experiments at CIP, which support the work of the Breeding and Genetics Department, chromosome numbers are doubled, i.e., triploid to hexaploid, by regeneration from petiole segments. A more detailed knowledge of the regeneration mechanisms involved helps us to develop routine cell and tissue culture techniques that are important to the many routine activities of CIP's tissue culture laboratory.

Genetic Stability In Vitro

The use of tissue culture methods to produce so-called *somaclonal variation* (genetic variation induced by regeneration from somatic cells that have undergone chromosomal changes) has drawn attention to the overall stability of tissue culture methods for propagation and conservation of potato genetic resources. In collaboration with Dr. Michael Jones of the Rothamsted Experimental Station in the United Kingdom, CIP is investigating the genetic stability of all its in vitro methods. Potato plantlets propagated and regenerated by a wide range of in vitro methods are being tested by various methods to determine the level of somatic mutation in tissue culture material.

A number of biochemical methods, some conventional and some nonconventional, can be used to look for these major or minor genetic changes. Plants can appear to be unchanged by morphological analysis; however, more detailed biochemical analysis can detect minor genetic changes. Electrophoresis involves the analysis of soluble proteins from potato tubers. Figure 4 shows an electropherogram in which the pattern of bands is highly individual and minor changes can be noted.

An even more sensitive method of analyzing DNA structure has been developed in a number of laboratories over recent years, this highly sensitive system is known as *restriction fragment length polymorphism* (RFLP). In simple terms, RFLP involves cutting the DNA of potato chromosomes into small fragments, separating the fragments on a gel, and looking at the pattern. Figure 5 shows a RFLP pattern gel obtained through a CIP research contract. This project will help us to develop methods that will ensure the genetic stability of potato germplasm held by CIP and other national programs.

Pathogen Elimination

The present system of pathogen elimination used routinely at CIP and in most national programs that have a pathogen elimination program is thermotherapy of whole plants for four to six weeks at 36° C followed by meristem isolation and culture. In collaboration with Dr. Stephen Slack of the University of Wisconsin in the United States, CIP is developing in vitro methods for thermotherapy and chemotherapy. Plantlets already in an in vitro germplasm collection, but still contaminated with viruses, are treated in vitro by both chemotherapy (i.e., with Virazole) and by thermotherapy in test tubes, which are placed in a small heat chamber. Shoot tips and meristems are then excised, cultured, and indexed. Results from Wisconsin indicate that this method is highly efficient as a method of virus eradication. The adoption of this methodology at CIP will allow an increase in the amount of material that can be freed from viruses. Consequently, CIP will be able to provide more material much more quickly to national programs for both breeding and seed programs.

Conclusions

CIP has developed and continues to develop a number of collaborative research projects in tissue culture and genetic engineering, some of which have already yielded information and methods that can be adopted as part of CIP's routine laboratory methods. These methods are now being transferred to national programs through training courses at CIP headquarters and in the regions. Some of the projects are medium-term (3-5 years) and still need further refinement and development before the technology or improved germplasm will be ready for transfer to national programs. A few of the projects are technically complex and should be considered as long-term (5-8 years), but they should eventually yield improved germplasm of importance to national programs. Collaborative research in these areas has allowed CIP to maintain an up-to-date link to new technologies and will continue to enable CIP to facilitate the transfer of these technologies to national programs. In areas where research progress is rapid, collaboration with experts worldwide allows CIP to assist national programs in the most effective manner possible.

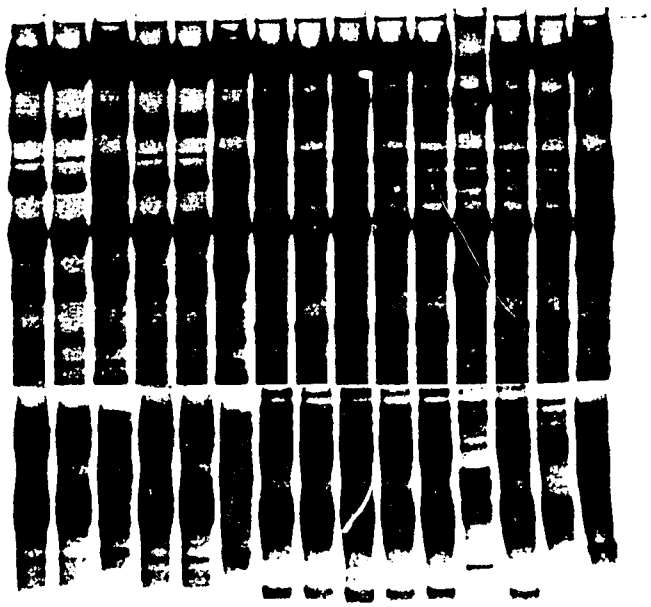


Fig. 4. Electrophoretic protein patterns of potato clones produced by analysis of soluble protein analysis on polyacrylamide gel.

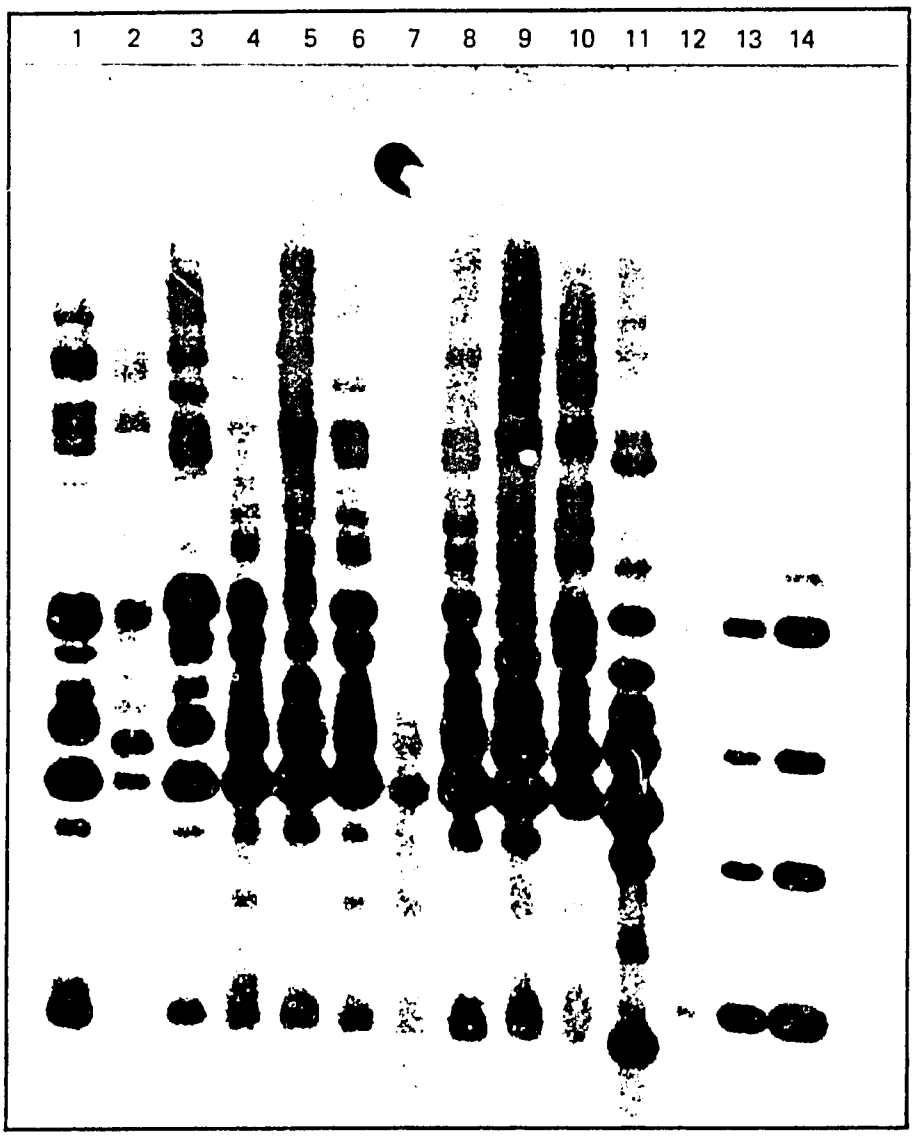


Fig. 5. A DNA fingerprint of 11 different potato clones and 3 molecular marker standards. The DNA was cut with one restriction enzyme and hybridized with the patatin gene probe. The different patterns of bands show clearly the genetic differences among these clones (Potter and

References

Espinoza, N. O., S. F. Yang, J. M. Jaynes, and J. H. Dodds. 1987. Plant cell tissue and organ culture. Scientific American. In press.

Espinoza, N. O., S. F. Yang, J. M. Jaynes, and J. H. Dodds. 1986. Plant cell tissue and organ culture. Trends Biotechnol. 4(12):314-320.

Galun, E., and D. Aviv. 1983. Cytoplasmic hybridization: genetic and breeding applications. p. 358-392. *In* D. A. Evans et al. (eds.). Handbook of plant cell culture, vol. 1. New York: Macmillan Publishing Co. 970 p.

Galun, E., P. Arzee-Gonen, R. Fluhr, M. Edelman, and D. Aviv. 1981. Cytoplasmic hybridization in Nicotiana: mitochondrial DNA analysis in progenies resulting from fusion between protoplasts having different organelle constitutions. Molec. Gen. Genet. 186:50-56.

Potter, R., and M. G. K. Jones. 1987. Development of molecular fingerprinting to study stability of potato during micropropagation and storage. Progress report of CIP collaborative research project with Rothamsted Experimental Station, Herts, U.K.

Schilde-Rentschler L., and P. E. Schmiediche. 1984. Tissue culture: past, present and future. CIP Circular 12(1):1-6. International Potato Center, Lima, Peru.

Sonnino, A. 1985. Anther culture and its uses in potato breeding. p. 319-335. *In* Innovative methods for propagating potatoes. Report of 28th CIP Planning Conference, December 10-14, 1984. Lima, Peru: International Potato Center. 342 p.

Tovar, P., R. Estrada, L. Schilde-Rentschler, and J. H. Dodds. 1985. Induction and use of in vitro tubers. CIP Circular 13(4):1-6. International Potato Center, Lima, Peru.

Wenzel, G., and H. Uhrig. 1981. Breeding for nematode and virus resistance in potato via anther culture. Theor. Appl. Genet. 59:333-340.