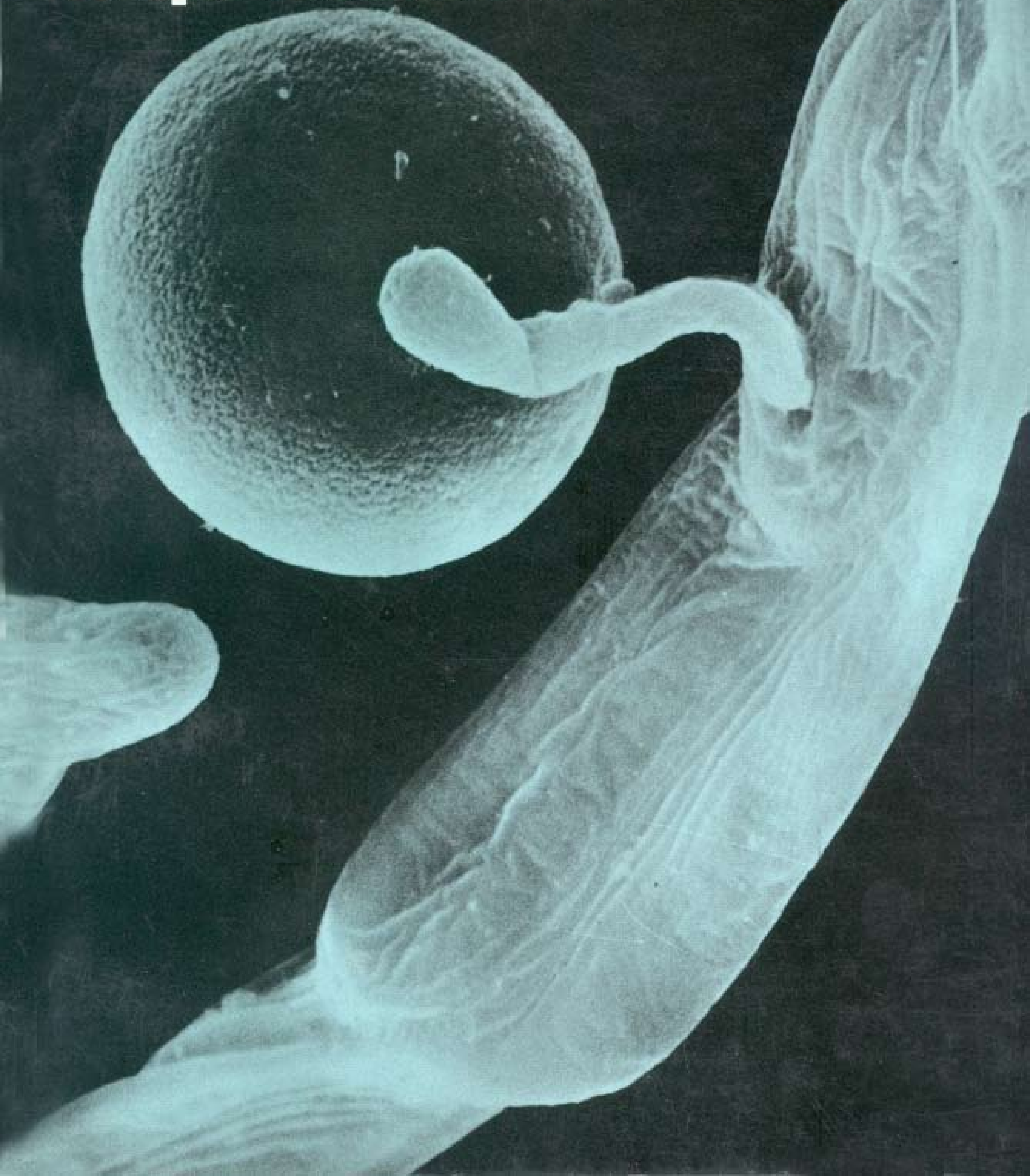
Biotechnology in Tropical Crop Improvement



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Cover: Biotechnology at ICRISAT Center: a scanning electron micrograph depicting pearl millet pollen germinating on the stigma.

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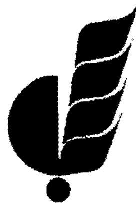
Proceedings of the International Biotechnology Workshop 12-15 Jan 1987, ICRISAT Center, India



ICRISAT
International Crops Research Institute for the Semi-Arid Tropics
Patancheru, Andhra Pradesh 502 324, India

1988

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Foreword

The objectives of the International Workshop on Biotechnology were to consider the feasibility of using biotechnological techniques to improve grain quality, yield potential, yield stability, and utilization of ICRISAT's mandate crops, i.e., sorghum (*Sorghum bicolor*), pearl millet (*Pennisetum glaucum*), chickpea (*Cicer arietinum*), pigeonpea (*Cajanus cajan*), and groundnut (*Arachis hypogaea*).

The possibilities of the application of somaclonal and gametoclonal variation, protoplast fusion, embryo and anther culture, clonal propagation, molecular detection, and genetic transformation to improve the agronomic fitness of cereals and legumes were assessed, and they were found to be of possible immediate use.

Publication of the papers presented is intended to provide readers with an understanding of the variety of techniques being employed, their relevance and usefulness, the dietetic and processing problems, prognostications on the future benefits, and uses of each of the main techniques of biotechnology in higher plants.

The papers fall into two main categories:

1. Research into **genetic manipulation** techniques relevant to ICRISAT-mandated legume and grain crops, such as direct genetic transformations, use of Ti plasmids, other methods of gene transfer, somaclonal/gametoclonal variations, molecular identification, and tissue culture.
2. Research that deals with the crops **products and their uses** and condition after harvest.

J.M.J. deWet
Scientific Editor

Preface

L.D. Swindale[1]

ICRISAT was created in 1972 by the CGIAR to carry out research to improve the quality of life for the people of the rainfed semi-arid tropics (SAT). Nearly 15 years later, in 1987 there was ample evidence that ICRISAT has been a success. Improved germplasm and breeding lines of all our mandate crops have been developed at ICRISAT and are now in the hands of national researchers. Some finished cultivars are already being used by farmers and are contributing significantly to increasing productivity. A technology to make better use of the resources in the Vertisol, black soil areas of India, has been developed and is spreading slowly. Most significant of all has been the marked strengthening of National Agricultural Research Systems (NARS) in the SAT.

Success has so far been greater with the two ICRISAT cereals than with the legumes. The same can be said universally for most other cereals and there is, for the time being, an oversupply of cereal grains that threatens the very success achieved by farmers.

Because sorghum and millet are the only cereals that can be grown successfully, in most years, in those hot, dry regions with erratic rainfall that constitute the SAT, we cannot abandon them. ICRISAT must improve the nutritional value of sorghum and millet and their suitability for a variety of alternative uses. ICRISAT must exploit existing research techniques for these purposes and try new techniques, particularly those that promise to enable genetic change.

Legume crops suffer, in contrast to cereals, from a shortage of supply. Because they complement the cereals so well, both in terms of the ecological regions in which they can be grown and in terms of the contributions they make to human diet and income, we need to find ways to increase their production by increasing their yield potential. To do this, we must deal more effectively with the causes of yield reductions: i.e., diseases, insects, and edaphic/environmental stresses.

The new techniques of biotechnology promise to contribute both to improving the nutritional value and alternative-use potentials of the cereals and the productivity of the legumes. But can that promise be revealed? That is the main question to which this group of experts addressed themselves and will help ICRISAT to answer. Other questions were:

1. Can we use recombinant DNA, protoplast transformation, monoclonal antibodies and tissue culture to bring about some of these needed changes in the next 5 years?
2. What are the probabilities of success with these techniques?
3. What are the betting-odds that in 1992 we will end up with as little progress as we have had with, for example, associative N-fixation in cereals?

A large effort in speculative research in biotechnology simply cannot be afforded. Because ICRISAT is an applied research institute, and because our donors expect us to produce visible and useful results, a fairly conservative stance must be adopted. Ideally one should start with work that has the highest chances of success, such as the use of monoclonal antibodies for identification of virus diseases. The proponents of any new projects in biotechnology must be prepared to state not only their objectives, but also the criteria to be used in, say, 5 years' time, to measure their success or failure.

It has already been decided to create a facility for biotechnology research within ICRISAT and to hire staff. This workshop was therefore most timely, and we expect to implement many, if not all, of its recommendations. For the new facilities must be used effectively.

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1. Director General, ICRISAT, Patancheru, A.P. 502 324, India.

Biotechnology in Agricultural Research

Sir Ralph Riley[1]

I see my task as that of trying to set the agenda for the Workshop. But first I should say something about my background in relation to biotechnology. I am a cytogeneticist and a plant breeder and, for a good deal of my career, I have been interested in the transfer of alien genetic variation from wild relatives into cultivated wheat. So in 1968, I attended a Rockefeller Foundation meeting in New York which addressed the question of wide crosses as an aid to crop improvement. This interest was generated because of work on *Triticale*, which seemed at that time to be of great promise. A reference was made at the meeting to the creation of wide cell hybrids in animals by fusion, using inactivated Sendai virus, and I wondered why something similar could not be used to effect wide hybridization of plants. In 1969, the Rockefeller Foundation called another meeting under the title "Crop improvement through the techniques of cell and tissue culture." People attending had names to conjure with in plant cell biology. There were Gamborg, Cocking, Yamada, D'Amato, Ericksson, Carlson, Morel, Braun, Torrey, Reinart, Nickel, Hildebrant, Roberts, and myself.

It became clear, at that time, that we would soon have the opportunities of creating protoplasts by the enzymatic digestion of plant cell walls. Interestingly, Cocking, who had worked on protoplasts for some time previously, had separated them mechanically from their walls. So the availability of enzymes from Japan at the end of the 1960s and in the early 1970s was a big step forward. This meeting was in May 1969 in Bellagio, and by 1972 the first somatic hybrid plant was produced by Carlson, Smith, and Dearing; and there have been many subsequent examples using PEG and electrofusion in particular. Although somatic hybrids have been successful in many combinations, so far as I am aware, they have had no influence on crop production as yet. The first somatic hybrid was produced in 1972 and it is now 1987.

There are, of course, species that used to be called recalcitrant, the ones that were difficult to regenerate as plants from protoplast, particularly the cereals and to some extent the legume crops. But recently there has been a breakthrough in rice, where numbers of people have demonstrated the possibility, at least in *Japonica* rices, of producing plants from protoplast. Cocking, Yamada, Oono, Toriyama, and the Mitsui group are in the forefront of this development. All this happened in 1985 and the way forward for transformation, of rice at least, now looks to be more possible than it was some years ago.

Of course, tissue-culture techniques have been used in crop improvement for a long time in the form of micropropagation. Indeed, it is as long ago as 1957 that Kassanis first demonstrated that, by the use of meristem culture, paracrinkle virus could be cleared from potato. That kind of biotechnology has been available for a considerable time.

Anther culture was first demonstrated by Guha and Maheshwari in 1964 in *Datura* and that is more than 20 years ago. Nitsche in 1974 was the first to demonstrate the creation of haploid sporophytes from isolated pollen. But the impact of this technique on breeding has been limited. Those who employ anther culture in breeding have so far been able to produce a maximum of about 10 000 plants per season. In plant breeding terms, that is hardly startling. In a conventional wheat breeding program, my colleagues at the Plant Breeding Institute look at in excess of 1.5 million F_2 plants per season. They need to look at such large numbers to have a reasonable probability of spotting elite recombinants. These inadequacies of scale cause me to worry about anther-culture in breeding programs.

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Now let me turn to the other aspect of biotechnology, raised both by Dr Swindale and Dr Kanwar, that of recombinant DNA transfer. In 1953 the structure of DNA was determined by Watson and Crick. Decoding the language took place between 1962 to 1966. In 1970 the first endonuclease restriction enzyme was produced. In 1973 the first foreign DNA was cloned into *E. coli.* In 1980 DNA clones of plant genes were produced for the first time. In 1983 it was shown that foreign genes will express in host plants. The progress of discoveries in the area of plant molecular biology has been rapid indeed.

The task of genetic transformation in higher plants was greatly facilitated by the development of the binary vector system in *Agrobacterium.* The Ti plasmid of *Agrobacterium* has a virulence region (*vir*) that is necessary for transfer of the T region of the DNA to the plant. But this *vir* region is not itself transferred. Consequently, two plasmids have been made and used together, one containing the *vir* region that drives the transfer of DNA and the other containing DNA that one intends to transfer into the plant. Foreign genes to be transferred must be inserted between the borders of the T region. Transfer can also be accomplished using electroporation, first discovered by Zimmerman in 1982. As an example, Walbot and her colleagues have electroporated foreign DNA into maize cells. In 1987 Grimsley and colleagues have shown that *Agrobacterium* can insert valid information into maize. The *Agrobacterium* system may become effective in monocots as well as dicots.

To illustrate what can be done, let me talk about two transformations made by my colleagues at the Plant Breeding Institute. The first concerns the work by Baulcombe and others on the cauliflower mosaic satellite sequences. The cauliflower mosaic virus can have, independent of the genome, a satellite sequence of DNA of about 335 bases in length. When the satellite is present, the expression of symptoms of the virus is often greatly reduced. DNA for the cauliflower mosaic satellite sequence was transformed into tobacco. When satellite DNA was a part of the genome of tobacco, there was essentially absence of viral symptoms on infection. Moreover, these plants showed about 20% less virus than plants without the satellite. It thus appears that useful things can be accomplished in relation to disease resistance in plants by this kind of transformation.

In another example, Flavell, Thompson, and others at the Plant Breeding Institute put the promoter for the storage protein glutenin of wheat in front of a gene-determining chloramphenical transferase. This construct was vectored into tobacco. The enzyme is expressed only in the tobacco seed, so the promoter prevents expression in the rest of the plant. The time course of the development of the product of the gene is such that it is only present in the seed about 14 days after pollination. A promoter of genetic activity can/may thus be isolated, and demonstrated to function when included in a foreign background, in the same place and in the same stage of development as it does when it is controlling the production of glutenin in the wheat seed.

This means that active genes can be constructed using DNA from various sources, and the expression of a foreign gene can be directed in a plant by having a promoter operating at the place and time wanted. Furthermore, it was shown that this activity is inherited in a simple Mendelian manner. Can such transformations be used in major crop plants? The plain answer is that we do not know and in my belief we are some way from knowing. First of all we do not really know what we ought to incorporate. This derives from our ignorance of the intermediary metabolism of plants. For example, in relating to many disease systems, because the products that are directly involved in the creation of resistance are unknown, critical DNA sequences cannot be isolated. So there is still a lot of work that remains to be done and my advice is to be cautious.

If one looks at history for what happened in the application of new technology in crop breeding, it becomes obvious that the time cost of application is often quite considerable. In 1984 I gave a talk at the UPOV (International Union for the Protection of New Varieties of Plants) conference in Geneva. I was asked to talk about "Biotechnology, dream or reality". I told that conference that biotechnology in crops had reality but was not immediate. The example that I chose to give about the speed of technology uptake related to the sugar beet crop of north America and Europe. This is a highly technical crop. It is triploid and hybrid. Hybridity

depends upon using cytoplasmic male sterility and hybridity helps by bringing in two genomes from a normal tetraploid pollen parent to cover some of the genotypic deficiencies of the monogerm genotype. These varieties came in widespread use only in the early 1970s. Let us trace the history of this development. Colchicine was discovered by Blakeslee and others in 1937. The first tetraploid sugar beet was produced in 1938 using colchicine. Between 1938 and early 1970 there was about 3 decades' interval for the acceptance of polyploid sugar beets on a wide scale by farmers. Turning to monogermy, this condition was first discovered in the Soviet Union in 1934. It was rediscovered by Savitsky in 1948 in the United States. So, from 1948 to the 1970s, again, it was 25 or more years before the technology for the monogerm condition was actually brought into wide-scale use. Cytoplasmic male sterility was discovered in sugar beet in 1942. It again took 25–30 years between 1942 and the 1970s in order to produce the hybrid sugar beet.

What I would like to do is to be realistic about the problems that we face, and to say to you that the discoveries we are making in cell and molecular biology of plants will not find any easy and ready application in crop improvement. There is a great deal of work still to be done and there are practical problems of great significance to agriculture, such that it would be unwise to rely too heavily on the 'magic' of biotechnology. But biotechnology is sure to help transform agriculture in the next century, and we should ensure that our R&D is preparing us for this transformation.

Biotechnology and ICRISAT

J. S. Kanwar[1]

Over the last 4 decades, remarkable strides have been made in our understanding of the chemistry and coding of genetic material in living organisms. These developments led to a justified enthusiasm, among scientists, to alter the genotypes of plants and to solve some problems that proved to be difficult to solve using conventional techniques of genetic improvement. This new field of science, biotechnology, has opened up enormous potentials to improve agriculture in general and food crop production in particular. The number of seminars and workshops that have been held on biotechnology over the last 2 decades is an indication of the worldwide interest in this field. International Agricultural Research Centers (IARCs) are equally interested in advancing the frontiers of knowledge in this field. The 1984 workshop on biotechnology held at IRRI is evidence of this interest, and so is this workshop, which attempts to look at the relevance and potentials of biotechnology in fulfillment of ICRISAT's mission. While not ignoring the importance of biotechnology in contemporary science, the seminar participants will be well advised to bear in mind some inherent pitfalls in this field as ably highlighted by the eminent biotechnologist, Elmer Garden.

The genetic improvement of sorghum, pearl millet, pigeonpea, chickpea, and groundnut is pivotal to the Institute's mandate. While the breeding objectives in respect of all these crops emphasize high and stable yields, concurrently equal attention is paid to resistance to insects, diseases, and abiotic stresses and to improve the grain quality to meet the acceptance of consumers.

The External Program Review Committee (EPR) which reviewed ICRISAT's programs in 1984, suggested that ICRISAT should expand its work on germplasm enhancement. The committee also recommended an intensification of research on breeding for resistance to the major diseases and insect pests, and improving the grain quality and food characteristics of the cereal and legume crops. The Technical Advisory Committee (TAC) of the CGIAR, while reviewing the research priorities of IARCs, endorsed the recommendations of the EPR for ICRISAT, but in addition emphasized the need of incorporating long-term sustainability of agriculture, and protection of the environment under dry farming systems.

Unlike wheat, rice, and maize, ICRISAT's mandate crops, especially pearl millet, pigeonpea, and chickpea, receive relatively little attention from scientists in developed countries. Though there is a vast scope for improvement of these crops through exploitation of existing genetic variation and use of conventional techniques of plant breeding and genetics, there are some problems in breeding for resistance to biotic and abiotic stresses that are difficult to solve by conventional breeding. Even more difficult is the improvement of grain quality without sacrificing yield.

The Technical Advisory Committee of the CGIAR recognizes that the CG system employs a multidisciplinary research approach in crop improvement by adopting four interrelated strategies: (1) to increase yield potential, (2) to narrow the gap between potential and actual yield, (3) to improve yield stability, and (4) to mitigate the erosion of crop yields by diseases and insects. TAC further recognizes that the CG system may gradually shift its emphasis from applied research to strategic and basic research. The development of improved technologies in dryland farming does entail a sustained effort on a wide range of ecological and biotic constraints, such

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as soil, water, and energy, as well as constraints related to insects and diseases. The solution of some of these problems may involve fundamental studies in crop physiology in the area of plant response to stresses. The appropriate strategy is to remain in close touch with recent advances in basic sciences, and to deploy these findings in the generation of new technology.

There clearly is a need to step up research in crops on which less strategic research has hitherto been done. TAC emphasizes the urgency to improve productivity of pigeonpea and groundnut by taking advantage of the recent advances in tissue culture and molecular biology. Part of the resources available to the CG system could be earmarked for new ventures in different, but equally important, areas of concern to crop improvement.

Conventional breeding techniques have contributed substantially to transfer of resistance to various biotic and abiotic stresses into the high-yielding genotypes. However, advance has been small in some cases, as for example with tolerance of borers and *Striga* in sorghum, of *Heliothis* in pigeonpea, and of ascochyta blight in chickpea. Even where high levels of resistance have been achieved through conventional breeding, as with downy mildew in pearl millet, the durability of such resistance has been far from satisfactory. It therefore remains the challenge of biotechnology to provide more rapid and durable progress in providing resistance to various stress factors. There is considerable reason for optimism in breeding for resistance to drought and salinity by employing tissue-culture techniques.

In groundnut, ICRISAT scientists have succeeded in crossing wild and cultivated species in an effort to transfer genes of resistance to foliar diseases. They are now interested in combining yield potential, earliness, and virus and insect resistance in these cultivars. It is generally believed that opportunities for incorporating virus resistance through biotechnological techniques are great. Dr D.V.R. Reddy and Dr J.P. Moss will disccuss the problems and possibilities. I hope this workshop will help them in developing improved technologies for achieving a breakthrough in both productivity and stability of production in groundnut. We believe that grain legumes, besides providing proteins and fats that are in short supply, can also help in diversification of agriculture and in improving the economy of many developing countries.

This consultants' meeting is being held in order to promote an interaction between ICRISAT scientists and the world community of biotechnologists, as a means of improving the yield potential, stability of production, and grain quality of the ICRISAT-mandated crops. The possible exploitation of somaclonal and gametoclonal variation, protoplast fusion, embryo and anther culture, clonal propagation, molecular detection, and genetic transformation will be critically assessed. The distinguished scientists assembled here today are leaders in different aspects of biotechnology. We are certain that they can impart information that will enable ICRISAT scientists to make rapid progress in the improvement of sorghum, millet, pigeonpea, chickpea, and groundnut.

It is fortunate that an international unit for research on biotechnology relevant to agriculture is being established at New Delhi. ICRISAT looks forward to active collaboration with this organization in areas of mutual interest. This may provide an opportunity to develop a coordinated program of research on the use of biotechnology for ICRISAT-mandated crops.

I have drawn your attention to some of the concerns of ICRISAT and placed before you some of the challenges for biotechnological research. We hope that this consultation will help us to focus our attention on priority areas of research that offer promise for rapid improvement in the production of sorghum, millet, pigeonpea, chickpea, and groundnut in the semi-arid tropics.

I wish this workshop success.

Genetic Manipulation

Gene Vectors for Plant Transformation

R.J. Shepherd[1]

Abstract

*A recombinant DNA vector can be defined as an agent that brings about genetic transformation of plants with foreign DNA. The vector must be active in transferring the DNA into the plant in such a way that it is maintained through cell division and plant propagation and that it be expressed as a new genetic factor. Regulatory regions that are active in plants to bring about expression of foreign DNA as new functions (phenotypes) are an essential feature of gene vectors. Consequently, well characterized plant regulatory regions are an important preliminary step in the development of plant gene vectors. Two types of plant gene vectors have been developed: (1) the integrating type, based on the Ti plasmid-*Agrobacterium *system and (2) the independently replicating type (nonintegrating) based on cauliflower mosaic virus (CaMV); both are illustrated and discussed.*

Introduction

Plants, as higher organisms, have one great advantage over most animals for genetic manipulation. Many of them can be regenerated from single cells whereas most animals cannot. With the recent advent of recombinant DNA vector technology, this difference is having a great impact on plant biology.

Gene transfer systems are needed for the introduction, propagation, and expression of foreign genes in plants. The direct transfer of cloned and defined DNA sequences into plants to cause genetic changes is the only way to assess the biological effects of specific sequences. These approaches of recombinant DNA technology with bacteria and yeast, have led to rapid advances in our knowledge of the structure and regulation of their genetic material. Undoubtedly, similar manipulations with plants will be equally revealing.

The only well developed gene transfer system for higher plants is the Ti plasmid of *Agrobacterium tumefaciens.* This system has recently become widely used by plant biologists because of its simplicity and effectiveness. Sequences inserted by the Ti vector become integrated into plant chromosomes (integrative transformation) and, as a result, are carried to succeeding generations through seed. This vector is having a great impact on fundamental studies of plant gene structure, organization, and expression. Eventually this knowledge will contribute to plant improvement for agricultural and aesthetic purposes. Ti plasmid vectors and the various manipulations involved in their use are discussed under the next heading.

Other types of gene vectors for plants are desirable for a variety of reasons. It would be useful, for example, to have extrachromosomal (nonintegrative) vectors for transformation of cells or whole organisms. These have been the types most useful for animal and bacterial transformations. Such a vector in coordination with Ti plasmids would allow sequences to be inserted into both cis and trans positions for evaluating regulatory sequences in plants.

In addition, it would be desirable to have gene vectors that produce very rapid transformations of plants. Ti vectors transform cells from which whole plants must be regenerated in tissue culture. The process of regeneration from one or a few totipotent cells, or from leaf discs, requires several weeks before adequate tissue or whole plants are available for evaluation. Virus-derived vectors that carry foreign DNA from cell-to-cell in intact plants are an attractive alternative for rapid transformations on the whole plant level. The prospects for developing useful virus-based gene vectors are discussed below.

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Transformation of Plants with Ti Plasmid Vectors

The most effective and widely used gene transfer system for plants is one based on the Ti (tumor-inducing) plasmids of *Agrobacterium tumefaciens*, a soil-inhabiting bacterium. This bacterium causes crown gall disease of dicotyledonous plants. It invades wounded plant tissues and causes hypertrophy which appears macroscopically as an undifferentiated gall at the wound site. The biology and molecular basis for crown gall has been reviewed recently by Nester et al. (1984).

Naturally occurring *Agrobacterium tumefaciens* contains large Ti plasmids (200 kbp) of which a portion, referred to as the "T-DNA" (Fig. 1), is transferred into the plant chromosome to cause a genetic change. This change, or transformation, is referred to as "tumorgenesis", since it leads to gall formation.

Tumorous tissue with integrated T-DNA consists of undifferentiated tissue which continues to proliferate in the absence of the inciting bacterium. It has undergone a genetic change leading to independent growth even in the absence of externally supplied hormones (auxins and cytokinins) which are generally required for growth. In addition to producing its own requirement of hormones, tumor tissue also produces one, or more, of several possible types of foreign compounds called "opines". These consist of modified amino acids that are not found in unmodified tissue. The most common opines are opaline and octopine. The synthesis for these compounds, like synthesis of auxins and cytokinins, is specified by T-DNA of Ti plasmid origin. Hence, wild-type Ti plasmids are generally of nopaline or octopine types depending on which opine they encode. Opines are utilized metabolically by *Agrobacterium*. The functions for opine utilization are also encoded on Ti plasmid DNA. Consequently *Agrobacterium*, but no other microorganism, is able to use opines metabolically.

Figure 1 shows a genetic map for each of the two wild types of nopaline and octopine Ti plasmids that have been studied more intensely than other types. Only a small portion of these plasmids, the T-DNA portion, is transferred from the bacterium to the plant cell. Membrane hybridization experiments, using plant DNA from transformed tissue, have shown that T-DNA is an integral part of plant chromosomal DNA. Other types of experiments have shown that T-DNA is transcribed to RNA, probably by plant RNA polymerase II. With integrated T-DNA from an octopine Ti plasmid, eight polyadenylated transcripts have been identified. Some of these transcripts probably arise from some of the various loci that control tumor morphology. These loci are designated tumor morphology shoot (*tms*) and tumor morphology root (*tmr*). The *tms* locus is involved in auxin metabolism and the *tmr* locus is involved in cytokinin metabolism. It has been shown, for example, that endogenous levels of auxin and cytokinins are altered by *tms* and *tmr* mutations. Double mutants that inactivate *tms* and

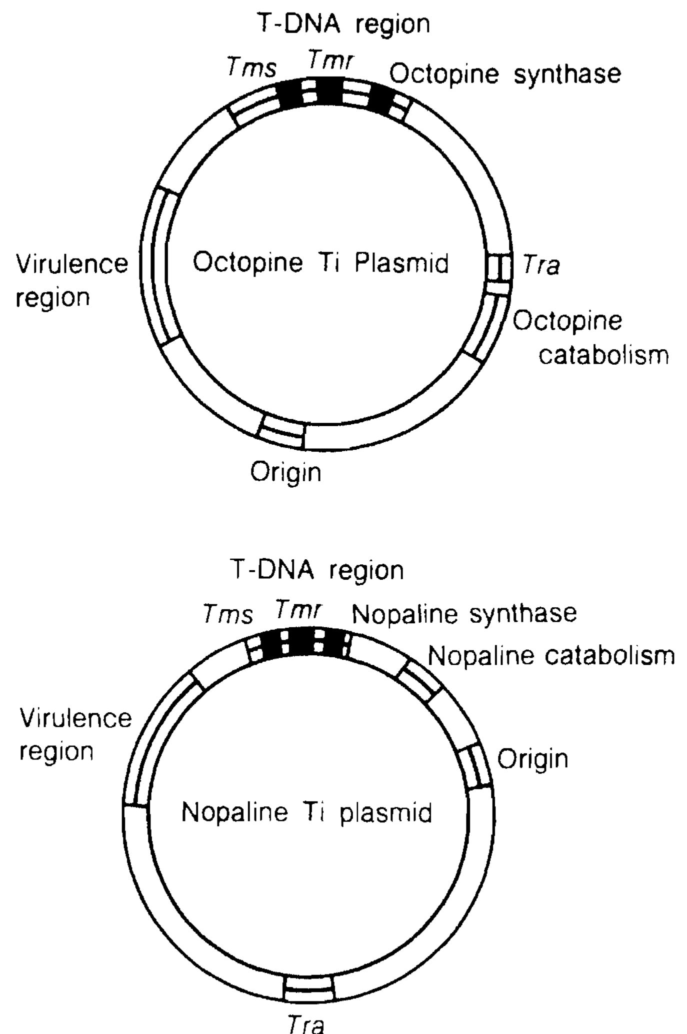

Figure 1. Genetic maps of two types of Ti plasmids. The types shown here, octopine- and nopaline-producing plasmids, represent the best characterized types. Note that the T-DNA region represents only a fraction of these large plasmids (200 kbp). Loci controlling opine synthesis, either octopine or nopaline in these examples, and tumor morphology appear on the T-DNA portion. The virulence functions (*vir* region) appears to the left of T-DNA in both plasmids. Other loci that govern catabolism of the opines appear elsewhere on the Ti plasmid, e.g., oncogenisis regions *tmr* and *tms*.

tmr functions are incapable of tumor formation even though T-DNA transfer to susceptible host cells occurs normally. Consequently, it is possible to disarm (attentuate) these plasmids by removing or inactivating these functions. This can be done without affecting the ability of T-DNA to transfer and integrate into plant chromosomes.

The T-DNA element that is transferred to the plant cell does not encode any of the products necessary for the transfer process. Transfer is brought about by trans acting functions specified by virulence loci of *Agrobacterium* and the Ti plasmid. The bacterial chromosome contains at least two loci, *chv* A and *chv* B, involved in attachment or binding of the bacterium to plant cells. The Ti-plasmid virulence loci are: *vir* A, *vir* B, *vir* C, *vir* D, *vir* E, and *vir* G. These specify the various events in T-DNA transfer and occupy about 35 kb of the Ti-plasmid that is separate from T-DNA. Unlike the T-DNA, this 35 kb *vir* region is never integrated into plant chromosomes.

Another element needed for T-DNA transfer is the left and right border sequences (LB and RB, see Fig. 2) that flank the T-DNA element. These border sequences are almost perfect, 25-base pair direct repeats. Deletion of RB prevents T-DNA transfer. The same is not true of LB as T-DNA often terminates internal to LB. The border sequences function as sites for recombination between bacterial and plant DNA during the integration of T-DNA.

In experiments with a nopaline Ti plasmid, it has been demonstrated that although RB alone is adequate for T-DNA transfer, it is functional in only one orientation, that of the native T-DNA. Hence it appears that T-DNA transfer has a definite polarity and, in this regard, is similar to DNA transfer during bacterial conjugation.

Some recent information bears on the regulation of the functions involved in T-DNA transfer. The *chv* loci that mediate attachment of *Agrobacterium* to plant cells are expressed constitutively and are not regulated. In contrast the *vir* loci of the parent plasmid of T-DNA are tightly regulated. Only *vir* A and *vir* G are expressed significantly in vegetative *Agrobacterium*. When exposed to plant cells, however, the other operons are induced to high levels. The signal molecules released by plant cells that bring about the induction of these functions have recently been identified as two phenolic compounds, acetosyringone (AS) and hydroxy-acetosyringone (OH-AS). Cultivation of *Agrobacterium* with either compound results in activation of the *vir* functions B, C, D, E, and G. These phenolics are believed to be

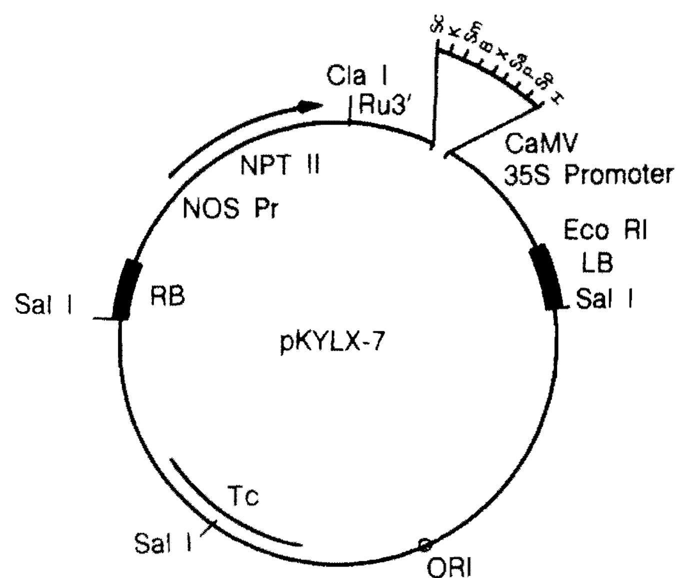

Figure 2. Genetic map of pKYLX-7, an example of a binary vector for gene transfer. The chimeric gene for a dominant selectable marker, neomyin phosphotransferase type II (NPT II) contains a nopaline synthetase promoter (NOS). The right and left border sequences (RB and LB) are indicated. A multiple cloning site downstream from the 35S cauliflower mosaic virus (CaMV) promoter is suitable for insertion of the coding regions of many foreign genes. It is followed by a terminator (3') region from the pea rubisco gene (Ru3'). The wide host range origin of replication derived from RK2 plasmid is indicated (ORI) as well as the sequences that lead to conjugative transfer (*tra*) from one bacterium to another. This vector was developed by Arthur Hunt, Department of Agronomy, University of Kentucky, Lexington.

the natural signal stimuli released by wounded plant cells. Products of *vir* A and *vir* G are believed to activate the other loci. *Vir* A may encode a membrane-bound protein that transmits the extracellular signal to an intracellular regulatory protein (*vir* G) to cause generalized activation of the *vir* regulon. An analysis of the nucleotide sequence of *vir* G has revealed one open reading region that encodes a protein of 30 000 daltons. It is homologous to eight known bacterial proteins including *omp* R of *E. coli*. The latter performs a regulatory role for other membrane proteins, suggesting that *vir* G has a similar function in the regulation of the *vir* functions (Stachel and Zambryski 1986a, Winans et al. 1986). In a leaf disc transformation system with a binary vector, that is described later, genetic analysis has shown that *vir* C may not be essential for T-DNA transfer (Horsch et al. 1986).

It has recently been found that activation of T-DNA transfer of *Agrobacterium* by the phenolic signal compound acetosyringone leads to the formation of a single-stranded linear DNA molecule with a particular orientation. This intermediate in the T-DNA transfer process has been termed "T-strand" (Stachel et al. 1986). It is a molecule analogous to the conjugative intermediate produced during bacterial mating. T-strand transfer from bacterial to plant cells may occur by a process very similar to bacterial conjugation.

The T-strand corresponds to the bottom strand of T-DNA as defined by the left and right border sequences. Its 5′ and 3′ ends map to the right and left T-DNA borders. Consequently, T-strand polarity corresponds to border polarity. The production of T-strand is through single-stranded cleavage of Ti plasmid DNA at specific sites that correspond to the border sequences. This cleavage is probably from specific single-stranded nicking by a protein encoded by *vir* D (Stachel and Zambryski 1986a and 1986b).

Development of Plasmid-based Gene Transfer Vectors for Plants

The binary vectors now in wide use by plant geneticists are 2nd-generation vectors. They employ a different strategy from that of the first type of Ti-plasmid based vectors to be developed. The difference in these two types is that no recombinational event is required between a newly fashioned vector construct and a Ti plasmid. The binary vectors are somewhat easier to use than the 1st-generation co-integrate vectors and are the only vector type described herein.

Binary strategy for vector construction was first developed by Hoekema et al. (1983), who showed that *vir* functions were active when supplied on a separate plasmid from that containing T-DNA. This allowed the in-vitro transfer of T-DNA to small plasmids that infect *E. coli* and their restructuring by conventional recombinant DNA techniques. After restructuring (insertion of foreign genes) the small plasmid is introduced into an *Agrobacterium tumefaciens* strain carrying a *vir* region on a Ti plasmid to bring about the transfer and integration of the T-DNA (with foreign genes) into plant cells. The Ti plasmid serves as a helper since it contains the *vir* functions. This was a major development in plant transformation technology, because it allowed the restructuring manipulations to be done in small, simple-to-use plasmids that replicate in *E. coli*, the most convenient and well developed cloning system for recombinant and DNA manipulations. Native Ti plasmids of about 200 kbp are much too large and complex for restructuring and transformation experiments. For example, it is not possible to clone directly into native Ti plasmids because of their large size.

The binary vector system, as the name implies, uses two plasmids. One is the gene vector and the other is the Ti plasmid helper that provides the *vir* functions. A binary vector has several important features; six are listed below.

1. The vector plasmid contains the right and left border sequences (RB, LB) from T-DNA. These are the only sequences of T-DNA that are required for transfer and integration into plant chromosomes. Other sequences, particularly those for oncogenesis (*tmr* and *tms*, Fig. 1), are eliminated since these prevent the normal differentiation of transformed plant cells.
2. A chimeric gene that functions as a dominant selectable marker in plant cells is included in the vector. Neomycin phosphotransferase II (NPT II) (Fig. 2) is an example. It confers kanamycin resistance to plant cells. The chimeric gene consists of the coding region of NPT II ligated to an appropriate 5′ regulatory region to promote transcription in plants. The promoter most frequently used has been that of nopaline synthetase (NOS) (Fig. 2).
3. A wide host range origin of replication enables the vector to replicate in both *E. coli* and *Agrobacterium tumefaciens* (Fig. 1 and Fig. 2). Some vectors contain two origins of replication, one from Col El derived plasmids (e.g., pBR322) and one from wide host range plasmids (e.g., RK2). The wide host range origin in pKYLX-7 (Fig. 2) is derived from the plasmid pTJS75. The latter replicates in a wide variety of bacterial hosts (Klee et al. 1985). The plasmid is derived from RK2 (Figurski and Helinski 1979).
4. The vector must also contain an appropriate origin for conjugation transfer so that it can be mobilized into *Agrobacterium* by a triparental mating (Fig. 3). These sequences involved in conjugative transfer are also designated *tra* or *bom* in various plasmids. The triparental mating procedure consists in mixing cells of *E. coli* containing the plasmid vector with cells of *E. coli* containing pRK2013. This mixture of cells is then added to a suspension of freshly grown *Agrobacterium* containing the helper Ti plasmid (to provide the *vir* functions). The three types of cells are filtered out

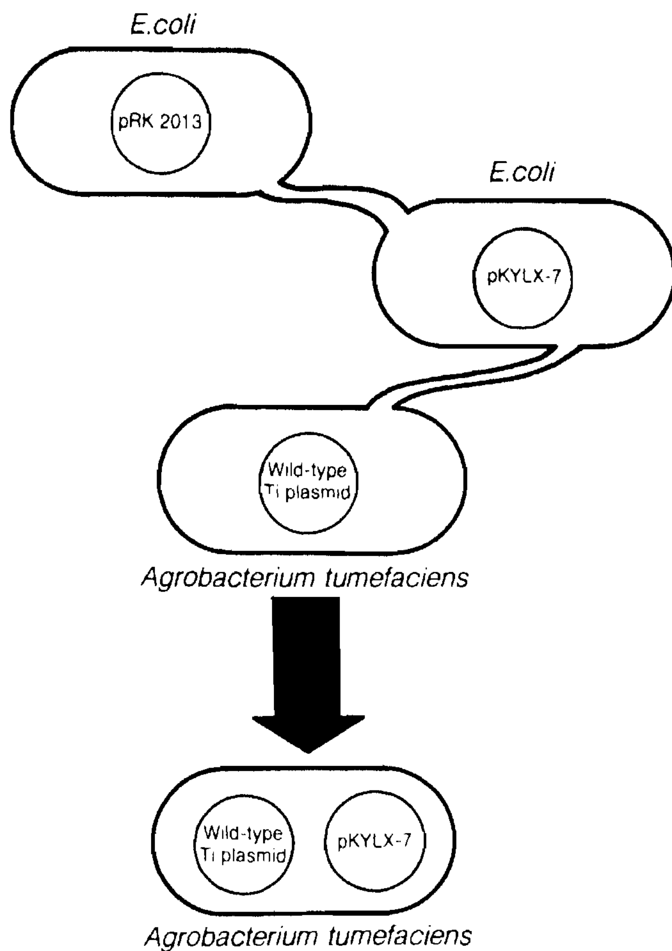

Figure 3. The triparental mating procedure for conjugal transfer of a gene vector plasmid from *E. coli* to *Agrobacterium*. Cells of *E. coli* with pRK2013 are mixed with cells of *E. coli* containing the gene vector (pKYLX-7), and these are then mixed with *Agrobacterium* with a wild-type or disarmed Ti plasmid. The cells are then collected on a permeable filter to bring the cells into close proximity. The pRK2013 plasmid, which contains transfer proteins for conjugation, mobilizes into *E. coli* containing pKYLX-7. It then supplies these same functions for mobilization of pKYLX-7 into *Agrobacterium*.

of solution and the filter placed on a rich agar medium to allow the 3-way conjugation to occur. The pRK2013 plasmid mobilizes into *E. coli* containing the vector. Within the vector *E. coli* cell, pRK2013 provides RK2 transfer proteins and other factors that act on the origin of transfer to bring about mobilization of the vector into *Agrobacterium*. Rogers et al. (1986) give details of the triparental mating procedure.

5. Some binary vectors also contain the alpha complementation sequence of the pUC series of plasmids (Vierra and Messing 1982). This allows screening for inserts on X-gal plates (uncolored versus colored bacterial colonies). The binary vector pEND4K (Klee et al. 1985) is an example.
6. In addition to the above features, some binary vectors have been engineered to contain the *cos* site of lambda phage (see below). Some cosmid vectors will accommodate DNA inserts of 30–40 kd since the lambda cohesive ends (*cos*) can be utilized in an in-vitro packaging system (Vollenweider et al. 1980).

The vector, illustrated in Figure 2, has been derived from the plasmid pGA472 described by An et al. (1985). It has been modified by removal of the *cos* site and Col El origin and insertion of the 35S promoter of cauliflower mosaic virus (CaMV), followed by a multiple cloning site (shown in Figure 2 as Sc K...Sp H) and the rubisco terminator sequence (Ru 3'). It contains a wide host range origin of replication and an origin of transfer for conjugative mobilization into *Agrobacterium.*

Procedures for Plant Transformation and Selection of Transformed Individuals

Procedures for transformation (Fig. 4) of leaf discs and suspension cell cultures are described by Horsch et al. 1985; An et al. 1986; An 1985; Rogers et al. 1986; and Lichtenstein and Draper 1985. The usual procedure is to culture leaf discs, or cells, briefly with freshly grown *Agrobacterium tumefaciens*, followed soon thereafter by transfer to a medium containing an antibiotic (carbenicillin) to kill the bacterium. The tissue or cells are then transferred to a kanamycin-containing medium to select out NPT II transformants. After 2–6 weeks on this medium the shoots that develop are excised from the calli and transplanted to an appropriate root-inducing medium containing carbenicillin (500 mg mL^{-1}) and kanamycin (100 μg mL^{-1}). After roots appear these are observed for necrotic tips or inhibited growth. Plants that have roots growing into this medium without discoloration are then transplanted into soil.

Antibiotic-resistant plants are assayed for the expression of neomycin phosphotransferase activity. If the vector contains intact octopine or nopaline genes, octopine and nopaline synthetase assays should be carried out. Step-by-step details of these assays are given by Lichtenstein and Draper (1985). Southern blot hybridization assays will confirm that the plant genome contains the foreign DNA.

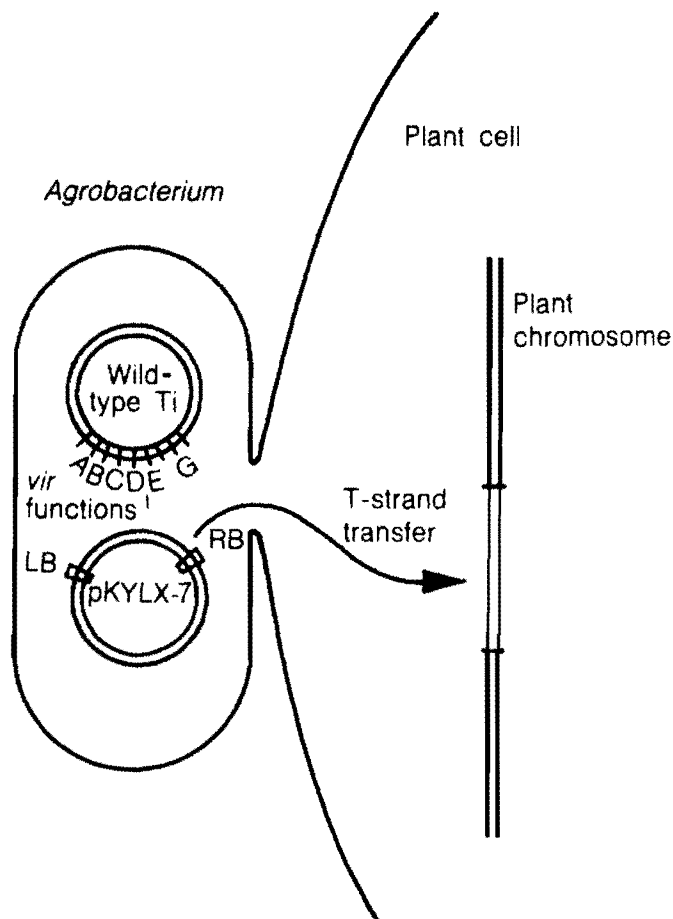

Figure 4. Attachment of *Agrobacterium* to plant cells and the movement and integration of the gene vector (pKYLX-7) into the plant genome. Functions for formation, transfer, and integration of T-DNA are supplied by the *vir* functions of a wild-type Ti plasmid, RB and LB, etc., as described for other Figures.

Plant DNA Viruses as Potential Gene Vectors

Some of the DNA viruses have been demonstrated to be useful as gene vectors. The cauliflower mosaic virus (CaMV), for example, has been engineered to carry the bacterial dihydrofolate reductase gene throughout plants to confer resistance to some drugs (Brisson et al. 1984). Both the double-stranded DNA viruses (caulimoviruses) and single-stranded DNA viruses (geminiviruses) are potentially useful as vectors although not much has been done yet with the latter group.

The major advantage of the DNA viruses, as vectors, is that their DNA becomes rapidly amplified and they move swiftly among cells to carry genetic material throughout intact growing plants. If they can be developed as vectors it may be possible to avoid the time-consuming tissue-culture steps required for plant transformation. The potential of these viruses as gene vectors has been reviewed recently (Shepherd 1986).

Figure 5 shows the genetic organization of the CaMV genome. Several strains have been completely sequenced to establish this structure. The genome consists of 8000 bp of DNA organized into six major genes and two intergenic regions. A large intergenic region of about 800 bp occurs between genes VI and I, and a small intergenic region between genes V and VI (Fig. 5). Transcriptional promoters occur in each of these regions.

The identity of some of the viral genes is known. Gene II is active in insect transmission, gene IV encodes the coat protein, gene V specifies viral DNA polymerase (reverse transcriptase) involved in viral DNA replication, and gene VI encodes the matrix protein of viral inclusion bodies. Biologically gene VI specifies a major host range determinant of CaMV (Schoelz et al. 1986). The functions of genes I, III, and VI are not known.

A prerequisite for using the genomes of caulimoviruses in gene vector construction is the molecular cloning of these viral DNAs in bacteria. Only in this way is it possible to achieve the versatility in DNA manipulation required for gene vector construction. With cloning it is easy to propagate and isolate large quantities of infectious viral DNA or noninfectious subgenomic pieces of the DNA for modular construction of vectors to be introduced into plants.

The first requirement in development of a vector of this type is to identify nonessential regions of the viral genome where insertions of foreign DNA can be made without inactivating the virus.

Gronenborn et al. (1981) identified a nonessential region in the CaMV genome by making insertions into the unique XhoI site in the center of open-reading region II (Fig. 5). A small deletion, including loss of the β_2 interruption (Fig. 5) had been observed in a mutant of the virus, and sequencing had shown that 321 bp of region II was missing from this strain (Howarth et al. 1981). Among a variety of insertions placed in the XhoI site of an infectious clone of CaMV, one of 65 bp was infectious. The insert was retained through several successive transfers of virus from systemically infected plants. Tolerance of a fragment of this size provided good evidence for a nonessential region in the CaMV genome.

Among larger inserts of 256, 531, and 1200 bp only the first was stable in CaMV strain CM1841 (Gronenborn et al. 1981). Although DNA with the 531 bp insert was infectious, there was a long delay in

symptom development, with 5–6 weeks required for systemic development of disease rather than the 2 weeks usually required. When viral DNA was recovered from infected plants and analyzed, most or all of the insert had been deleted, and often the deleted portion extended into the flanking regions of gene II. This suggested that, although CaMV will tolerate insertions in region II, the size must be no larger than about 250 bp if the recombinant virus is to be stably propagated. Inserts of this size are too small, however, to be useful as a gene vector.

Daubert et al. (1983) have made insertions in the

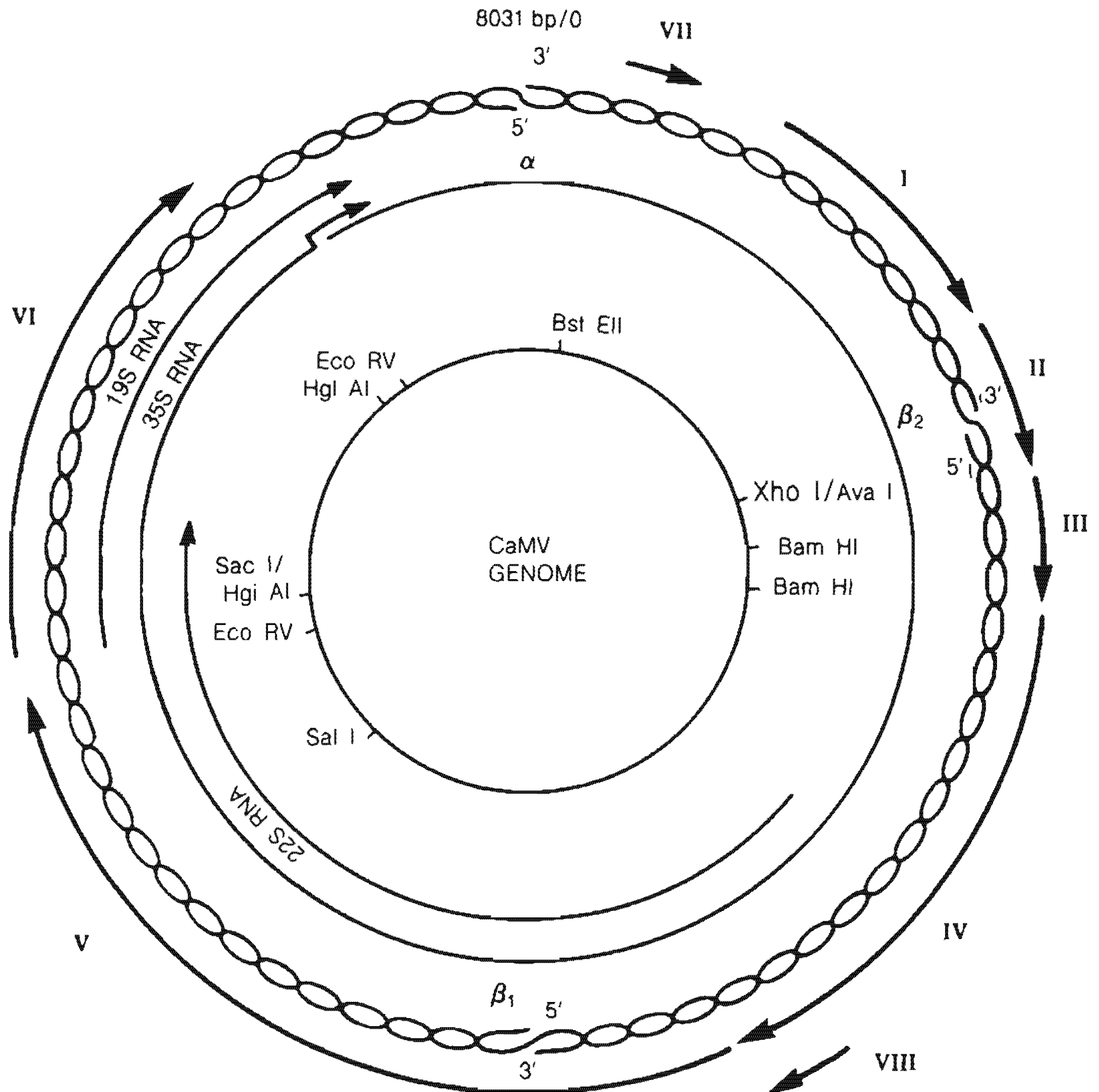

Figure 5. A schematic map of the cauliflower mosaic virus CaMV genome. The double-stranded DNA of 8000 bp is indicated by the innerwoven line. The zero position is at the top as indicated by 8031 bp/0. The DNA found in virions is a relaxed, circular molecule with three single-stranded interruptions (overlaps) at specific locations. The transcribed or minus strand has a single interruption at point α. The complementary, or plus strand, has two interruptions at points β_1 and β_2. A supercoiled, actively transcribed form of the DNA occurs in the nucleus of infected cells. The two main RNA transcripts are indicated by the lighter, inner lines labeled 19S RNA and 35S RNA. The innermost circle shows the location of various restriction sites useful in restructuring viral DNA.

large intergenic region of the CaMV genome. The intergenic region to the right of the zero map position (Fig. 5) is probably not functional since extensive insertions can be made in this region, or it can be completely deleted, without affecting biological activity of the virus. The behavior of larger insertions in region II or the large intergenic region suggests there is a much more stringent limitation on encapsidation of additional DNA in the viral chromosome than there is for replication. When inserts of 1200 bp are made into region II and plants are inoculated with this DNA, a systemic infection develops after a long delay. For example, disease development in these plants may require 2 months as opposed to the usual 2 weeks. When the DNA is examined, some of the inserted DNA is still present in region II, though most has been deleted including portions of region II. The DNA inoculated into plants several weeks earlier had obviously persisted in some manner in the initially infected cells. To account for this delay one might surmise the DNA is replicating since it is difficult to envision how the DNA would persist, and undergo spontaneous deletion, unless it was actively replicating.

The reason for the unstable nature of large insertions in the CaMV genome is unknown. It may be a reflection of a packaging limitation for virus particle formation. It seems reasonable that a size limitation may be a precondition for encapsidation in coat protein and for completely encapsidated DNAs to move well cell-to-cell to establish systemic infections. If so, this could account for the severe limitation on the size of insertions that are propagated stably during virus replication and movement.

The first successful use of one of the DNA viruses as a gene vector for higher plants was that of Brisson et al. (1984). They substituted a methotrexate-resistant dihydrofolate reductase (*dhfr*) gene of bacterial origin for region II of CaMV and mechanically inoculated this into plants as the cloned DNA. They found that the virus carried the *dhfr* gene systemically throughout the plant. Such plants became resistant to methotrexate sprays that are ordinarily very toxic. This successful expression of a *dhfr* gene in plants demonstrates the potential of DNA viruses as vectors on the whole-plant level.

Brisson et al. (1984) constructed CaMV region II replacements with the *dhfr* gene, leaving as small a nontranslated sequence as possible between its flanking genes. These genes are very closely packed in native viral DNA. Only 1-2 nucleotides occur between the termination codon of one gene and the initiation codon of the next. Consequently, these investigators reasoned that this feature of CaMV open-reading regions may be necessary for the translation of the full-length transcript as a polycistronic messenger RNA. They kept the leader sequences between translational, initiation, and termination codons to a minimum. Two plasmids containing the CaMV genome were constructed and tested. In one case, region II had been almost completely removed except for five codons, plus a stop codon, before the coding region of the *dhfr* gene. In a second case, the distance between the region I stop codon and *dhfr* initiation codon was reduced even further, to 9 bp. With both plasmids only 1 bp existed between the *dhfr* stop codon and the start codon of region III.

When the viral genome was excised and the DNA mechanically inoculated to plants, both engineered DNAs caused infections that developed systemically. Both were stably propagated and maintained in infected plants through the first infection cycle. However, the *dhfr* gene in the DNA with the longer spacer sequence between coding regions was gradually lost during a second and third infection cycle in plants. The other DNA construct with the shortest spacer between genes I and II was stable during successive transfers in plants. Other constructions with longer intergenic spacers at either the 5′ or 3′ ends of the *dhfr* coding region were considerably less stable, indicating that a close packing arrangement of genes may be a crucial factor in stability and expression in a CaMV vector (Brisson et al. 1984).

The methotrexate-resistant phenotype was demonstrated by spraying transformed and nontransformed plants with methotrexate. Healthy plants, and those infected with virus without the methotrexate-resistant genes, developed symptoms of severe senescence. Those infected with viral genomes containing bacterial *dhfr* showed few effects of toxicity.

Complementation Experiments with Defective Viral Genomes

Several attempts have been made to circumvent the low limit on the amount of DNA that can be accommodated by a caulimovirus vector. One approach has been to construct complementing systems in which essential functions would be removed from each of two components required for replication, encapsidation, and movement. Each component of the system would share functions needed for replication and systemic movement throughout the plant.

The missing functions in each component could then be replaced by foreign DNA. Such a system would be akin to the split genome of the white fly-borne geminiviruses that is probably a modification by nature to put new functions into a small genome limited in size by its ability to encapsidate in viral-coat protein. The same split-genome trait is also a common feature of many RNA plant viruses where it is very likely, again, an evolutionary adaption for packaging a larger amount of genetic material in units sufficiently small for cell-to-cell movement through plasmodesmata.

If artificial split-genome systems can be fashioned with the caulimoviruses, it may be possible to increase greatly the amount of foreign DNA that can be encapsidated for cell-to-cell movement. Investigators have prepared clones of CaMV with lethal insertions, or deletions, in various regions of the genome and tested these for complementation. When inactive clones with lethal modifications in different regions of the virus genome are inoculated into plants in pairs, infections frequently develop as if complementation had occurred. However, when the viral DNA in such plants was analyzed, only the wild-type virus genome was found. These could have arisen only as the result of recombination between mutant genomes. There is no evidence in any of these experiments that complementation occurred. Investigators have concluded that recombination precludes any chance for complementation between constructs made with a single virus genome.

Certain caulimoviruses show little, if any, hybridization with CaMV. Hybridization tests have indicated there is relatively little homology between the DNAs of CaMV and carnation etched ring virus, dahlia mosaic virus, figwort mosaic virus, mirabilis mosaic virus or thistle mottle virus. There is, however, homology between the DNAs of CaMV and the horseradish latent virus, a less distantly related virus. Lack of homology suggests the other caulimoviruses might be less prone to recombine with CaMV in mixed infections. Consequently, a need exists for testing these viruses for recombination in mixed infections.

Another approach worth investigation is to transfer virus functions to the host chromosome. This can be done easily now using the Ti plasmid-*Agrobacterium* system. The removal of one or more essential virus genes and then implanting these in the host genome would allow the replacement of deleted regions of the virus with foreign DNA. A ploy of this sort would be analogous to the COS cell system developed with SV40 virus as a vector for mammalian cells. In this case, early virus functions (large T-antigen and permissivity factors) required for SV40 DNA replication were transformed into mammalian cells that subsequently expressed these functions.

It remains to be seen how useful the caulimoviruses will be as recombinant DNA vectors. Unless complementing systems using partially deleted genomes of two different viruses can be developed, it seems unlikely that these viruses will be useful gene vectors. However, they represent one of the few sources of DNA sequences that are highly amplified in plants. Vectors of this sort for gene transfer in plants are desirable and further efforts to explore the vector potential of these viruses is well justified.

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Direct and Indirect Gene Transfer Using Pollen as Carriers of Exogenous DNA

D. Hess[1]

Abstract

The pollen system of gene transfer, proposed by us in 1974, offers several advantages: it can be practicable with cereals, it avoids somaclonal variation, and is easy to use. In direct gene transfer studies using genomic DNA, and especially transducing phages, full evidence of a gene transfer was achieved. In more recent experiments the possibility of an indirect transfer was investigated. Mutual interactions between pollen tubes of Petunia *and* Agrobacterium *were detected, including the activation of the bacterial* vir*-region by factors excreted from the pollen tubes. In progenies obtained from cocultured pollen, the regeneration pattern changed towards a more undifferentiated growth. Some calli induced by a special cotyledon assay system maintained growth on hormone-free media and showed nopaline synthase activity, indicating the transfer of tumor genes from the* Agrobacterium.

Introduction

In the early 1970s we were engaged in protoplast work and succeeded, for instance, in regenerating *Petunia hybrida* plants from isolated protoplasts or in introducing DNA and phages into protoplasts (Hess 1976). Our aim was to use protoplast systems as an alternative to the procedures used in the first transformation experiments with higher plants (Hess 1969). Even in this phase of protoplast research, limitations of protoplast-based transformation systems became apparent. It soon turned out that regeneration from protoplasts is difficult, if not impossible, in cereals. Furthermore, somaclonal mutations may mask the expression of the transforming gene.

In 1974 I proposed to use pollen as 'supervectors' to introduce foreign genetic material (Hess et al. 1974, Hess 1975). This pollen system of transformation avoids the above-mentioned limitations. In the last decade we studied the practicability of this so-called 'pollen transformation' in direct and indirect gene transfer experiments.

Gene Transfer using Pollen as 'Supervectors'

When we started our experiments, the role of *Agrobacterium tumefaciens* in transferring part of its DNA into plant cells was not yet fully understood. Therefore, in the last decade we were engaged in direct gene transfer experiments using genomic DNA, transducing phages and plasmids as donor systems. The results obtained were reviewed recently (Hess et al. 1985, Hess 1986, 1987).

In early studies, pollen of some solanaceous species were incubated for 5 hours with radioactively labeled bacterial DNA, e.g., pollen of *Nicotiana glauca* with *Rhizobium* DNA. Following a short DNase treatment to remove superficially bound DNA, rhizobial DNA could be re-extracted from the pollen tubes. Furthermore, autoradiography revealed labeling within the pollen tubes, and pollen tubes that had been burst during preparation showed radioactivity just before the opening (Fig. 1). The data are consistent with uptake of DNA into the growing pollen tubes (Hess et al. 1974).

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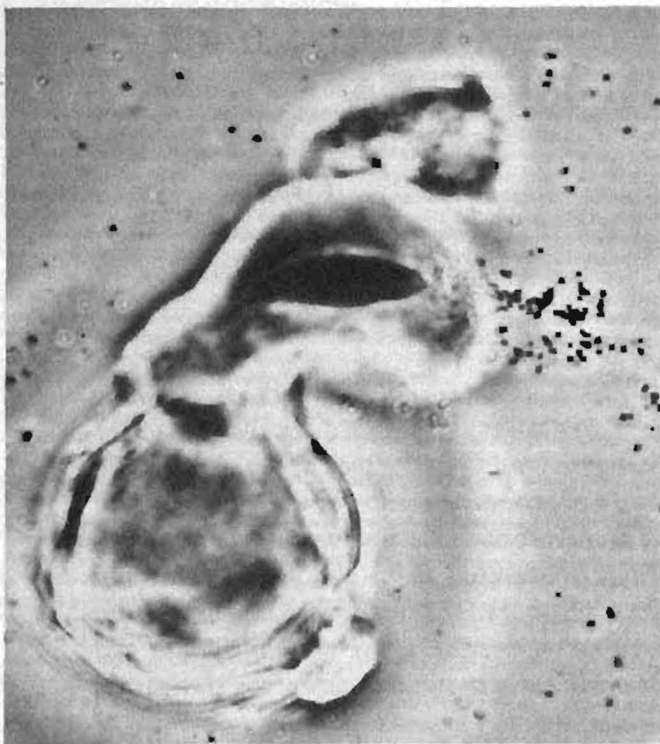

Figure 1. Autoradiograph of *Nicotiana glauca* pollen following incubation with ^{14}C-labeled DNA from *Rhizobium leguminosarum*. The pollen had been incubated for 3 hours in a germination medium containing 50 μg mL^{-1} bacterial DNA, spec. activity 0.176 μc mg^{-1}. Incubation was followed by a 5-min DNase treatment and autoradiography. There was detectable radioactivity that had been released from the pollen-tube tip burst during the preparation (Hess et al. 1974).

There are two possible ways for DNA uptake into pollen. First, in an assumed leaky phase of the intine immediately after contact of the air-dry pollen with the stigmatic fluid or with fluid in general (Heslop-Harrison 1980), and secondly via the cell wall-free tip of the growing pollen tube (de Wet et al. 1986).

Genomic DNA was used to transfer tumor genes from *Nicotiana langsdorffii* into *N. glauca* (Hess et al. 1976), and to correct a white flowering mutant of *Petunia hybrida* to anthocyanin synthesis (Hess 1980). Considering the levels of evidence for gene transfer obtained in these experiments, only phenotypical and formal genetical data could be obtained.

There are several reports dealing with transformation of maize. Mixtures of genomic DNA and maize pollen were used by de Wet et al. (1985, 1986) and Ohta (1986). It should be mentioned that none of these investigations gives full proof of a gene transfer. In a transformation study in *Zea mays* and *Solanum lycopersicum*, Sanford et al. (1985) were unable to get any results at all. This publication has to be mentioned, however, only because it demonstrates what should be avoided in pollen transformation experiments (Hess 1987).

When we started transfer experiments, using transducing phages as vectors, nothing was known about specific eucaryotic promotors. Nevertheless, we used phages of well established expression in eucaryotes (Merril et al. 1971, Horst et al. 1980). In addition, we found that nuclei isolated from the receptor *Petunia hybrida* were able to transcribe at least linear bacterial DNA (Blaschek and Hess 1977). Therefore, the basic prerequisites for successful gene transfer were available.

Using phages transducing the *E. coli* β-galactosidase (E.C.3.2.1.23) gene, we obtained phenotypical, formal genetical, and enzymological evidence for an integration of the foreign gene into chromosomal DNA of *Petunia hybrida* (Fig. 2). The bacterial β-galactosidase could be clearly differentiated from the *Petunia* enzyme. Despite this, it seemed to be advantageous to transfer a gene that had no plant counterpart.

The *E. coli* transferase (E.C.2.7.7.12) gene seemed to meet these requirements, because at the time we started our investigations no plant transferase was known. We thoroughly investigated the *Petunia* enzymes of galactose metabolism (Hess et al. 1979, Komp and Hess 1981, Dressler et al. 1982). Using enzyme microassays (Merril et al. 1972) we detected a *Petunia* transferase (Dressler et al. 1982) so that we were confronted, unfortunately, with the situation that there was a plant counterpart of the bacterial gene to be transferred.

An additional difficulty was the comparatively low expression of the bacterial transferase gene in transformed petunias. The screening procedure was based on an improved growth of transformants on galactose media (Fig. 3). By selfing heterozygous transformants, only 20% of the offspring showed an improved growth on galactose. Transformation homozygotes showed a stable percentage of more than 30% of better growing plants over their selfed generations (D. Hess: unpublished data).

Despite these difficulties, several levels of evidence of gene transfer were achieved. Phenotypical evidence is presented in Figure 3. Formal genetical evidence was obtained by selfing selected transformants over 5 generations and by reciprocal crosses. Figure 4 demonstrates the increase of plants with improved growth over 5 selfing generations starting from one single transformant. This increase, accompanied by an increase in transformation homozygotes, indicates that the original transformant was heterozygous. Corresponding results had been ob-

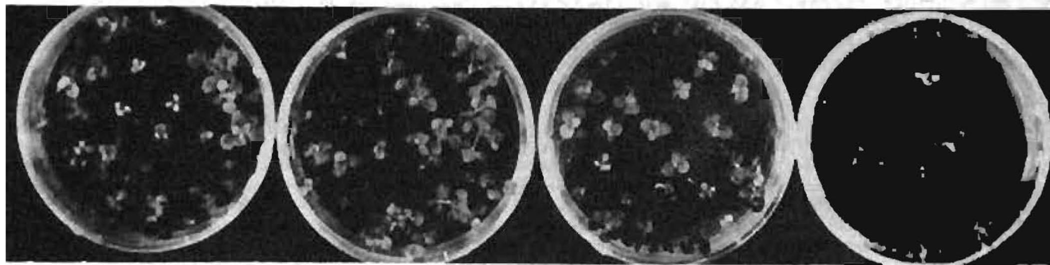

Figure 2. Evidence of the transfer of the *E.coli* β-galactosidase gene into *Petunia*. In the experiment pollen had been treated with phages transducing the bacterial β-galactosidase gene, in the control with homologous *Petunia* DNA. From an original transformant and from a control plant, selfing progenies were obtained. A 3rd-generation transformant (T) and a 3rd-generation control plant (C) were selfed, T×s and C×s, and reciprocally crossed. The progenies were screened on 4% lactose medium for seedling development and β-galactosidase activity under conditions optimal for the bacterial enzyme.

At the top of each column, photographs of representative petri dishes are shown. The growth differences between T×s and C×s, and between T×s and C×s and their reciprocal crosses are significant at $P<0.1\%$. The growth differences between C×T and T×C are not significant. For β-galactosidase activity, standard deviations are indicated as simple T lines. The figure demonstrates phenotypical (growth behavior), formal genetical (stability of the newly acquired trait over the generations, no maternal inheritance, e.g., localization on a chromosome), and enzymological (bacterial β-galactosidase activity) evidence of a gene transfer (Hess 1978).

tained in reciprocal crosses (Fig. 5). Especially, there was no maternal inheritance so that the transferred bacterial gene material has to be localized on a chromosome (D. Hess: unpublished data).

Plants with improved growth on galactose showed a significant higher transferase activity under conditions that had been optimal for the bacterial enzyme (Fig. 6). In addition, this activity was as heat-sensitive as the bacterial transferase (Hess and Dressler 1984). Southern blot analysis detected a 4.8 Md fragment in homozygous transformants that was absent from all controls (Fig. 7). This molecular

evidence completes the proof of a successful gene transfer using transducing phages in our pollen system.

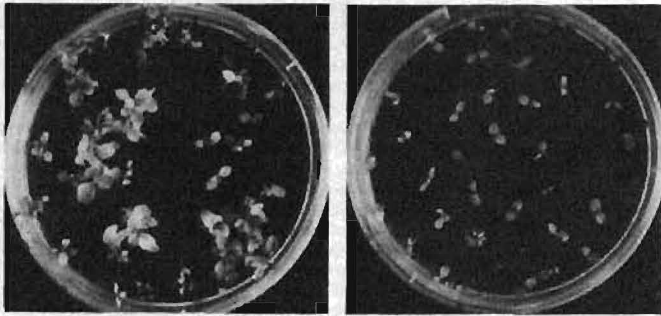

Figure 3. Phenotypical and formal genetical evidence of the transfer of the *E.coli* transferase gene into *Petunia*. In the experiment pollen had been treated with phages transducing the bacterial transferase gene, and in the control with phages transducing a mutated bacterial transferase gene. From an original transformant, and from a control plant, selfing progenies were obtained. The figure demonstrates the growth behavior of the third selfed offspring on 0.1% galactose medium: Transformant, experiment (left), control (right) (Hess 1979).

As a rule, pollen preparations show nuclease activity. However, using genomic DNA, enough carrier DNA is present to neutralize this activity, and, using phages, the gene material to be transferred is protected within the phage head (at least in our *Petunia* pollen preparations no protease activity could be detected).

Using plasmids, however, one could expect difficulties. The first attempt using a trait of plasmid origin was as early as 1975 when we tried to transfer kanamycin resistance from *E. coli* into *Petunia* (Hess 1975). This was the first attempt to use kanamycin resistance as a marker. This, unfortunately, was the only positive aspect of our plasmid work. The results were inconclusive, and also when we repeated the experiments with improved methods (Hess 1981). Similar conclusions were reached using plasmid DNA of various origins (Negrutiu et al. 1986, Sanford and Skubik 1986).

There are several reasons for this failure. Sanford and Skubik (1986) used no suitable screening system. In addition they neglected the possibility of a

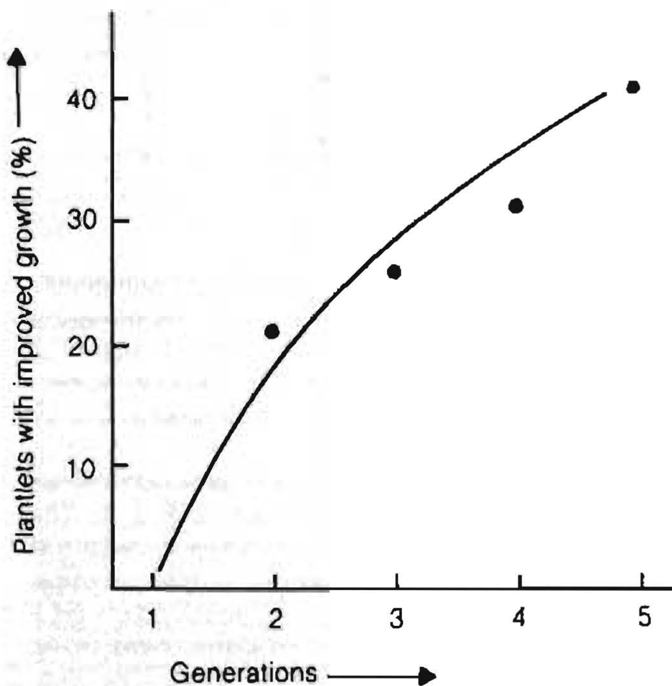

Figure 4. Formal genetical evidence of the transfer of the *E.coli* transferase gene into *Petunia*: increases in the percentage of plants with improved growth on 0.1% galactose medium in selfed progenies derived from one original transformant. The control values had already been substracted (D. Hess: unpublished data).

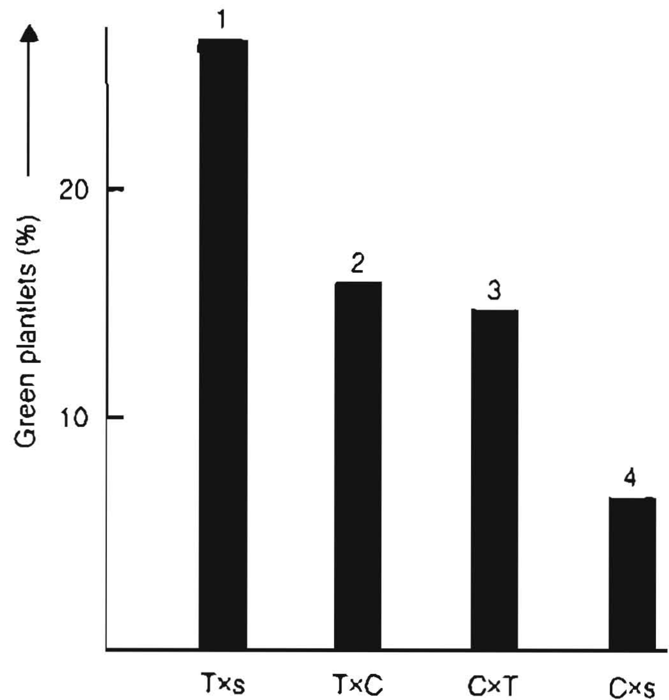

Figure 5. Formal genetical evidence of the transfer of the *E.coli* transferase gene into *Petunia*: selfing and reciprocal crosses of a 3rd-generation transformant (T) with a control plant (C), which had been derived by selfing from an original transformant (T×s) and a control (C×s) plant, respectively. The resulting offspring was screened on 0.1% galactose. The differences 1/2 and 3/4 are significant at $P<0.1\%$; difference 2/3 is not significant (D. Hess: unpublished data).

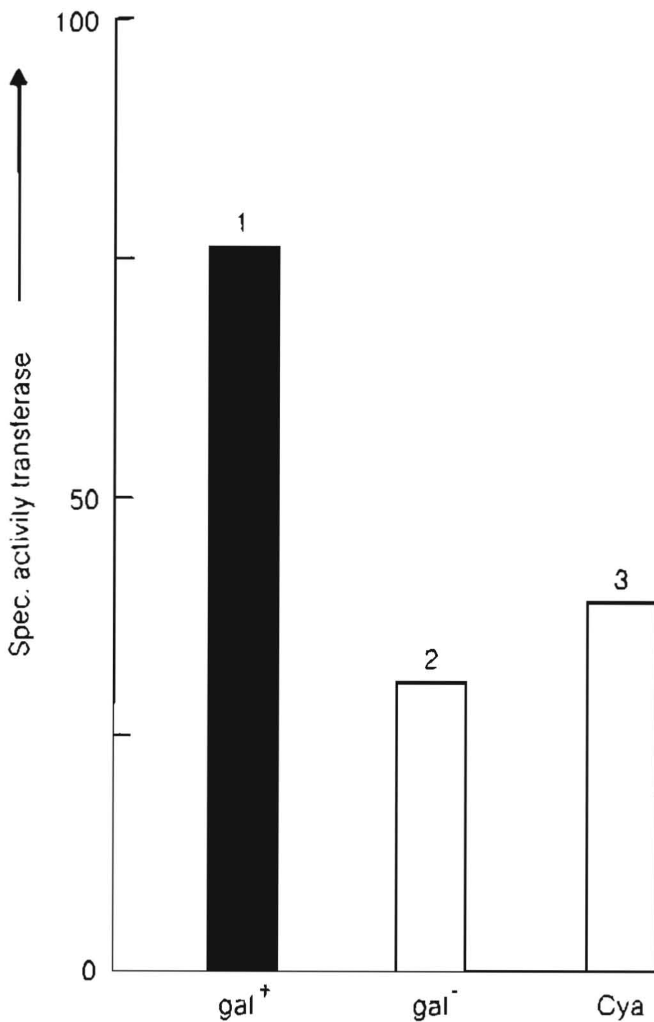

Figure 6. Enzymological evidence of the transfer of the *E.coli* transferase gene into *Petunia*: spec. transferase activity under conditions optimal for the bacterial enzyme. Third-generation plants derived by selfing from an original transformant (gal$^+$:pollen treated with phages transducing the transferase gene), and from two different controls (gal$^-$:pollen treatment with phages transducing the mutated transferase gene; Cya: pollen treated with homologous *Petunia* DNA) were assayed. Difference 1/2 is significant at $P<0.1\%$; difference 1/3 at $P<0.1\%$; difference 2/3 is not significant (Hess 1987).

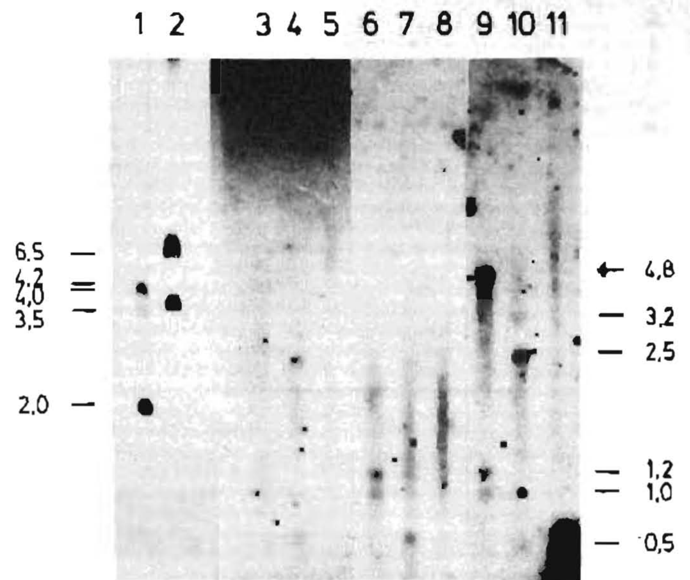

Figure 7. Molecular evidence of the transfer of *E.coli* transferase gene into *Petunia*. Southern blotting onto Gene Screen Plus and hybridization to a nick-translated probe synthetisized from total dvgal 120 plasmid DNA.

1,3,5,7,: restricted with Hind III,
2: restricted with EcoR1;
4,6,8: uncleaved.
1,2: dvgal;
3,4: Cya (control, cf. Fig. 6);
5,6 gal$^-$ (control, cf. Fig. 6);
7,8: gal+ (transformant, cf. Fig. 6).

The size of the restriction fragments is indicated in Md. The arrow indicates a fragment containing bacterial DNA which was found in transformants only (Hess and Dressler 1984).

nuclease degradation of the Ti plasmids they intended to use. Negrutiu et al. (1986) tried to remove nucleases by washing the pollen, but possibly nucleases between exine and intine or within the cavities of the exine were not removed. Other possibilities for their failure are the very high kanamycin concentrations they used and that the pollen following washing was no longer able to take up DNA (Hess 1987).

Mutual Interactions in Cocultures of Pollen with *Agrobacterium*

A coculture medium was developed in which both *Petunia* pollen and *Agrobacterium* showed optimal growth and development. Following 5 hours of coculture, the pollen/*Agrobacterium* mixture was plated onto Cefotaxim® agar to kill the bacteria, and pollen-tube growth was measured over the next 48 hours. Coculture with both kanamycin-sensitive and resistant *Agrobacterium* strains stimulated pollen-tube growth on kanamycin-free media considerably, apparently by growth factors excreted by the bacteria (Fig. 8). On kanamycin (KM) media, only the kanamycin-resistant *Agrobacterium* strain was able to promote pollen-tube growth. This effect was not obtained by media filtrated through membranes that permitted the passage of proteins and nucleic acids, but not of bacteria.

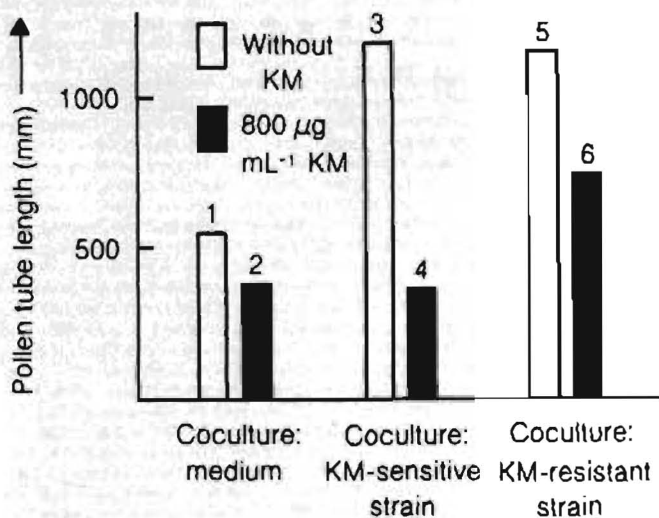

Figure 8. Stimulation of *Petunia* pollen-tube growth by coculture with both a kanamycin-sensitive and a kanamycin-resistant strain of *Agrobacterium tumefaciens*. Pollen-tube length was measured 24 h after plating the pollen/*Agrobacterium* mixture on Cefotaxim® medium, with and without kanamycin (KM). All the differences except 3/5 and 2/4 are significant at $P<1.0$ or $P<0.1\%$ (D. Hess and J. Suessmuth, University of Hohenheim, personal communication 1987).

Activation of the *vir*-region is needed before *Agrobacterium* is able to transfer DNA into plant cells. This activation is mediated by defined phenolic compounds excreted by plant material. To study the activation of the *vir*-region, it was combined with the *E. coli* β-galactosidase gene. In this construction β-galactosidase activity indicates the activation of the *vir*-region (Stachel et al. 1985). FITC-labeled® *Agrobacteria* fix onto the growing pollen tube in a polar manner. On the tip of the pollen-tube cell wall no bacteria were detected, probably because there were no receptor sites.

Using the β-galactosidase assay, we were able to demonstrate *vir*-activation by preparations of pollen ingredients, but not by stigmatic preparations and not by exudates from pollen nor stigma (Fig. 9). HPLC (High-Pressure Liquid Chromatography) and MS (mass spectometric) studies, however, revealed the presence of kaempferol-3-glucosyl-galactoside, the main factor responsible for *vir* activation in all four sources of *vir*-activators mentioned above. The highest concentrations were found in preparations of pollen ingredients. In the other preparations the amounts of the four factors were so low that the β-galactosidase test was not possible. This does not exclude an activation of *Agrobacteria* fixed onto the pollen tube or after pollination onto the stigma, because they are exposed to much higher levels of the substances just excreted. These results demonstrate that, in cocultures, pollen/*Agrobacterium* activation of the *vir*-region is a prerequisite for gene transfer, and is possible.

A mixture of *Petunia* pollen and *Agrobacterium tumefaciens* C58 (wild type) was used for pollination of *Petunia*. The seeds were grown under sterile conditions on Whites (WH) medium. Callus formation was induced in several ways. The most efficient was to remove a cotyledon from plantlets at about 2 weeks old and to place it stalk upwards onto WH medium. On the cut surface of the stalk, a callus developed within about 3 weeks. Part of the calli and of the stalk tissue underneath the calli showed differentiation into roots and shoots.

In both the experiment and controls, about 80% of the cotyledons developed calli. In the experiment, however, more calli maintained growth and fewer roots and shoots differentiated from the calli and cotyledons (Fig. 10). Therefore, in the 1st generation derived from cocultured pollen, undifferentiated growth had been favored. Bacterial growth factors might first trigger this effect, too, but it will be difficult to explain its perpetuation from pollen via the seedlings to the calli (Hess 1987).

Teratomic, or tumor-like, formations were detected in unwounded seedlings, in experiments as well as in controls, and are due to culture conditions.

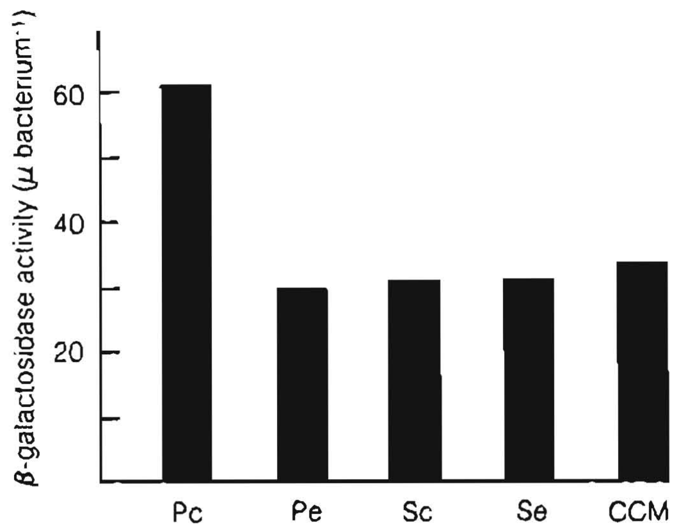

Figure 9. Activation of the *vir*-region of *Agrobacterium tumefaciens* C58 (wild type) by preparations from the pollen (Pc) and the stigmata (Sc), and by pollen (Pe) and stigma (Se) exudates of *Petunia*. The differences between Pc and the other values are significant at $P<1.0\%$. The other differences are not significant. CCM: Coculture medium (R. Zerback and D. Hess, personal communication 1987).

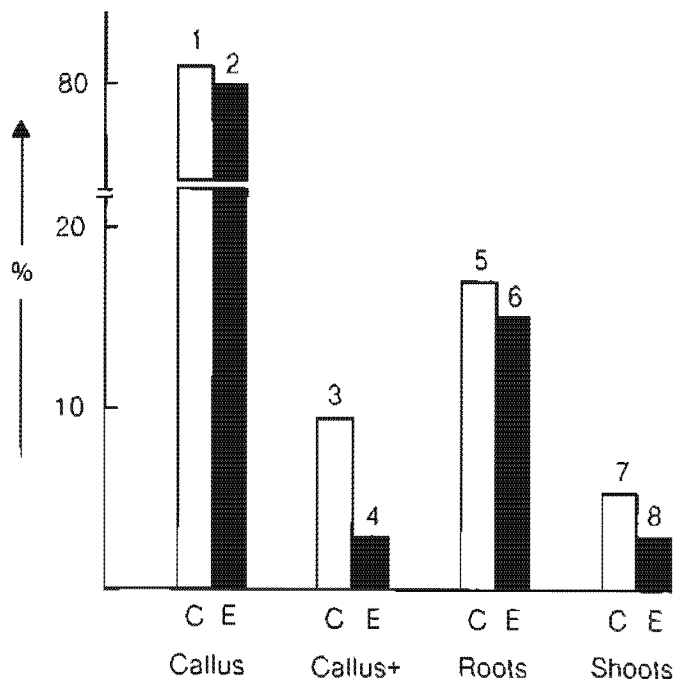

Figure 10. Callus formation and differentiation from cotyledons of the first progenies derived from pollen that had been cocultured with *Agrobacterium tumefaciens* C58 (wild type) in the experiment (E) and with coculture medium in the control (C). In both experiment and control, respectively, ca 800 cotyledons had been assayed. Callus: callus from the cut surface of the cotyledons stalk, dead or alive, with and without differentiations. Callus + : dead callus. Shoot and roots: those that had differentiated from the calli or from the adjacent tissue. The difference 1/2 is not significant; all the other differences are significant at $P<0.1\%$ (Hess 1987).

Therefore, we first induced callus formations by wounding, and thereafter the calli obtained were screened on hormone-free modified MS medium. The callus material we used were the cotyledonar stalk calli just mentioned.

Some of the experimental calli maintained growth on hormone-free modified MS medium in nine subcultures over a period of more than 1 year. Some showed nopaline synthetase activity. Davey et al. (1980) and Lorz et al. (1985) believe that growth on hormone-free media and nopaline synthetase activity are sufficient evidence for gene transfer. Despite this, we plan to initiate a southern blot analysis as soon as we have enough callus material. It seems difficult, however, to explain our results without a transfer of tumor-inducing bacterial genes (Hess 1986).

Conclusion

Studies are under way to improve the transformation frequency by supplying *vir*-activating factors produced by our plant material. In the meantime our investigations have been extended to summer wheat. Wheat tissues also excrete *vir*-activating factors. It therefore seems worthwhile to evaluate the indirect pollen system using wheat.

Acknowledgment

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Genetic Transformation of Cereals

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Abstract

The most efficient delivery system in genetic transformation of higher plants is the Ti plasmid of Agrobacterium tumefaciens. *However, this system does not work in cereals. Although the cereal protoplasts can be directly transformed, regeneration of functional plants from the cereal protoplasts does present some technical difficulties. Problems associated with plant regeneration from cells in culture can be overcome by transforming the male or female gamete and allowing fertilization to take place with one or both of the transformed gametes involved. The male gametophyte of maize can serve as a carrier of naked DNA to the embryo sac, where egg transformation takes place as a consequence of fertilization. Caryopses so developed can germinate and produce transformed plants.*

Introduction

There are three pathways to efficient transformation of plants. First, transformation of cells or protoplasts in culture followed by regeneration of functional plants. Second, transformation of the sperm and/or egg followed by fertilization and embryogenesis. Third, transformation of the zygote followed by embryogesis. The most efficient delivery system of exogenous DNA is the Ti plasmid of *Agrobacterium tumefaciens* (Horsch et al. 1985). This transformation system works in many dicotyledoneous and some monocotyledoneous species (Hernalsteen et al. 1984; Hooykaas-Van Slogteren et al. 1984). *Agrobacterium* infects maize (*Zea mays*) callus (Grimsley et al. 1987). Cereal cells can also be directly transformed and pollen can be used to transport naked DNA to the embryo sac where transformation is achieved during fertilization. Techniques being used to transform cereals are discussed first for cells in culture and then for gametophytes.

Transformation of Cells in Culture

Plant protoplasts, cells from which the cell wall has been removed, are readily transformable by coculture with naked DNA (Paszkowski et al. 1984). The technique used is similar to that developed for transformation of protoplasts with *Agrobacterium* (Krens et al. 1982). Protoplasts in a medium consisting of 0.1 mg L^{-1} 2,4-D, 1 mg L^{-1} NAA, and 0.2 mg L^{-1} BAP (Nagy and Maliga 1976) with 13% w/v (weight/volume) PEG are incubated at room temperature for 30 min with plasmid DNA. Developing cell clones are transferred to solid medium, transformed calli are selected, and regenerated into plants following standard culture techniques (Paszkowski et al. 1984). Transformation efficiency is enhanced by encapsulating the DNA in liposomes (Caboche and Deshayes et al. 1985; Deshayes, et al. 1985), or by introducing the DNA into protoplasts by electroporation (Hashimoto et al. 1985; Fromm et al. 1986). Cauliflower mosaic virus DNA (Odell et al. 1985), brome mosaic

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virus RNA (French et al. 1986), as well as the Ti plasmid allow for expression of recombinant genes in plant cells. The major problem with direct transformation in cereals is plant regeneration from protoplasts. Yamada et al. (1986), however, succeeded in regenerating functional plants from protoplast-derived callus of rice (*Oryza sativa*). Techniques will probably be developed to facilitate plant regeneration from protoplasts of other cereals.

Gametophyte Transformation

The egg or zygote can be transformed by microinjection. This is readily achieved in animals. Palmiter et al. (1983) transformed mice by microinjecting excized zygotes with a DNA fragment containing the metallothionein I gene fused with the structural gene coding for human growth hormone. Treated eggs were transplanted into surrogate mice mothers. Transformed offspring had elevated levels of growth hormone, and grew larger than their normal siblings.

The female gametophyte (embryo sac) of higher plants is difficult to remove intact from the ovule, and attempts to induce embryogenesis in egg or zygote of exised embryo sacs have so far failed (Allington 1985). Exogenous DNA can, however, be introduced into the ovary loculus (Hepher et al. 1985). Soyfer (1980) injected developing caryopses of a waxy mutant of barley (*Hordeum vulgare*) with total cellular DNA of a barley genotype having normal starch production. The offspring produced pollen that segregated for normal and waxy. Similarly, Zhou et al. (1983) achieved transformation in upland cotton (*Gossypium hirsutum*) when ovules were injected with cellular DNA of sea island cotton (*Gossypium barbadense*) soon after pollination. The progenies of transformed plants simulated genetic introgression between the two species, and segregated for various traits through 3 subsequent generations. These experiments indicate that naked DNA introduced into the embryo sac can affect transformation of the egg.

A convenient method of introducing exogenous DNA into the embryo sac is to use the developing male gametophyte (germinated pollen grain) as a carrier of tranforming DNA. Hess (1975) demonstrated that bacteriophage was absorbed from a culture medium by germinating pollen. Subsequent studies (Hess et al., 1985) found that phage transducing the *Escherichia coli* β-galatosidase or galactose-1-phosphate uridyltransferase genes are successfully transferred to offspring of *Petunia* plants that were selfed with phage-treated pollen. Sanford et al. (1985), however, failed to induce transformation of tomato (*Lycopersicon esculentum*) or maize (*Zea mays*) following pollination with DNA-treated pollen. Negrutiu et al. (1986) demonstrated that in tobacco (*Nicotiana tabacum*) little DNA is taken up by germinating pollen. Sanford and Skubik (1986) found that, although *Agrobacterium tumefaciens* infects tobacco pollen, pollination with treated pollen failed to produce clear evidence of transformation. Be that as it may, pollen-mediated transformation has been achieved in maize, and it will probably also work in sorghum (*Sorghum bicolor*) and pearl millet (*Pennisetum glaucum*).

Maize was transformed using this technique by de Wet et al. (1985, 1986), and Ohta (1986). The technique used is simple. Ohta (1986) used a 0.3 Mol sucrose solution as a pollen germination medium. Fresh pollen of the recipient genotype was either mixed with DNA in a buffer, and applied to the pollen germination medium on the stigmas, or DNA was added to the pollen germination medium before the solution was transferred to the stigmas. The method preferred by de Wet et al. (1986) is to germinate pollen in a petri dish before DNA is added. It was found that a pH above 7 protects DNA from becoming degraded in the pollen germination medium, and that high salt concentrations in the medium facilitated DNA uptake by the pollen tube at pollen germination. Fresh pollen is collected soon after anthesis and germinated in a petri dish on a 0.3-mm layer of pollen germination medium (PGM) consisting of 15% sucrose, 1.3 mMol calcium nitrate and 1.6 mMol boric acid in water. After 3–5 min at 30–35°C, approximately 80% of the 60–100 thousand grains used to pollinate 10–20 ears show signs of pollen-tube growth (as determined by microscopic investigation). Incubation is initiated at this time by adding 9 mL of PGM and 40 μg DNA in 0.5 mL SSC buffer (0.15 Mol NaCl, 0.015 Mol sodium citrate at pH 7.2). This pollen slurry is directly transferred with a pipette to stigmas that were previously cut, protected from stray pollen by a shoot bag, and allowed to grow for 15–24 hours. Pollination of each ear requires approximately 1 min, and PGM evaporates in about 15 min on stigmas of ears covered with paper bags.

Ohta (1986) used a maize genotype homozygous for a series of dominant endosperm characters on a full background of aleurone pigmentation as DNA donor, and the same genotype with the recessive alleles as the recipient. Using the pollen technique, marker genes were transferred to the offspring of the

Table 1. Effects of pollen germination and of pollen incubation with exogenous DNA on selfed offspring of maize inbreds B73 and DP194.

Treatment	Numbers of				
	Ears	Kernels	Seedlings	Mutated	Transformed
Selfed B73	70	20051	19940	21	0
Germ. pollen	568	5879	5869	6	0
B73 DNA	568	4985	4421	238	0
Tripsacum DNA	568	5172	4362	211	3
Selfed DP194	80	20221	19540	28	0
B14 A DNA	2169	11001	9186	406	2

recipient genotype in the embryo, endosperm, or both endosperm and embryo. Rate of transformation ranged from 0.00 to 9.29% for individual marker traits. The highest rate of transformation was obtained when a pollen/DNA paste was prepared and transferred to DNA in buffer on the stigmas.

In experiments by de Wet et al. (1986) maize inbreds B73 and DP194 were self-pollinated with pollen incubated in total cellular DNA of maize genotype B14 A carrying the marker gene for rust resistance, or of *Tripsacum dactyloides*. Data obtained are summarized in Table 1.

Seed-set was low following treatment of pollen with DNA. A majority of ears produced no seeds, and those that did produce seed had 1–10 kernels per ear. Low seed-set is due to breakage of pollen tubes during pollination, and failure of pollen tubes to penetrate stigmas. Seeds, however, germinate and usually produce functional seedlings.

Treatment of pollen with cellular DNA has a mutagenic effect on the offspring of the recipient genotype. Approximately 5% of seedlings were weaker than their siblings and a majority of the weak seedlings either died because of developmental abnormalities such as chlorophyl deficiencies, or developed into small and barren mature plants. This was true following self-pollination with alien or self DNA. Treatment with alien DNA resulted in transfer of marker genes from the DNA donor to the recipient genotype. Transformation rates (number kernels : number transformed) for specific marker genes were 0.00–0.02–0.06% (Table 1). Transformation occurred in both endosperm and embryo of the same kernel or in the one or the other. Genetic studies indicated that one or more copies of the transforming gene became incorporated into the recipient genome, and that insertion may be on more than one chromosome.

Transformation using the pollen technique can be unequivocally demonstrated only by the presence of alien DNA sequences introduced through pollen during fertilization in offspring of treated plants. Several cloned genes are available for transformation studies. The bacterial APH II gene (kanamycin resistance), under control of enkaryotic promoters and polyadenylation signals, was selected for transformation of maize.

The APH II gene encodes in bacteria a protein product, that by a process of phosphorylation deactivates members of the aminoglycoside family of antibodies typified by neomycin, kanamycin, and G148 (Rothstein and Reznikoff 1981). When the bacterial expression control sequences at the 5′ end of this gene are replaced by eukaryotic expression controls derived from *Agrobacterium tumefaciens* opine synthase genes, or from the 19S or 35S promotor region of cauliflower mosaic virus (CaMV), and transcription termination and poly(A) addition signals from one or other of these sources are added to the 3′ end of the gene, it is capable, following transformation, of conferring antibiotic resistance on a wide range of higher and lower eukaryotic cells (Fraley et al. 1983; Herrera-Estrella et al. 1983; Horsch et al. 1984; Hooykaas-Van Slogteren et al. 1984; Horsch et al. 1985). In a recent report by Paszkowski et al. (1984), such a construct using the CaMV gene VI (19S RNA) promotor coupled in a translational fusion with the APH II gene and terminated with the CaMV RNA termination and poly(A) addition signals, all maintained in an *E. coli* vector, was shown to be capable of transforming cells of *Nicotiana tabacum* to kanamycin resistance through direct uptake of DNA by protoplasts.

This plasmid (pABD I) was made available to us by Ingo Potrykus of the Friederich Miescher Institute at Basel. For transformation assays we used maize inbred B73 or DP194. We have shown that

seedlings of these maize inbreds turn chlorotic and eventually died when treated with kanamycin at the time of germination. To provide a second selection marker, a plasmid (pCW 45) was used that contains the right and left border regions of a Ti plasmid, bracketing an intact nopaline synthase gene together with the APH II gene, linked to nopaline synthase gene controlling sequences. This provided an opportunity to assay for activity of the nopaline synthase gene, in embryos about 20 days after pollination.

Plants were grown and pollinated in nurseries. Pollen was germinated in a petri dish, and 15 mL of pollen germination medium and 5 mL of plasmid DNA in buffer were added to make a concentration of approximately 3,4,8 or 12×10^9 plasmids per mL. Two mL of this mixture were used to pollinate each maize ear. Ten or fewer kernels were produced per ear. Data obtained are summarized in Table 2. All concentrations of plasmid DNA used effected transformation, both maize inbreds B73 and DP194 were transformable, and both plasmids, pABD I (APH II under control of CaMV VI), and pCW 45 (APH II flanked by the right and left border regions of a Ti plasmid) expressed in the maize genome.

To screen for kanamycin resistance, seeds were germinated between layers of blotting paper that were kept moist with a solution of 300 μg/million kanamycin in distilled water. Seedlings appeared normal at germination. They were transplanted to soil 5 days after germination and watered normally. Two to 3 weeks later leaves of susceptible plants started to turn white, and all new leaves lacked chlorophyl. These seedlings eventually died. Seedlings that were resistant to kanamycin remained green and continued to grow normally. Some surviving seedlings produced leaves with white streaks, suggesting that individual meristematic cells can lose the ability to resist kanamycin. Green seedlings are recognized as having been transformed. The rate of kanamycin-resistant transformation was 2.2% in maize inbred DP194 and 1.7% for inbred B73 with plasmid pABD I, and 1.0% in B73 with plasmid pCW 45.

Seedlings that did not show chlorophyl deficiencies after 4 weeks were transferred to individual pots. Total cellular DNA of these plants was extracted and purified, and used in Southern blot experiments with ^{32}P-labeled plasmids (Southern 1975). These experiments confirmed the presence of the APH II gene in the genome of kanamycin-resistant B73 and DP194 maize derived from pABD I-treated pollen, and suggested that multiple copies of the DNA were present. Comparable studies using the pCW 45 transformed resistant plants gave equivocal results indicating no, or low, copy number integration. The pollen transformation technique is now being tested with sorghum and pearl millet. Hybrid seeds of these cereals are produced by using isogenic male-sterile and male-fertile lines as parents. In transformation experiments, a male-sterile line is pollinated with treated pollen of the isogenic male-fertile line. Pollen germination of both species is rapid, and fertilization is achieved within 1 hour after pollination.

Table 2. Seed-set and transformation of maize inbreds DP194 and B73 that were selfed after pollen was treated with plasmids containing the bacterial APH II kanamycin resistant gene.

Treatment[1] plasmid	Plasmids mL^{-1}	Inbred	No. tested kernels	Seedlings resistant	% Transformation
pABD I	3×10^9	DP194	280	4	1.43
pABD I	3×10^9	DP194	650	17	2.62
pABD I	4×10^9	DP194	300	8	2.67
pABD I	4×10^9	B73	100	0	0.00
pABD I	4×10^9	B73	400	0	0.00
pABD I	8×10^9	B73	141	2	1.42
pABD I	8×10^9	B73	141	3	2.13
pABD I	8×10^9	B73	350	6	1.71
pCW 45	8×10^9	B73	1100	3	0.27
pCW 45	8×10^9	B73	700	15	2.14
pCW 45	12×10^9	B73	104	1	0.96

1. Experiments with the same plasmid and concentration were repeated on at least two different days.

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Plant Molecular Breeding

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Abstract

Plant molecular breeding involves the selection of transformants from the offspring obtained after the introduction of either isolated gene(s), or the total exogenous DNA containing the target gene(s). Both techniques show considerable promise for genetic improvement of crop plants. This recombinant DNA technology has several limitations in its application to agriculture. The knowledge of molecular genetics of most crop plants is rudimentary. A successful application of this technique entails substantial investment in equipment and trained personnel. On the other hand, the technique of direct introduction of exogenous genomic DNA into plants is relatively simple, and it has been applied successfully in China: new varieties of cotton and rice are described. Breeding lines with increased yield, quality, or disease resistance have been obtained with this technique. It is demonstrated that some of these acquired traits are retained for at least 6–10 generations.

Introduction

Plant molecular breeding involves the introduction of recombinant DNA or total genomic DNA into the plant, and screening the progeny for the chosen traits (Zhou et al. 1983; Huang et al. 1981; Zhou 1978). Complexities owe much of their origin to an inadequate understanding of what genes to transfer, how to select, and eventually what impact the new genotype may have on agriculture. This paper deals with the Chinese experience in genetic improvement of cotton and rice using the technique of plant molecular breeding. The technique used was the introduction of exogenous DNA into the embryo sac after pollination.

The critical part of this technique is for the exogenous DNA to be correctly placed, and timed so that the DNA can pass through the pathway of the pollen tube to enter the embryonic sac and transform the egg, zygote, or early embryonic cells. Following such a treatment, the seeds are collected, planted and, after growth, the offspring selected for transformed traits. The method and timing of the introduction of DNA vary with different plant species.

The pathway of the pollen tube between the microphyle and embyonic sac is formed physiologically in a certain time after pollination. It is much wider than the space inside a pollen tube. Actually it is a tunnel or a 'free way' for the pollen tube(s) to pass through the nucellus and enter the embryonic sac. Therefore, it is a natural pathway to be applied to introduce the exogenous DNA (gene) into the embryonic sac and transform the germ line cell(s). The transformation rate could be as high as 10^{-2}. Hess (1980), de Wet (1985), and Ohta (1986) used DNA-treated pollen or mixed the DNA with the pollen to pollinate the plant. The DNA were probably carried by the pollen tube instead of the pathway of the pollen tube we used. There is one more step in their method than in ours for the introduction of exogenous DNA (gene).

The introduction of exogenous DNA (gene) by the pathway of the pollen tube should be performed precisely at the time after the pathway has appeared and before it becomes blocked by the developing pollen tube. If the flower is big, as in cotton, it is

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convenient to inject the DNA from the top of the ovary, in order to have an even distribution of the DNA in the ovules. If the flower is small, as in rice or rape, it is best to cut off the stigma, to retain as short as possible a section of style, drop the DNA onto the cut surface, and allow the DNA to be drawn into the ovary.

The success with this technique has been demonstrated in cotton with *tritium*-labeled DNA. It was shown that, 30 min after DNA injection, some ovules have DNA in their embryo sacs. From 2 to 4 hours later, 80% of the ovules have exogenous DNA. It is clear that exogenous DNA can only pass through the nucellus from the microphyle to the embryo sac, by the pathway of the pollen tubes.

The egg cell, zygote, and the early embyonic cells are without normal cell-wall cells. So they behave as protoplasts and take up exogenous DNA. To demonstrate this, recombinant DNAs of M13 (mp7) with different common sequences from the receptor were injected into cotton ovaries to allow it to transform the zygote in the embryo sac. A Sau 3A restricted Southern blot of the DNA from mature seeds showed the integration of the M13 DNA which was not found in the original cotton DNA. Kanamycin-resistant gene plasmids (kindly sent by Dr Simpson and Dr Sun of ARCO Plant Cell Research Institute) were respectively recombined with common sequences of cotton and rice (Shen and Zhou 1985), and introduced into cotton and rice using the appropriate technique for each plant. Some of the 1st-generation recovered plant showed higher resistance to kanamycin than did their parents.

Introduction of Exogenous DNA into Cotton after Pollination

During 1978-86, 133 combinations were made in the Institute of Industrial Crops, Jiangsu Academy of Sciences. Among them 71 combinations passed from their 2nd to their 10th generations (D_2 to D_{10}, D meaning DNA). They were combinations with self DNA and DNA from intraspecies, interspecies, intergenus, and interfamilies, respectively. We used two upland cottons (*G. hirsutum*: one glandless and the other with red leaves) and one sea island cotton (*G. barbadense* 416) to accept their own DNA. No variation has been seen among their offspring during 6 generations. They were inbred and homozygous, as were the original acceptors.

In intraspecies transformation of DNA from different varieties of *G. hirsutum*, no big variations were seen among the offspring although there were some quantitative alterations in the phenotypes, e.g., the size of the leaves, and bolls, deeper or lighter green in the leaves, etc. But if a disease-resistance donor DNA was put into a sensitive receptor, e.g., wilt diseases caused by *Fusarium oxysporum*, or *Verticillium dahliae*, the resistance transference was obvious. In the interspecies transformation of *G. barbadense* (416) DNA added to *G. hirsutum* (glandless), the offspring showed extensive variation, and sterility was common. Some variant offspring stabilized in the 1st generation, or few segregated for selected traits in a non-Mendelian fashion. The latter category might have been effected by transposable elements.

Intergeneric transformation was also performed with *Abutilon avicennae* DNA added to *G. barbadense*. One offspring was obtained. It resembled *G. barbadense*, but grew taller and yielded better than its parent.

Twelve genotypes of wild cotton were used as DNA donors on *G. hirsutum*. Three of the combinations gave positive results in phenotypic variation. Resistance to *Fusarium oxysporum* or *Verticillium dahliae* were introduced into *G. hirsutum* through the use of DNA from within and between species. The rate of variation following introduction of DNA ranged from 0 to 10% among offspring, depending on the combination and environmental factors.

Sixty microinjections of *G. hirsutum* with DNA from *G. barbadense* 416 resulted in five bolls. There are on an average 35 ovules per boll and 175 seeds were expected. We obtained only 37 seedlings, 28 of which had the hairless characteristic of the donor. Seventeen of the D_1 plants showed changes in size and petal number of the flowers, or shape and number of anthers from their parents. The donor and the acceptor used were inbreds that were maintained by controlled selfing. No variation has been found in the offspring of the controls.

Molecular Breeding of Cotton

By using the technique of exogenous DNA introduction after pollination, a series of new cotton varieties were produced. Here we present three examples.

Intraspecies combination of wilt (*Fusarium oxysporum*) transfer

The wilt-resistant donor was *G. hirsutum* (52-128). The acceptor, Jiangsu 1, a disease-sensitive *G. hirsu-*

tum cultivar from Jiangsu province, has a higher yield and a better quality of lint than (52-128). DNA from (52-128) was introduced into Jiangsu 1 in 1979. One D_1 plant was selected because it showed wilt resistance; it was given the number 3072. This genotype was multiplied in the field for 7 generations (1980-86). The level of resistance among offspring was as high as in the donor (52-128). The yield of cotton fiber of 3072 was similar to that of Jiangsu 1 in a normal, uninfected field, but 30% higher in *Fusarium*-infested fields. The quality of the fiber was the same as that of Jiangsu 1.

Interspecies combination of wilt (*Verticillium dahliae*) transfer

The resistant donor was *G. barbadense* (7124). The *Verticillium*-sensitive acceptor was *G. hirsutum* (9101), which is a *Fusarium oxysporum* resistant variety. The introduction of DNA was performed in 1980. A *Verticillium*-tolerant D_1 was selected and the genotype was multiplied. This new variety (1138) yields 20% higher than the acceptor. The bolls are larger and the fibers are stronger than those of either parents. It has been genetically stable since D_1, that is 7 generations.

Intergeneric combination

The DNA donor was an *Abuliton avicennae* and the acceptor was *G. barbadense* (416). This work was performed in 1978. One plant derived from this experiment differed phenotypically from its siblings. The phenotype of this plant was very much like the DNA acceptor, except for the plants being taller, bolls being bigger, and yield of fiber higher. There were no qualitative alterations but only quantitative changes in the variant. Except for height, which is decreasing, the other characteristics have been retained up to D_8.

Introduction of Exogenous DNA into Rice

This work was performed in the Institute of Crop Breeding and Cultivation, Chinese Academy of Agricultural Sciences, Beijing. The donor plant was the grass *Spartina*. The DNA of this donor was injected into rice ovules 2-4 days after selfing. Among 1st-generation offspring, a single plant had a compact growth habit with broad, short, stiff, and erect leaves with only three nodes above the ground. Protein content and amino acid composition in the seeds of the 3rd-generation offspring were significantly higher than those in the recipient parent.

Exogenous DNA was also introduced into rice through the pathway of the pollen tube. The style was cut off 2-3 hours after pollination, and DNA of a rice genotype with purple glumes was introduced at the place of excision. Several plants with purple glumes appeared among the offspring.

A new rice variety was obtained by introducing maize (*Zea mays*) DNA into *Oryza sativa*. The phenotype of this variety (Jing-Yin 47) is principally that of the acceptor, except for reduced stature, earlier maturity, and greater tolerance of drought. This variety is suitable for cultivation in the northern part of China (Duan and Chen 1985).

Conclusion

Introducing donor DNA that carries gene(s) of interest into plants is simple. Breeders can use this technique to improve crops. The strategy for development of plant molecular breeding, as outlined in this discussion, emphasizes two levels of introducing gene(s). One is the use of recombinant DNA, and the other is the direct use of donor DNA.

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Potential of Complementary DNA Techniques for Detection of Viruses

D.J. Robinson[1]

Abstract

Spot hybridization tests, in which labeled complementary DNA is used to detect viral nucleic acids in plant extracts, are particularly useful in situations where serological tests are unsatisfactory. For viruses that show extreme serological diversity among isolates, such as tobacco rattle virus and peanut clump virus, it may be possible to choose probes that will detect a wide range of isolates. Probes can also be designed to detect groups of viruses, such as the white fly-transmitted geminiviruses. Spot hybridization can also diagnose infections where no viral coat protein is produced, as with NM-forms of tobacco rattle virus, some African cassava mosaic virus isolates, and viroids. Other potential applications include the identification of virus isolates that contain satellite nucleic acids, and the detection of infections with viruses for which only virus-related nucleic acids but not virus particles can be isolated.

Introduction

Of the established methods for virus detection, serological procedures are usually the most satisfactory, and among these, enzyme-linked immunosorbent assay (ELISA) is the most widely used. In sensitivity, cDNA techniques are comparable to ELISA, and in their simplest form are no more complex. However, to achieve this sensitivity and simplicity, it is necessary to use ^{32}P-labeled probes, and, because use of this isotope requires properly designed facilities and well trained personnel, ELISA is likely to be the method of choice for many applications. Nevertheless, cDNA tests can be designed to have levels of specificity that are not obtainable in serological tests, and it is in situations such as these that cDNA methods have their greatest potential. The examples that follow are intended to illustrate those kinds of circumstances in which cDNA tests are likely to prove most useful.

Extreme Serological Variation between Virus Strains

Tobacco rattle virus in narcissus

Tobacco rattle virus (TRV) is a rod-shaped virus with an extensive natural host range and a worldwide distribution. It occurs as a large number of strains, many of which are only distantly serologically related to one another (Harrison and Robinson 1986). Thus, an antiserum raised against any one strain will not detect the whole range of field isolates (Harrison et al. 1983). However, the coat protein gene is in the smaller of the two genome RNA species (RNA-2), which differs greatly in sequence between strains, whereas the larger genome part (RNA-1) is of similar sequence in all strains (Robinson and Harrison 1985). Thus a cDNA probe representing a part of RNA-1 will detect all strains of TRV.

A very simple method of sample preparation

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proved adequate for detection of TRV in narcissus. Leaf samples were ground with 50 mMol phosphate buffer pH 7.5, and 5 μL samples pipetted onto sheets of nitrocellulose. After baking in a vacuum oven at 80° C for 2 hours to fix the spots, these 'dot-blots' can be stored at room temperature until needed. The probe, cDNA representing about 2 kb of RNA-1 from TRV strain SYM cloned in a bacterial plasmid, was labeled with ^{32}P by nick-translation. Tests on a series of samples with symptoms suggestive of TRV infection from farms in eastern Scotland clearly differentiated those that were infected with TRV from those that were not (Fig. 1). For each sample, biological and serological tests either confirmed the presence of TRV in those that were positive in the cDNA test, or demonstrated the presence of a different virus to account for the symptoms in those that were negative. Thus, a simple cDNA test provides a reliable method of diagnosis for TRV in narcissus.

Peanut clump virus in groundnut

Peanut clump virus (PCV) is a rod-shaped virus, isolates of which may have little or no serological relationship to one another (Reddy et al. 1985). However, strong nucleotide sequence homologies were detected among three strains of PCV from India, and less strong homologies were found between these Indian isolates and one from West Africa. Thus, whereas serological tests for PCV are strain specific, a cDNA test should at least detect all Indian isolates.

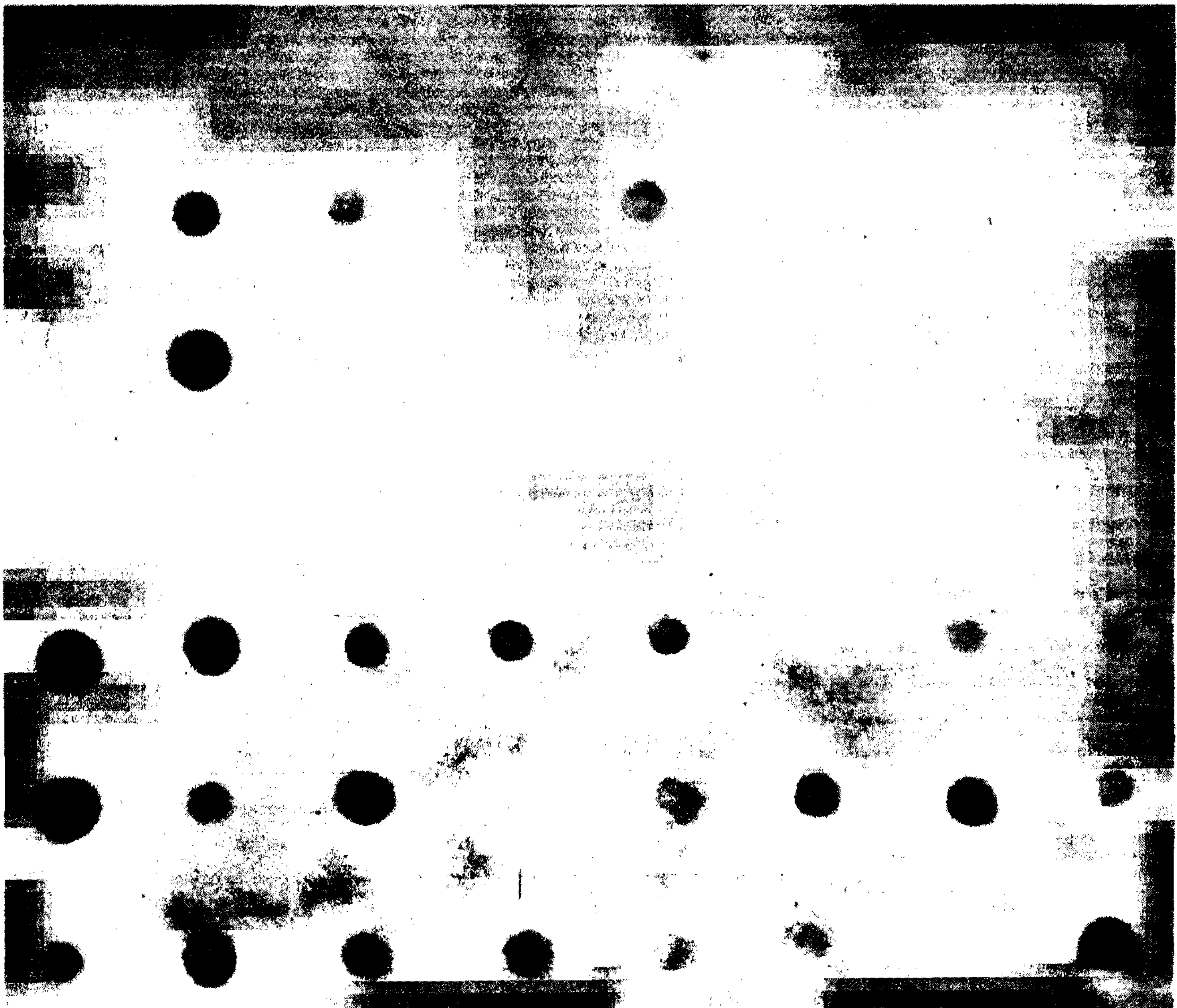

Figure 1. Spot hybridization tests on narcissus samples, probed with a tobacco rattle virus (TRV) RNA cDNA clone. Twenty-five samples react positive (dark) for TRV infection and 23 negative.

Because cloned PCV cDNA was not available, a probe was prepared by random-primed reverse transcription from PCV RNA. In initial tests, using dot-blots prepared from PCV-infected *Nicotiana benthamiana* leaves in the same way as those from TRV-infected narcissus, this probe detected three serologically distinct Indian isolates (Fig. 2). However, in order to detect PCV in groundnut, in which it does not reach such high concentrations as in *N. benthamiana*, increased sensitivity was required. The use of a vacuum filtration device that enables more leaf extract to be applied per unit area of nitrocellulose, allowed detection of the heterologous strains just at the limit of sensitivity. Further improvement in sensitivity is needed to make this test reliable for routine screening; such improvement is likely to be at the expense of simplicity. However, the prospect of a single test, for a wide range of PCV isolates, will compensate for the greater complexity.

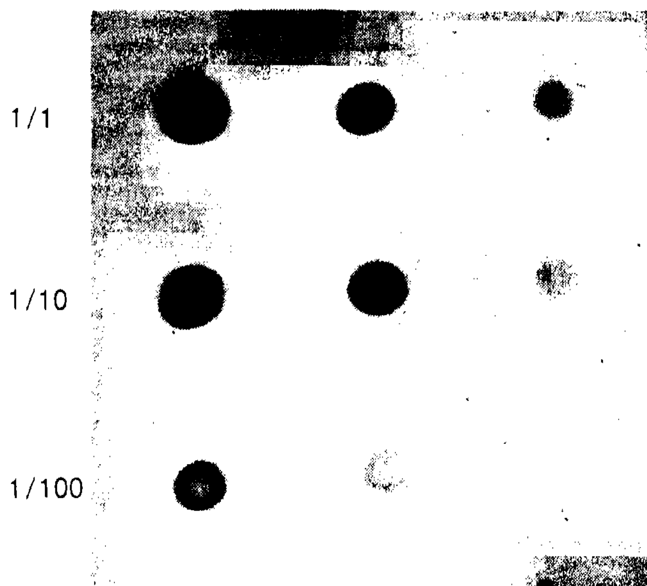

Figure 2. Reaction of three Indian isolates of peanut clump virus PCV (strains B, H, and L) in a spot hybridization test, probed with random-primed cDNA to PCV strain B. Figures on the left indicate dilutions of the extracts.

Tests to Detect Groups of Viruses

In screening programs, it may sometimes be useful to employ a test of wide specificity that will detect any one of a group of viruses, and then to apply additional tests only to those samples that are positive in the first test to identify the individual virus involved. It is possible to devise a group-specific probe for any group of viruses that have a substantial amount of common sequence in their genomes, such as the white fly-transmitted geminiviruses.

The geminiviruses have DNA genomes that consist of circular, single-stranded molecules of 2.5-2.8 kb (Harrison 1985). The genomes of viruses of beet curly top (BCTV), maize streak (MSV), and wheat dwarf (WDV), which are transmitted by leaf hoppers, consist of one such DNA molecule. However, other geminiviruses are white fly-transmitted and possess two DNA species of similar size but largely different sequence, both of which are required for systemic infection of plants.

For work with DNA viruses, we find it best to denature plant extracts by treatment with 0.1 Mol NaOH for 10 min, followed by neutralization, before spotting onto nitrocellulose in the usual way. For probes, we have used cloned double-stranded versions of the two genome parts of African cassava mosaic virus (ACMV), labeled by nick-translation. Such probes, whether representing DNA-1 or DNA-2, detect not only the homologous Kenyan-type strain of ACMV, but also strains from Angola and Nigeria (Robinson et al. 1984). However, only the DNA-1 probes react with extracts of plants infected with ACMV isolates from the coastal region of Kenya or from the Indian subcontinent. Moreover, ACMV DNA-1 probes also react with DNA from several other white fly-transmitted geminiviruses (Roberts et al. 1984) (Table 1), and these reactions can be distinguished from those with Kenyan coastal and Indian isolates of ACMV because they are abolished by stringent washing of the blots. Of particular interest among viruses that react in this way are horsegram yellow mosaic virus (Muniyappa and Veeresh 1984) and mung bean yellow mosaic virus (MYMV) (Honda et al. 1983), both of which occur in the Old World and infect a range of legume species, including groundnut and pigeonpea. ACMV DNA-2 probes react only weakly with Kenyan coastal and Indian ACMV isolates and with MYMV, but not with any of the other white fly-transmitted geminiviruses. No reaction of either probe with any leaf hopper-transmitted geminivirus has been detected.

Thus, probes representing DNA-1 of ACMV, or of other white fly-transmitted geminiviruses, will detect a range of such viruses. Similar group-specific detection is possible using serological tests, since these viruses are serologically related to one another. However, probing with DNA-2-specific probes can discriminate the homologous virus from others in the group, and it may be preferable to a battery of quantitative serological tests.

Table 1. Nucleotide sequence homologies with African cassava mosaic virus (ACMV) DNA species revealed by spot hybridization.[1]

Virus	ACMV DNA-1	ACMV DNA-2
African cassava mosaic (Strains T, A & N)	+++	+++
African cassava mosaic (Strain C)	++	+/++
Indian cassava mosaic	++	+/++
Mung bean yellow mosaic	+	+
Bean golden mosaic	+	-
Horse gram yellow mosaic	+	-
Tomato golden mosaic	+	-
Tomato leaf curl	+	-
Tobacco leaf curl	+	-
Beet curly top	-	-
Maize streak	-	-
Wheat dwarf	-	-

1. +++ Strong, homologous reaction.
++ Weaker reaction, resistant to stringent washing.
+ Weaker reaction, abolished by strigent washing.
+/++ Weaker reaction, effect of stringent washing not tested.
- No reaction.

Infections where no Virus Coat Protein is Produced

Tobacco rattle virus in potato

Infections with TRV RNA-1, or with particles that contain RNA-1, in the absence of RNA-2 lead to systemic infections of plants. However, because RNA-2 contains the coat protein gene, no coat protein is produced in these so called NM-type infections (Harrison and Robinson 1986). Infections of this kind occur in nature. Indeed, most TRV isolates from potato in Scotland are NM-type (Harrison et al. 1983). Serological tests are obviously not applicable in such circumstances, but a spot-hybridization test using an RNA-1 specific probe as for TRV in narcissus can be used. However, spotting buffer extracts of infected plants onto nitrocellulose is unsatisfactory for NM-forms of TRV. Instead, leaves are ground with buffer and phenol, and the separated aqueous phase used for spotting. Undiluted phenol-saturated extracts spread somewhat when applied to nitrocellulose, but this can be overcome and greater sensitivity achieved by use of the vacuum-filtration device. Moreover, in this instance, a further increase in sensitivity results from substituting a modified nylon membrane for nitrocellulose. With these improvements, the cDNA method will detect TRV NM-forms in leaf tissue, but is still not sufficiently sensitive for use with potato tubers, where concentrations of virus RNA are very low and extracts may contain large amounts of carbohydrate.

African cassava mosaic virus in cassava

Sequeira and Harrison (1982) described mosaic-affected cassava plants from Angola in which ACMV was not detectable by serological or mechanical inoculation tests. However, Robinson et al. (1984) showed that, in spot hybridization tests, both ACMV DNA-1 and DNA-2 probes reacted with extracts from these plants. Thus they contain isolates of ACMV that are apparently defective for particle production. Although such isolates seem not to be particularly widespread, the possible occurrence of similar phenomena with other viruses needs to be borne in mind when screening programs are designed.

Viroids

Viroids are small, infective, single-stranded, circular RNA molecules that code for no detectable protein products. The first spot hybridization method described for the detection of a plant infective agent was for potato spindle tuber viroid (Owens and Diener 1981). This method, or adaptations of it, has remained the most effective means of detecting viroid infections.

In recent years, much interest has centered on the development of a nonradioactive method of labeling hybridization probes. Of those so far described, one of the best for simplicity and sensitivity is the photobiotin method of Forster et al. (1985). They found that the method had a limit of sensitivity of about 4 pg (5×10^{-17} moles) of avocado sun-blotch viroid. This is equivalent to about 0.1 ng of a typical virus RNA of 2×10^6 molecular weight, or about 2 ng of rod-shaped or filamentous nucleoprotein particles. Thus, if extract equivalent to 2 mg of tissue is spotted, the detection limit for such viruses is of the order of 1 $\mu g\ g^{-1}$ tissue, which is considerably less sensitive than spot hybridization with ^{32}P-labeled probes or ELISA. Moreover, there are problems with false positive reactions from healthy material using photobiotin-labeled probes (Forster et al. 1985), although this may be overcome by using improved methods of

sample preparation. Nevertheless, nonradioactive probes have advantages in avoiding the use of ^{32}P and in being quicker to perform, because no autoradiography step is involved, although the possibility of extended exposure of the autoradiogram is an easy way of checking for weak reactions. Further developments in methods involving nonradioactive probes can be expected, which may make such methods more generally useful.

Satellite RNA Species

Some plant viruses have associated with them RNA species that are not part of the virus genome, but which are dependent on the helper virus for their replication. These satellite RNA species, which typically occur in some virus isolates and not in others, may modify disease expression in infected plants. Thus, it may be important to know whether a particular virus isolate contains a satellite RNA or not. Because many satellite RNA species do not code for their own coat protein, spot hybridization would be the method of choice for their detection. We have used such a method for research purposes to detect satellite RNA in cucumber mosaic virus isolates, but, as far as I am aware, no application in practical pathology has been described.

Viruses that have Not been Purified

For some plant virus diseases, neither the particles of the causative agent nor its genome nucleic acid have been purified. In such instances, biological tests have, until recently, been the only diagnostic method available. However, it has been observed that when double-stranded (ds)RNA from virus-infected plants is electrophoresed in polyacrylamide gels, the patterns obtained are characteristic of the particular virus, and it has been suggested that this could form the basis of a diagnostic test for viruses whose particles have not been purified (Dodds 1986).

We have studied two such viruses, carrot mottle virus (CMotV) and groundnut rosette virus (GRV), both of which are sap-transmissible but dependent on luteoviruses for aphid transmission. Individual dsRNA species were extracted from gels, denatured, and cDNA made from them by random-primed reverse transcription. These cDNA preparations were used as probes to hybridize blots made by electrophoretic transfer of dsRNA from gels to Zeta-Probe membrane. The CMotV probes reacted with dsRNA species from plants infected with any of several isolates of CMotV, but not with GRV or other viruses with similar biological properties. Likewise, probes prepared from dsRNA of a chlorotic isolate of GRV reacted with dsRNA from both chlorotic and green rosette isolates, but not with dsRNA from other viruses.

These reactions could be used as the basis of a test for the identification of CMotV and GRV infections that is more positive than reliance on dsRNA electrophoretic patterns alone. However, for routine use it will be essential to clone the cDNA probes, and, in order to handle large numbers of samples, it will probably be necessary to simplify the complex dsRNA extraction and northern blotting procedures.

For any particular problem in virus detection, the ideal solution will depend on the circumstances of the problem and of the laboratory involved. I do not suppose that cDNA methods will be applicable in all instances, but I have tried to indicate, by way of examples, those kinds of situation in which I think cDNA will prove useful.

The most important characteristic of cDNA methods is that they are not dependent on virus coat protein, and thus can provide specificities that are not available using serological methods. Sample preparation methods can be as simple as those used for serological tests, but more complex procedures may increase sensitivity. Nonradioactive probes are easy and safe to use and give rapid results, but as yet they lack sensitivity. Further developments in this area can be confidently expected, and will expand the potential usefulness of cDNA techniques.

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Potential of Enzyme-linked Immunosorbent Assay for Detecting Viruses, Fungi, Bacteria, Mycoplasma-like Organisms, Mycotoxins, and Hormones

D.V.R. Reddy, P.T.C. Nambiar, R. Rajeswari, V.K. Mehan, V. Anjaiah, and D. McDonald[1]

Abstract

Enzyme-linked immunosorbent assay (ELISA) is one of the most widely employed serological tests. For example, the double antibody sandwich (DAS-ELISA) technique has been successfully employed for the detection of various rhizobia, to assess the quality of inoculum, and to assess the establishment of the inoculated rhizobial strains in soil. This technique can also distinguish between different strains of Rhizobium. *ELISA has successfully been adapted to direct antigen coating (DAC-ELISA) and protein A coating (PAC) procedures for the detection and assay of several peanut viruses. DAC-ELISA is a simple and useful tool for virus detection in field surveys and in seed samples. Both DAC- and PAC-ELISA are more convenient to use for the detection of large numbers of antigens than DAS-ELISA. More recently, a competitive ELISA procedure has been developed to detect up to 2 ng aflatoxin B_1 in groundnut seed samples.*

Introduction

Nakane and Pierce (1966) coupled low molecular weight antibodies to produce enzymatically active immunological conjugates, and visualized the immune reaction with histochemical-staining techniques. Utilizing this principle, enzyme immunoassays, of the type popularly referred to as enzyme-linked immunosorbent assay (ELISA), were developed by Engvall and Perlmann (1971) and van Weeman and Schuurs (1971). Subsequently, several modifications were introduced to the ELISA test procedures. ELISA is currently by far the most widely used test in serological diagnosis. It permits detection of antigens, of widely varying size and morphology, and is adaptable to a wide range of plant extracts and conditions. This paper discusses the uses of various forms of ELISA at ICRISAT for the detection and/or quantification of peanut viruses, mycoplasma-like organisms (MLOs), *Rhizobium* strains, and aflatoxin B_1.

Detection of Peanut Viruses

Most workers use the double antibody sandwich form of ELISA (DAS-ELISA) described by Clark and Adams (1977) for the detection of plant viruses. In this procedure the solid phase (often wells of polystyrene or polyvinylchloride microtitre plates) was coated with immunoglobulins (Ig), which were mainly immuno gamma globulins (IgG). Partially purified Ig were prepared from antisera by precipitation with either ammonium sulfate or sodium sulfate. Test samples were then added and the Ig trapped the virus antigen. Enzyme-labeled antivirus Ig were added, which attached to the trapped virus antigens (Fig. 1). Finally, a suitable substrate was added to produce colored hydrolysates, thus permitting visual scoring and quantitative measurement, by colorimetry, of the amounts of antigen trapped. The DAS-ELISA procedure is known to be highly specific and often detects closely related strains (Koening 1978). The main disadvantage of this test is

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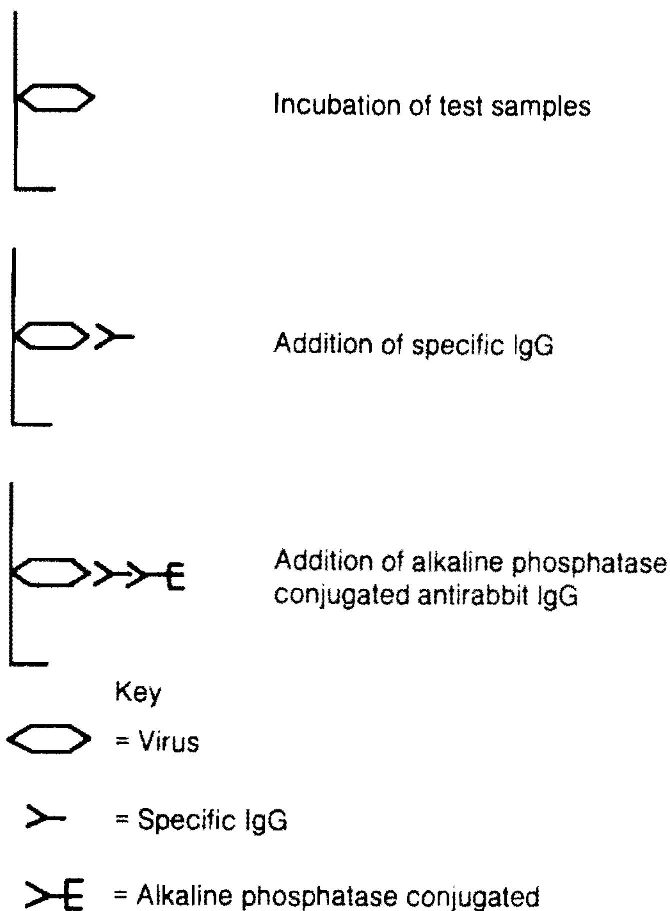

Figure 1. Principle of the double antibody sandwich (DAS) form of enzyme-linked immunosorbent assay (ELISA).

that it requires preparation of different Ig conjugates for each virus to be tested. To overcome this disadvantage we have tested several "indirect ELISA" procedures for the detection of three peanut viruses. Rabbit Fc specific Ig prepared in goats and conjugated with alkaline phosphatase was utilized in these procedures. A single conjugate could be utilized with antisera for different viruses. We describe below the two indirect-ELISA procedures that we have been able to apply successfully for the detection of several peanut viruses. They facilitated both the detection of a broad range of serologically related viruses, and the utilization of high dilutions of crude antisera.

For the direct antigen coating procedure (DAC-ELISA), the method developed by Mowat (1985) was used with minor modifications (Hobbs et al. 1987b). In the first step, plant extracts prepared in a carbonate buffer were applied directly to the wells. In the second step, diluted unfractionated antiserum was added. Ig attached to virus antigens were detected by the addition of enzyme conjugates of rabbit Fc specific Ig prepared in goats (Fig. 2). This method is by far the simplest of all the ELISA test procedures and can be completed within 3 hours. The main disadvantage of DAC-ELISA is that adsorption of the viral antigen to the well surface depends to some extent on the host components present in the plant extracts (Mowat 1985). We found DAC-ELISA to be very useful for virus detection in disease surveys (Reddy 1986, Hobbs et al. 1987b) since plant extracts could be used for coating plates without the necessity of using Ig for trapping viral antigens. The procedure has also been successfully used for detecting peanut mottle virus (PMV) in groundnut seed, thus facilitating screening of groundnut germplasm for seed-transmitted PMV. Small portions of cotyledon from 25 individual seed were mixed, ground in approximately 2.0 mL of carbonate coating buffer, and added to a well of an ELISA plate. As 8 plates, each with 96 wells, could be processed in a single day, we were able to test over 16 000 seeds per day for PMV presence.

Since host components tend to adsorb to the well surface, DAC-ELISA is not suitable for quantitative estimation of viruses present in crude plant extracts. DAC-ELISA is not suitable for investigating serological relationships, unless the virus is present in high concentration in crude extracts, and antisera having few, or no, antibodies against plant host components, are available. As a result we tried various indirect ELISA procedures described by Barbara and Clark (1982), van Regenmortel and Burck-

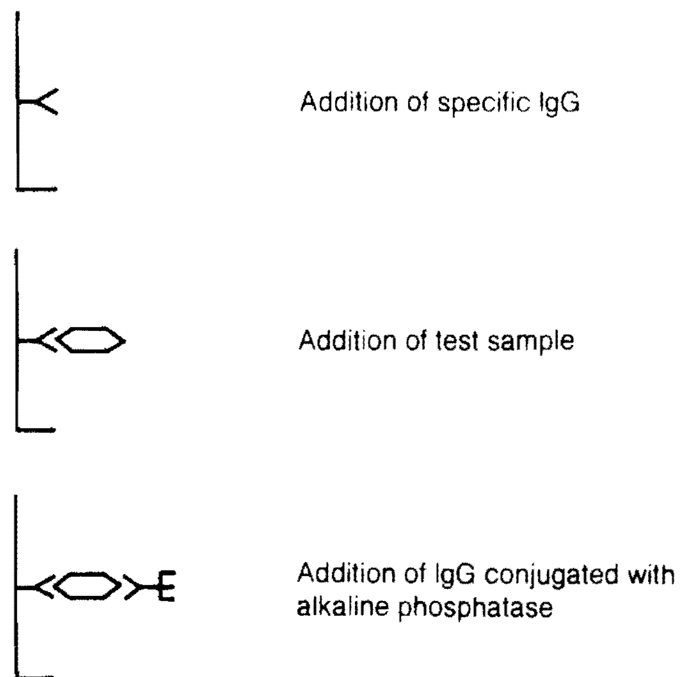

Figure 2. Principle of the direct antigen coating (DAC) form of enzyme-linked immunosorbent assay (ELISA).

ard (1980), and Edwards and Cooper (1985). We found that the protein-A coating ELISA (PAC-ELISA) procedure described by Edwards and Cooper (1985) was simpler to use and permitted cross absorption of antisera with healthy plant extracts in order to minimize nonspecific reaction. In the 4-step PAC-ELISA the plates were first coated with 1 ng mL^{-1} of protein-A. In the second step, high dilutions of unprocessed antisera were added. The Fc portion of IgG present in the antisera was bound to protein-A. In the third step, test samples were added and the $F(ab)_2$ portion of IgG trapped the virus. In the fourth step high dilutions of antisera, usually cross-absorbed with healthy plant components, were added. IgG present in antisera attached to the viral antigens. The Fc portion of this IgG was detected by the addition of conjugated antirabbit Fc specific antibodies produced in goats (Fig. 3).

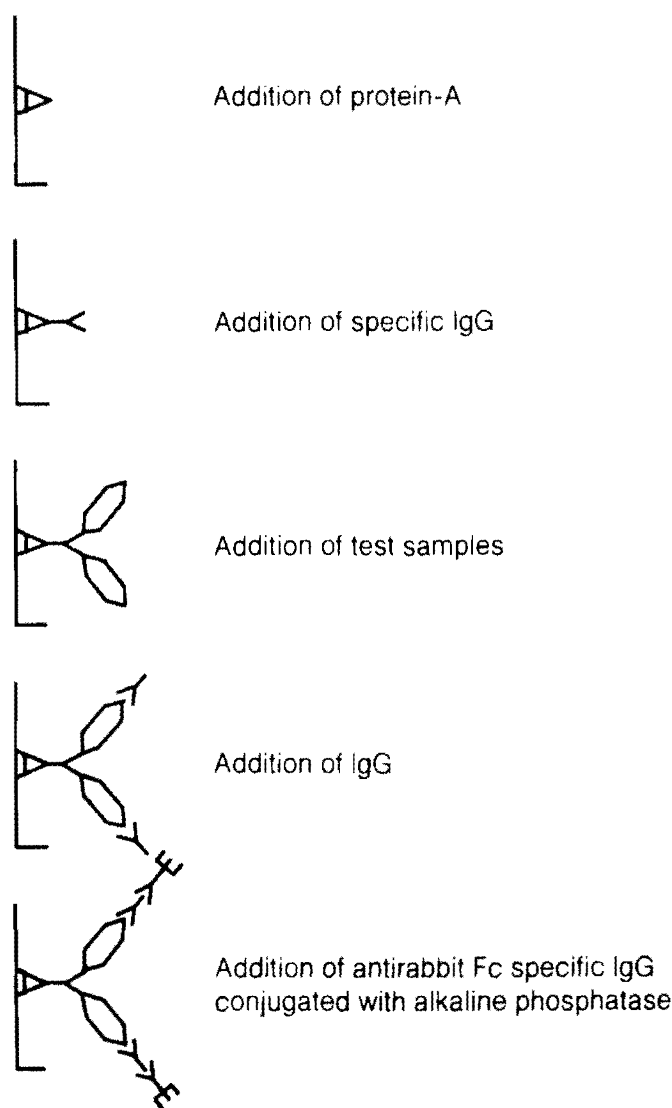

Figure 3. Principle of the protein-A coating (PAC) form of enzyme-linked immunosorbent assay (ELISA).

In Table 1, the results obtained on three peanut viruses (tomato spotted wilt (TSWV), peanut mottle (PMV), and peanut clump (PCV) viruses), using DAC-, PAC-, and DAS-ELISA procedures, are presented. Experimental details are reported by Hobbs et al. (1987b). Sensitivity of the DAC and PAC procedures was comparable to that of the DAS procedure, even under the short incubation period (45 min at 37°C for each step) conditions (Table 1).

Detection of Mycoplasma-like Organisms

We have utilized the PAC-ELISA procedure for detection of mycoplasma-like organisms (MLOs) in crude extracts of leaflets, stems, and pegs of groundnut plants showing witches' broom symptoms. The antisera produced for partially purified MLOs contained antibodies to host plant antigens. The PAC-ELISA procedure permitted cross-absorption of antisera to healthy plant antigens, thus minimizing the nonspecific reaction (Hobbs et al. 1987a). We have used this procedure for the detection of MLOs in several field-collected groundnut samples infected with witches' broom.

Detection of *Rhizobium*

The *Rhizobium* strain NC 92 was used to produce polyclonal antibodies in rabbits. In DAS-ELISA, *Rhizobium* numbers could be estimated utilizing optical density measured at 405 nm in peat-based inoculum (Nambiar and Anjaiah 1985). Estimates by the ELISA procedure were close to the values obtained from the plate count and plant infection techniques; even from samples stored at 37°C for 8 days (Table 2). The DAS-ELISA method is preferred to plant infection and antibiotic sensitivity tests, as large numbers of samples can be processed more economically and in a shorter time. Using an indirect ELISA procedure, utilizing $F(ab)_2$ fragments, *Rhizobium* populations were estimated quantitatively in several soil samples. With neither direct nor indirect ELISA procedure was it possible to estimate *Rhizobium* numbers when the populations were below 10^3 cells g^{-1} soil. Antiserum produced against *Rhizobium* strain NC 92 was also successfully used for the estimation of the percentage of nodules formed by this strain in soils inoculated with the NC 92 strain (Nambiar et al. 1984).

Table 1. Detection of three peanut viruses using the direct antigen coating (DAC), protein-A coating (PCA) and double antibody sandwich (DAS) form of ELISA.

	Absorption value—A410[7]		
Sample[1]	DAC	PAC	DAS
1. Groundnut seed[2]			
Healthy	0.07	0.11	0.08
PMV-infected[3]	1.39	0.97	0.96
2. Groundnut tissue[4]			
Healthy	0.08	0.13	0.08
PMV-infected[3]	1.50	1.25	0.69
3. Groundnut tissue[2]			
Healthy	0.19	0.10	0.28
TSWV-infected[5]	1.24	0.36	1.05
4. Groundnut tissue[2]			
Healthy	0.12	0.14	0.09
PCV-infected[6]	0.38	0.46	0.31

1. All dilutions were based on the original mess of the tissues.
2. 1:100 dilution.
3. Peanut mottle virus (PMV).
4. 1:1000 dilution.
5. Tomato spotted wilt virus (TSWV).
6. Peanut clump virus (PCV).
7. Mean absorbance A of three wells for each dilution in three separate experiments.

Table 2. Enumeration of rhizobia in peat inoculant, stored at 37° C for 8 days[1].

Enumeration method	Number of rhizobia estimated (10-8 cells g^{-1})
Plate count method	1.01
Plant infection method	2.41
Direct ELISA	1.37

1. Source: Nambiar and Anjaiah 1985.

Detection of Aflatoxin B_1

Aflatoxins are extremely potent hepatocarcinogens affecting a wide range of animals including man (Butler 1974). Of the four aflatoxins (B_1, B_2, G_1, and G_2) naturally occurring in crop produce, B_1 is the most potent and common. The method most commonly used for aflatoxin detection and quantification is thin-layer chromatography (TLC). This method is time-consuming, expensive, and permits analysis of only limited numbers of samples. Immunoassays have also been used for aflatoxin determination. They are sensitive, highly specific, and, being cheap, permit analyses of large numbers of samples.

Since aflatoxins are low molecular-weight compounds, they do not possess antigenicity. However, if the aflatoxin molecule is conjugated with a protein, such as bovine serum albumin (BSA), it can be used to produce a specific antiserum (Fig.4). The oxime derivative of aflatoxin B_1 (aflatoxin B_1—carboxymethyloxime) conjugated to BSA was found to be suitable for production of aflatoxin B_1 specific antibodies of high titre (Gaur et al. 1980). We describe below a competitive ELISA procedure which we have used successfully for the detection of aflatoxin B_1 in groundnut seed samples. In principle the method adopted is similar to that described by Morgan et al. (1986).

Commercially obtained hapten for aflatoxin B_1 (oxime-bovine serum albumin, BSA) was used to prepare a polyclonal antiserum in rabbits. The oxime-BSA was adsorbed to the wells of an ELISA plate (NUNC)®. BSA was then added to saturate the wells. Antiserum produced for oxime-BSA was diluted to 1:40 000 and mixed with various concentrations of pure aflatoxin B_1 standard, or samples of groundnuts with antisera containing various concentrations of aflatoxin, and then preincubated at 37° C for 1 hour. Prior to mixing, the groundnut seed samples were homogenized in 50% aqueous methanol, filtered, and 10-fold dilutions of the extract

in saline were used. Test samples and pure toxin, following incubation with antiserum, were added to ELISA plates, precoated with the oxime-BSA. Ig present in the antiserum, not neutralized by toxin, adsorbed to oxime-BSA. In the final step, Ig attached to oxime-BSA were detected by alkaline phosphatase conjugated rabbit Fc specific Ig. The intensity of color produced by the substrate, P-nitrophenyl phosphate, was inversely proportional to the concentration of toxin present. By employing a standard curve prepared for the pure toxin (Fig. 5) it was possible to estimate the aflatoxin B_1 concentrations in the test samples (Table 3). Results of the ELISA test were compared with those from analyses by TLC and there was general agreement between the tests.

Conclusions

At ICRISAT we have successfully used various forms of ELISA in the study of viruses, MLOs, *Rhizobium*, and aflatoxins. We have used only polyclonal antisera. Specific antisera, especially monoclonal antibodies, are essential for the differentiation of several native strains of rhizobia such as IC 6006 and IC 6009. Polyclonal antibodies for these strains cross-reacted with several native *Rhizobium* populations. Similar problems were experienced

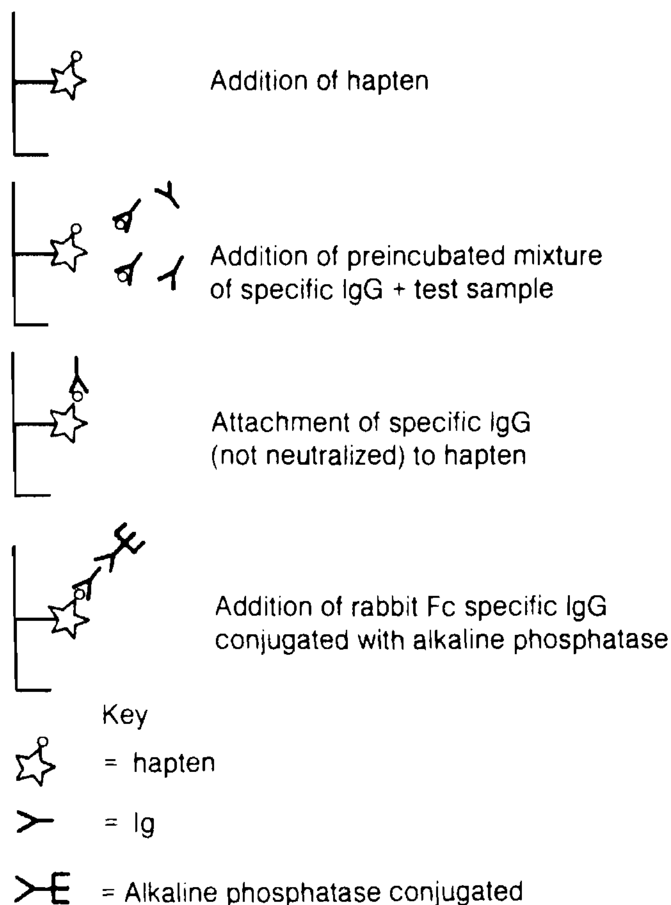

Figure 4. Principle of a competitive enzyme-linked immunosorbent assay procedure for aflatoxin B_1 estimation.

Table 3. Quantification of aflatoxin in groundnut samples by competitive ELISA.

Sample no. (naturally infected seeds)	Genotype	Toxin conc. ng g^{-1} seed	
		Mean	SE±
1	Ah 7223	1.73	0.461
2	J 11	0.52	0.161
3	U4-47-7	0.51	0.071
4	C55-437	1.19	0.499
5	UF 71513	2.75	0.999
6	PI 337394 F	1.37	0.482
7	JL 24	1.04	0.293
8	JL 24	2.39	0.723
9	TMV 2	2.19	0.602
10	EC 76446 (292)	4.13	0.887
11	EC 76446 (292)	2.43	0.531
12	NC Ac 17090	0.38	0.018
13	NC Ac 17090	2.06	0.525
14	NC Ac 17090	0.57	0.132
15	ICG 3204	1 758	588
16	ICG 3206	16 115	5 522
17	Breeding line	259 424	88 476
18	Breeding line	259 263	88 522

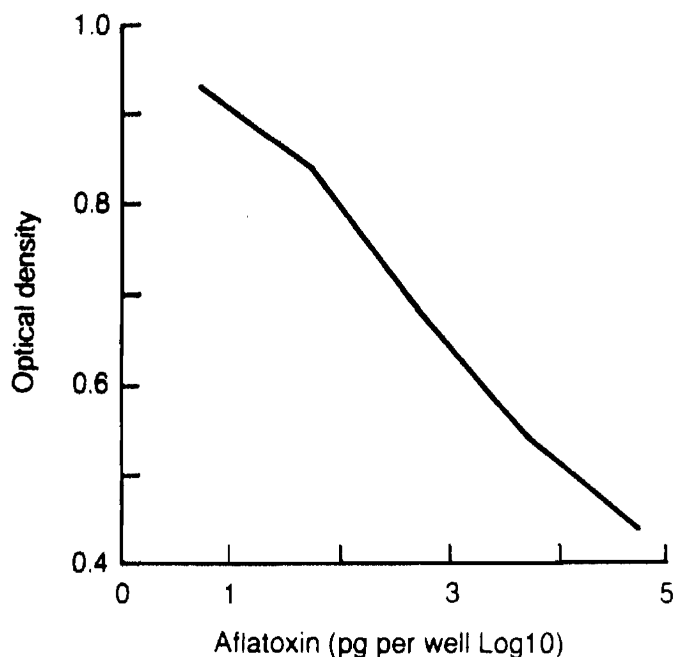

Figure 5. Competitive enzyme-linked immunosorbent assay for aflatoxin B_1. The standard competitive curve was prepared for toxin prepared in methanol-saline, as described in the text. The response was linear between 10 and 100 ng of the toxin. Results represent the average of five determinations.

when polyclonal antisera were used for detecting mycorrhizal fungi in roots. Since ELISA has already been used for distinguishing several species in the Endogonaceae (Aldwell et al. 1985), monoclonal antibodies (MAbs) offer immense potential for the detection of mycorrhizal fungi by ELISA.

We hope to extend our initial research results on aflatoxin B_1 to other aflatoxins and to other mycotoxins. In the competitive ELISA procedure adopted, small variations in absorption values resulted in wide differences in the estimates of toxin concentration. To overcome this setback we are testing other ELISA procedures: these include enzyme-labeled toxins, use of MAbs, and different enzyme and substrate combinations. We are also trying to improve the procedures for extracting the toxin from seed samples. For the detection of individual forms of a toxin, e.g., aflatoxin B_1, G_1 or G_2, MAbs are essential.

We are also interested in using MAbs for the estimation of gibberellins and other plant growth hormones in plant tissue.

Bacterial wilt of groundnut, caused by *Psuedomonas solanacearum* E.F. Smith, is an important disease in parts of southeast Asia. Since the bacterium is known to occur as several different, morphologically indistinguishable strains (Hayward 1986), the ELISA test combined with the utilization of MAbs offers excellent prospects for the detection of these strains.

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Application of Monoclonal Antibodies in Infectious Diseases and Cancer

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Abstract

The use of the hybridoma technique has revolutionalized biomedical research. Relatively little use is made of these techniques in research on plant pathogens. Variation and diversity of the antigenic constitution of the infectious agents render serodiagnosis based on conventional antisera exceedingly difficult. Both for therapeutic use, and for the production of vaccines, it is essential to identify the protective epitopes from the complex array of antigens displayed by pathogens. Some infectious agents are known to produce toxins that are responsible for pathogenesis. Monoclonal antibodies (MAbs) provide the means to (a) unravel antigenic variations among strains, (b) identify antigens useful for serodiagnosis, (c) identify protective epitopes, (d) purify or remove the specific antigens, and (e) conduct protection experiments using animal models. Advances made in these areas are described with specific medical examples.

Introduction

The two major ways in which a host defends itself against foreign, invading organisms are by the production of sensitized lymphocytes (cellular immune response), and by synthesizing specific proteins, called antibodies (humoral immune response). Both types of immune response specifically react with the foreign material (antigen) and eliminate them in a variety of ways (Good and Fisher 1971). Repetitive sequences of small polypeptides, or carbohydrates, present on antigens are referred to as 'epitopes'. They provide stimulus to specific B lymphocytes which differentiate and synthesize antibodies (immunoglobulins—lg).

The basic principle underlying the production of antibodies to unlimited numbers of antigens by vertebrate hosts was postulated by Sir Macfarlane Burnet (Burnet 1959). According to his clonal-selection theory, the circulating lymphocytes in higher vertebrates (including man), have, on their membranes, lg molecules of various 'fits'. These lg-bearing lymphocytes recognize the antigen of a right fit when they come in contact with them; they then get stimulated, increase clonally, and differentiate into antibody-producing plasma cells. However, these plasma cells have a finite life and, as soon as the triggering antigen is removed from circulation, they are slowly eliminated.

Since antigens may possess one or more epitopes that may stimulate a number of B cell clones, humoral immune response to an antigen results in the production of a diversified mixture of antibodies (polyclonal response). They react specifically with the epitope on the antigen that stimulated their production. Since several antibodies are present, it leads to dilution of a specifically desired antibody. The segregation of antibodies of desired specificity could be achieved by isolating the specific B cell, that secreted the antibody, immortalizing it by somatic cell hybridization, and cloning it further.

Kohler and Milstein (1975) developed the method of producing pure, highly specific antibodies by utilizing the hybridoma technique.

In this paper, essential features of developing MAbs, and their application for the detection of viral and bacterial pathogens and in cancer is discussed.

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Procedure of Immortalization

MAbs can be defined as products of a progeny of a single antibody-producing cell, fused with a cell that has the ability to grow indefinitely in tissue culture. For fusion, plasma cell tumors (myelomas) that possess the ability to grow continuously in tissue culture are used. The hybrid cell, thus generated, acquires the function of antibody production from the antibody-producing cell, and the ability to grow indefinitely, in tissue culture, from the myeloma cell.

Myeloma cell lines, currently used, originate from mice and rats. In order to get stable, antibody-producing hybridomas, the fusion partner should be lymphocytes from the spleens or lymph nodes of the same strain of animal, immunized with the relevant antigen. For the purpose of fusion, mutants of myeloma cells that do not synthesize or secrete their own immunoglobulins, have been selected (Schulman et al. 1978). Mutant cells are further selected for 8 Ag (Azaguanine) or 6 Thg (6-thioguanine) resistance. They lack the enzyme HGPRT (hypoxanthine, guanine phosphoribosyl transferase) and do not survive in the selection medium (Littlefield 1964) containing hypoxanthine, aminopterin, and thymidine (HAT). The presence of aminopterin restricts the biosynthetic pathway of DNA synthesis in myeloma cells. At the same time, they cannot utilize the hypoxanthine from the medium because of the absence of enzyme HGPRT. Hybrid cells, on the other hand, can selectively grow in HAT medium as they acquire the HGPRT gene from the genome of the antibody-secreting cell partner. Unfused lymphocytes from the immune donor animal have a finite life and do not survive in tissue culture.

In a typical fusion experiment, the spleen cells of hyperimmunized mice are mixed with myeloma cells in the presence of a fusing agent, polyethylene glycol, and plated in the wells of microtest tissue culture plates, on the supportive feeder layer of mouse peritoneal macrophages in HAT medium. When the selectively growing hybrid clones attain confluency, the culture supernatants are tested for the presence of specific antibodies. The specific antibody-secreting clones are further purified by recloning under the conditions of limiting dilution. Once stable antibody-producing clones are obtained these hybridoma cultures provide a rich and unlimited supply of 'monoclonal antibodies' (MAbs). Since the original myeloma cells have an ability to grow as 'ascites tumor' in the peritoneal cavity of the same strain of mouse, the hybridoma can also be grown as ascites in the mouse. The ascitic fluid from the tumor contains high concentrations of MAbs. The hybridoma cell lines can be stored in liquid nitrogen.

Because of the purity and uniformity of MAbs, they can be used as a powerful tool in the diagnosis and immunotherapy of infectious diseases. If specific reagents can be developed that define tumor-associated antigens, the monoclonals can be used in the detection, monitoring, radioimaging, and perhaps in immunotherapy of cancer. Diseases involving immune systems, such as immunodeficiency and autoimmune diseases, can be better understood and better managed with this dynamic tool. Because MAbs have the potential to define protective epitopes, they are likely to make a major contribution in the development of vaccines. Particularly, the combination of recombinant DNA techniques and MAbs may obviate the need for large supplies of protective antigens for the purpose of vaccine production.

Detection of Viral and Bacterial Pathogens

MAbs can be used:

1. for the classification of infective agents,
2. for serodiagnosis,
3. for serotyping of strain on isolates,
4. for passive immunization, and
5. as structural probes for the identification of protective or toxic epitopes.

MAbs exhibit a reduced background, nonspecific cross-reaction compared to polyclonal sera that are more commonly used. As a result, MAb kits are currently available for immunodiagnosis of infectious diseases (McMichael and Fabre 1982, Engleberg and Eisenstein 1984, Porterfield and Tobin 1984).

MAbs produced against highly conserved epitopes are being widely used for the identification of viruses. Several viruses show antigenic drift. For example, influenza virus (enveloped virus containing single-stranded RNA) exists in several different strains (Gerhard et al. 1980). Influenza virus A contains seven structural proteins which include two viral glycoproteins-HA (haemaglutinin) and NA (neuraminidase), and five viral coded proteins—NP (neucleoprotein), MP (matrix protein) and polymerases P1-P3 (Kilbourne 1975, pp 483-538).

The antigenic evolution in influenza virus occurs predominantly in HA and NA molecules. During the

past several years, various epidemics of influenza, throughout the world, have been caused by serologically distinct subtypes of the virus (Table 1).

Once the human population develops immunity against the new virus subtype, viruses with minor antigenic changes begin to arise. This phenomenon is called 'antigenic drift'. This drift might occur due to hypermutability of influenza virus HA and NA genes. MAb technology has tackled this issue fruitfully. It is now known that variant viruses, exhibiting antigenic alterations in individual epitopes of HA molecules, could be readily selected in vitro by MAbs. They represent point mutations resulting from changes in a single base in the HA gene. The changes occur predominantly at a critical locus, at four antigenic sites on the HA molecules.

On the other hand, measles and polio viruses show fewer structural variations, thus facilitating the production of MAbs for serodiagnosis and for vaccine production. MAbs were also utilized for the identifications of strains of viruses suitable for vaccine (Sabin-like), which can be distinguished from virulent strains (non-Sabin-like) (Porterfield and Tobin 1984).

Another example of a viral disease in which MAbs have made a remarkable contribution, is that of the rabies virus (Koprowski and Wiktor 1980). Rabies, a rhabdovirus, infects all warm-blooded animals. It is a neurotropic virus, never detected in the blood of infected animals. Infection occurs in most cases by bites or scratches from rabid animals. Only minor antigenic differences have so far been detected in the rabies group. Rabies virus has five major antigenic protein moieties. A number of MAbs have been generated against rabies and are being used to distinguish rabies virus strains and rabies-related viruses isolated from different parts of the world. Antinucleocapsid antibodies have been used to identify viruses causing rabies in man and animals. MAbs are also being used to select with care strains of the virus for vaccination. By implanting hybridoma cells in millipore chambers in animals, it is possible to ascertain the ability of MAbs to neutralize the virus in infected laboratory animals (Table 2).

Table 1. Influenza virus subtypes involved in four epidemics

Year of epidemic	Geographical region	Antigenic composition of subtype virus
1918	Pandemic	H1N1
1957	Asia	H2N2
1968	Hong Kong	H3N2
1971	Russia	H1N1

Table 2. Mouse protection studies against rabies.

Hydridoma clone implanted in millipore chambers	Challenge with rabies strain[1]		
	PM	ERA	AF (street virus)
101-1		0/5	1/5
110-3	3/6	0/5	5/5
234		5/5	5/5
Control	8/8	5/5	5/5

1. The slash separates the no. of mice dead from the no. of mice challenged.

Generalized bacterial infection in infants is a leading cause of neonatal mortality. The bacterial pathogens most frequently seen in infected infants are group B streptococci (GBS). The subtypes of GBS are identified from the structure of a cell wall polysaccharide (S substance). These subtypes are immunologically distinguishable. To facilitate a rapid diagnosis of GBS, MAbs have now been generated and used for identification of bacterial pathogens and to assess their protective effect in mice (Mitchison and Coates 1982; Polin 1980). Another important bacterial disease in developing countries is tuberculosis. *Mycobacterium tuberculosis, M. africanus*, and *M. bovis* are identified as highly pathogenic strains in man. These are generally classified by morphological and biochemical means and by phage typing. Currently, a panel of MAbs have been developed (Porterfield and Tobin 1984, Mitchison and Coates 1982) and utilized in the identification of individual strains (Table 3).

Leprosy is one of the major diseases in India. The disease occurs in two forms, the tuberculoid type

Table 3. Identification of strains of *Mycobacterium tuberculosis* using monoclonal antibodies.

Strain	Country	Reactivity with MAb	
		TB23	TB71
H37Rv	USA	+	+
H37Ra	USA	+	+
S1	Britain	-	+
6067	Britain	+	+
7219	India	+	+
M. bovis Valee	-	+	-
M. bovis BCG	-	+	-

with a low load of bacilli and good cellular immune response, and the lepromatous type with high bacillary contents and meager cellular response. Research in leprosy has been hampered because of difficulties in cultivating the causal bacterium *Mycobacterium leprae* in vitro. Recently MAbs have been developed for the phenolic glycolipids synthesized by *M. leprae*, and for polypeptides from the bacilli (Porterfield and Tobin 1984, Gillis and Buchanan 1982, Young et al. 1984). It is hoped that monoclonal antibodies to phenolic glycolipid of *M. leprae* may permit the early detection of leprosy.

Antibodies to Bacterial Toxins

Recently, MAbs have been prepared against a number of toxins from bacteria. This has helped in understanding the process of pathogenesis (Porterfield and Tobin 1984). Their use in therapy is yet to be assessed. A product of mouse/human cell fusion producing human MAbs has been shown to offer protection in mice (Ginliotti and Insel 1982). In a recent report, human B lymphocytes have been stimulated in vitro with pokeweed, *Phytolacca amexicana*, mitogen, and tetanus toxoid. This cell population has been further used for fusion to generate two monoclonal antibodies directed against different epitopes of tetanus toxoid antigen (Ziegler-Heitbrock et al. 1986). These antibodies, when administered together, offered protection in mice challenged with tetanus toxin.

Monoclonal Antibodies in Cancer

Cancer results from the host's cells that have lost the ability of controlled growth. As a consequence, the cells first grow at the cost of surrounding normal tissues by invading them, then get dislodged from the primary site, and thirdly disseminate to other organs through blood vessels and lymphatics. The major problem in cancer research is to identify, with confidence, any antigenic structure that is exquisitely present on human cancer cells and not on their normal counterparts. In spite of this, several observations in the clinics and experiments conducted in the laboratory have shown that the hosts can respond to cancer cells by producing specific cellular and humoral responses.

Conventional antisera against cancer cells, prepared in the heterologous animals, generally produce a host of antibodies directed against cellular components present in normal cells as well. These reagents, so far, have not been able to identify effectively, the putative tumor associated antigens (TAAs), which are meagerly expressed in cancer cells. The availability of monoclonal technology has opened the possibility of segregation and immortalization of those antibody-producing B cells that recognize the TAAs. Already, a number of monoclonals have been prepared against cancer cells, and the panel is ever increasing. Obviously, no MAbs are likely to be a 'pan-tumor-cell-reagent', even for the tumors arising at the same site and having the same histology. As enumerated in Table 4, attempts are being made to use these reagents for various purposes, such as in cancer diagnosis and in monitoring and treatment of cancer (McMichael and Fabre 1982, Sikora and Swedley 1984, Wright 1984, Kennet et al. 1980, Lennox 1984, Goding 1983, Haynes and Eisenbarth 1983, Boss et al. 1983, Mitchell and Oettgen 1982, Baldwin and Byers 1985).

Tumor Diagnosis

In the case of solid tumors, it has not yet been possible to obtain a totally specific MAb for a particular tumor, but a number of reagents have been

Table 4. Uses of monoclonal antibodies in cancer.

1. Tumor diagnosis and classification	1. Histological sections of primary tumors and metastasis 2. Cell phenotypes in leukemias and lymphhomas
2. Monitoring	1. Circulating tumor antigens for presence of metastasis and tumor load 2. Circulating levels of drugs and hormones 3. Tumor localization and identification of metastasis-radioimaging
3. Therapy	1. Direct cytotoxicity 2. Purging of bone marrow 3. Targeting of drugs, toxins and radionuclides 4. Augmentation of cellular cytotoxicity.

used to identify tumor cells in primary tumors and metastasis in breast cancer, melanoma, brain tumors, etc. (Wright 1984). Using a battery of MAbs, several antigens associated with melanomas have been identified and characterized (Wright 1984).

One of the major advantages of hybridoma technology has been the development of MAbs to cell phenotypes representing different stages of maturation of haemopoietic cells, especially T lymphocytes (McMichael and Fabre 1982, Sikora and Swedley 1984, Lennox 1984). These antibodies have been utilized in the subclassification of lymphomas and leukemias, and in the diagnosis of immunodeficiency diseases. Highly specific MAbs have also been utilized to induce immunosuppression in patients who underwent organ transplantation, and to reduce the host tissue destruction in bone marrow transplantation (Lennox 1984).

Monitoring of Cancer Patients for Efficacy of Treatment

Cancers of the gastro-intestinal (GI) tract often produce carcino-embryonic antigens (CEA), and liver cancers produce alpha-feto-protein (AFP). These foetal proteins are shed by cancer cells and are found in blood circulation. The levels of these TAAs in circulation correlate with the tumor burden on the host, and so can be used as markers for assessing the efficacy of the treatment. A large number of MAbs are now available against these antigens, in the form of kits, that are being used routinely for monitoring patients.

Radioimaging

During earlier years, xenogeneic transplants of human tumors grown in 'nude mice' (mice that lack thymus, and hence cannot build up the immune response) were used to study the localization of radiolabeled MAbs in tumor tissue. Recently, immunoscintigraphy is being conducted on a limited scale on patients, using a procedure called 'emission computerized tomography' (Mitchell and Oettgen 1982, Baldwin and Byers 1985).

Tumor Targeting

Theoretically, radioisotopes, toxins, and drugs attached to MAbs should offer direct interaction between the toxic agents and target tumor cells, thus obviating the need for large doses of chemotherapeutic agents in cancer therapy. In the case of toxins, similar problems exist as most of the mammalian tissue cells have receptors for the B chain of the toxin molecules. In the case of toxin, separation of their A and B chains and attaching the toxic A chain to the MAbs, which will direct the toxic chain to the target tumor cells, would be an ideal way of avoiding nonspecific damage to normal cells. Because of these fascinating possibilities, serious attempts are being made to use MAbs as "magic bullet-like" targeting agents (Mitchell and Oettgen 1982, Baldwin and Byers 1985). Most of the experiments conducted so far have been done in vitro or in animal models. Bone-marrow purging has been done in vitro to remove alloreactive T cells or cancer cells before transplantation (Wright 1984).

Radiolabeled antibodies have now been used to achieve remission in hepatomas and Hodgkin's lymphomas using an anti-Ferritin antibody. The conjugates of drugs with monoclonals have been shown to target in xeneogeneic transplants of human tumors (Mitchell and Oettgen 1982, Baldwin and Byers 1985).

Immunotherapy

Anti-T-lymphocyte MAbs have been used to treat patients with acute lymphoblastic leukemias and T-cell lymphomas (Boss et al. 1983). Patients with advanced GI-tract malignancies showed some beneficial effect after treatment with mouse MAbs of isotype IgG 2a (Boss et al. 1983). Herbermann et al. (1985) and studies in our laboratory have demonstrated that target-directed MAbs show augmentation of cytotoxicity of lymphocytes from cancer patients. These studies offer a potential for utilizing MAbs to boost cellular effector mechanisms that seems to be of primary importance in the fight against cancer.

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Somaclonal and Gametoclonal Variation

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Abstract

For several years it has been recognized that the introduction of plant cells into culture results in genetic changes in some cells. These genetic alterations can be recovered later in plants regenerated from these cell cultures. More recently it has been recognized that this method of introducing genetic changes into crop plants can be used to develop new breeding lines. Genetic variation, introduced by using cell cultures, has been termed somaclonal and gametoclonal variation. This paper reviews the history of this technology and offers genetic documentation of somaclonal cultivars, in tomato and tobacco. Somaclonal and gametoclonal variation are new tools at the disposal of the geneticist and plant breeder that not only permit reduction in the time taken for the development of a new variety but also provide access to new classes of genetic variation.

Introduction

The ability to recover intact plants from cultured cells was originally viewed as an efficient method of producing large numbers of clones, an expectation that has been realized. Many commercial laboratories now use tissue culture of shoot tips to propagate a wide range of ornamental plants, and some crop plants. As predicted, tissue culture propagation has been shown to be economically competitive with conventional propagation and has resulted, to a large extent, in clonal fidelity (Murashige 1974). However, it was recognized in early experiments that, if cultures were established from explants that did not contain a preorganized meristem, or if cultures were maintained as callus prior to plant regeneration, the regenerated plants were quite variable. In early reports most of the variation was attributed to the readily detected chromosome instability of cultured plant cells. In many of these studies, the degree of chromosome instability was reported to be proportional to the length of time the cells remained in culture. Recognition of the spontaneous variation inherent in long-term culture led to the use of cell culture for mutagenesis and selection of genetic variants and for direct recovery of novel genotypes from cell cultures via somaclonal variation. This paper describes the types of genetic variants that have been recovered from cell cultures via somaclonal variation and discusses the impact of this variation for crop improvement and recovery of new ornamentals.

Terminology

A specific genetic terminology to describe the variation recovered from cultured cells has evolved. Alterations have been referred to as phenotypic or genotypic changes. The **genotype** refers to the sum total of the genetic information, while the **phenotype**, is recognized to result from a combination of genetic (genotypic) and environmental factors. Phenotypic changes that are not the result of genetic alterations are termed **epigenetic** changes. Hence, in most cases, it is appropriate to characterize variation in the plant or plant cell phenotype as a genetic or an epigenetic change. The distinction between these two types of changes is conclusively demonstrated only by detailed genetic evaluation often requiring several sexual generations. Because of this requirement, very few reports of cell culture-induced variation have been conclusively shown to have a genetic basis.

Early variant plants regenerated from cell cultures

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of geranium were termed **callicones** (Skirvin and Janick 1976), while plants regenerated from protoplasts of potato were termed **protoclones** (Shepard et al. 1980). Larkin and Scowcroft (1981) promoted the use of the more general term **somaclonal** variation for the variation detected in plants derived from any form of cell culture. However, the type of genetic variation recovered in regenerated plants is, to a large extent, dictated by the genetic constitution of the particular cell population that is regenerated. It is necessary for genetic reasons to distinguish between plants regenerated from somatic or gametic tissue. The term **somaclone** refers to plants regenerated from cell cultures originating from somatic tissue, and **gametoclone** refers to plants regenerated from cell cultures originating from gametic tissue.

Similarly, mixed terminology has evolved to refer to sexual progeny of plants regenerated from cell cultures. The terminology proposed by Chaleff (1981) is adopted here. Plants regenerated from cell culture, irrespective of tissue or origin, are referred to as **R** or $\mathbf{R_0}$ plants. The self-fertilized progeny of **R** plants are referred to as $\mathbf{R_1}$ plants. Subsequent generations produced by self-fertilization are termed $\mathbf{R_2}$, $\mathbf{R_3}$, $\mathbf{R_4}$, etc.

Review of Literature

Although cloning of identical plants was originally believed to be the principal use for plant culture, it has become increasingly clear that under the appropriate cultural conditions, a great deal of genetic variation can be recovered in regenerated plants (Table 1). Results from early experiments documented genetic variability in plants regenerated from protoplasts (Shepard 1982). Growth of unorganized callus was thought to be necessary for the induction of variation (Skirvin and Janick 1976), but recent results suggested that genetic variability is present even in populations of plants regenerated directly from leaf explants in the absence of callus growth. Phenotypic variation has been reported in a number of plant species regenerated via organogenesis or embryogenesis.

Genetic variation was first detected as altered chromosome numbers in cultured plant cells. The onset of chromosome instability has been well characterized in *Daucus carota* (Smith and Street 1974) and *Haplopappus gracilis* (Singh et al. 1975). The most frequently reported variation has been polyploidy (Skirvin 1978), attributed to selective growth

Table 1. Documented cases of somaclonal variation in crop and ornamental species.

Species	Altered characteristics	Inheritance[1]	References
Apium graveolens (celery)	Isozyme variation; chromosome structure	No	Orton 1983
Chrysanthemum morifolium	Flower size and color, petal shape	No	Ben-Jaacov and Langhans 1972
Dendrobium sp	Flower size and color, petal shape	No	Vajrabhya 1977
Lactuca sativa (lettuce)	Plant height and vigor, fertility, leaf color, chlorophyl pigment	Yes	Engler and Grogan 1984
Lycopersicon esculentum (tomato)	Fruit color, growth habit, fertility, flower color, chlorophyl content	Yes	Evans and Sharp 1983
Medicago sativa (alfalfa)	Yield, fertility	Yes	Pfeiffer and Bingham 1984
Nicotiana alata	Time to flower, flower and leaf morphology, pollen viability, plant height	Yes	Evans and Bravo 1986
Pelargonium spp	Plant and organ size, leaf and flower morphology, oil constituents, fasciation, pubescence, anthocyanin pigmentation	No	Skirvin and Janick 1976
Saintpaulia ionantha	RuBCase activity	No	Bhaskaran et al. 1983
Solanum tuberosum (potato)	Chromosome structure, yield, disease resistance	No	Shepard 1982
Zea mays (maize)	Early tasseling, leaf spotting, curled leaves, vigor, tillering	Yes	Brook Houzen et al. 1984

1. Yes = Documented transmission of traits to self-fertilized progeny.

of normally nondividing polyploid cells pre-existing in the original explant (D'Amato 1977). It has also been reported that the frequency of polyploid cells is dependent on the concentration and type of cytokinin used in the culture medium. Bennici et al. (1971) reported that the frequency of polyploid cells in cultures of *H. gracilis* was dependent on the ratio of kinetin to naphthaleneacetic acid (NAA). Polyploid plants have been recovered in many commercially important plant species such as *Pelargonium zonale* (Skirvin and Janick 1976), *Nicotiana tabacum* (Brossard 1976), *N. alata* (Evans and Bravo 1986), *Lycopersicon esculentum* (Evans and Sharp 1983) and *Medicago sativa* (Reisch and Bingham 1981). Aneuploid changes, involving the gain or loss of a few chromosomes, have also been frequently reported in plant cell cultures. The accumulation of aneuploid cells has been attributed to ageing of cultures (D'Amato 1977). Older cell lines are often incapable of plant regeneration. However, aneuploid plants have been regenerated from cultures of *N. tabacum* (Sacristan and Melchers 1969) and *Saccharum* spp (Heinz and Mee 1971). Aneuploidy is often associated with sterility, but this is no handicap in plant species that are propagated asexually. In addition to these numerical chromosome changes, observation of anaphase bridges and fragments suggests that the structure of plant chromosomes is modified in cell culture (Singh et al. 1975). Chromosome rearrangements have been detected in clones of potatoes (*Solanum tuberosum*) regenerated from mesophyll protoplasts (Shepard 1982).

Evidence of recovery of single gene mutations produced via somaclonal variation has been presented. The progeny of tomato plants regenerated from leaf-derived callus were examined (Evans and Sharp 1983) and 13 distinct single gene mutations were recovered among 230 regenerated tomato plants. This frequency of visual somaclonal mutations (ca 1 in 18 regenerated plants) is substantially greater than the cell mutagenesis rate from several cell selection experiments (Maliga 1980). Several of these single-gene mutants of tomato have been well characterized and mapped to specific chromosomes (Evans and Sharp 1986).

Cytoplasmic genetic variation has been detected less frequently than gene mutations following plant regeneration from cell culture, although the importance of this class of variation has been emphasized (Sibi 1978). The most compelling evidence for cytoplasmic genetic variation among regenerated plants was presented by Gengenbach et al. (1981), who used restriction enzyme analysis of isolated mitochondrial DNA to detect spontaneous variant maize plants regenerated from cell culture. These authors monitored variation in cytoplasmically controlled male sterility, a mitochondrially encoded character. Hence, somaclonal variation has resulted in numerical and structural chromosomal changes in nuclear genetic modifications and in cytoplasmic genetic variation. This broad spectrum of variation suggests that, by using appropriate selection methods, all classes of genetic variation could be recovered and used for crop improvement.

No detailed systematic studies have been devised to ascertain the source of somaclonal variation. However, it is possible, by examining published literature, to gain information regarding the source of variation. Skirvin and Janick (1976) systematically compared plants regenerated from callus of five cultivars of *Pelargonium.* Plants obtained from geranium stem cuttings in vitro were uniform, whereas plants from in vitro root and petiole cuttings, and plants regenerated from callus, were variable. This suggests that some variability is correlated with donor explant, and pre-exists in the tissue used to establish cell cultures. On the other hand, evidence from several laboratories suggests that variability is dependent on hormone concentration of the culture medium. Variability is most likely to be the result of both pre-existing genetic instability and genetic alterations induced during the process of cell culture and subsequent plant regeneration.

Recent indications of somaclonal variation in several crop plants have stimulated interest in applications of this method for crop improvement. Initial studies with sugarcane suggested that clones with disease resistance could be regenerated from callus induced from susceptible plants. Krishnamurthi and Tlaskal (1974) isolated clones of regenerated sugarcane with resistance to Fiji disease virus and downy mildew. These selected resistant clones did not show reduced sucrose yield. Similarly, variants of sugarcane have been isolated with resistance to eyespot disease (Larkin and Scowcroft 1981). Most sugarcane variants have been attributed to changes in chromosome number. Researchers in several laboratories have examined plants regenerated from protoplasts of potato following the recovery of somaclones resistant to late blight (Matern et al. 1978). Potato variants have been isolated with resistance to early blight and with altered growth habit, tuber shape and color, and maturity date. These somaclonal variants of potato were attributed to changes in chromosome number (Karp et al. 1982) and chromosome structure (Shepard 1982). While these var-

iants can be stably propagated asexually, the genetic inheritance of this variation in sugarcane and potato has not been determined. Variation with potential agricultural application has been detected in several plant species that are sexually propagated. These include yield improvement in tobacco, TMV (tobacco mosaic virus) resistance in tobacco, Southern leaf blight (*Helminthosporium maydis*) resistance in maize (*Zea mays*), and leaf shape in geranium (Evans and Sharp 1986). Somaclonal variants have also been described in rapeseed for blackleg disease susceptibility (Sacristan 1982), and for growth habit, fruit color, and male sterility in tomato (Evans and Sharp 1983). All of these variants demonstrate the potential of somaclonal variation for the production of new breeding lines.

As detailed genetic analyses have been completed for only a few somaclonal variants, it is not surprising that this method has not been extensively exploited by plant breeders. Most variants reported to date have been chromosomal. While chromosomal changes can be tolerated in asexually propagated crops such as potato and sugarcane, nuclear gene and cytoplasmic variation may be much more important for crop improvement of sexually propagated crops.

Tomato Improvement

Evans and Sharp (1983) and Evans et al. (1984) published a description of experiments designed to generate somaclones of tomato and to ascertain the genetic basis of somaclonal variation. Tomato seeds of a well-characterized, open-pollinated tomato variety, UC82B, were sown in a greenhouse. Young, fully expanded leaves were removed from plants, sterilized, and placed onto a culture medium known to permit regeneration of plants from cultured leaf explants (Padmanabhan et al. 1972). Callus that developed from the cultured leaf explants regenerated shoots within 4 weeks. Regenerated shoots were rooted on a medium containing NAA (napthalene acetic acid), then transferred to the greenhouse for maturation and fruit collection. Self-fertilized seed was collected from mature fruits on R_0 plants. To evaluate the R_1 generation, seed was sown in the greenhouse where seedling characters, such as chlorophyll deficiency, anthocyanin content, and leaf shape were monitored. R_1 seedlings were transplanted to the field to classify mature plant characteristics such as pedicel type, fruit shape, and fruit and flower color. In addition, preliminary data on agronomic characters were collected in the R_1 field evaluation.

Variation in chromosome number, particularly tetraploidy, 2n=48, was detected. Sterile aneuploid lines were also detected. However, since emphasis was directed to analysis of R_1 progeny of regenerated plants, most aneuploid lines were discarded because little or no R_1 seed was collected. Several R_1 progenies were observed to segregate for morphological characters. These included recessive mutations for male sterility, jointless pedicel, tangerine virescent leaf, flower and fruit color, lethal chlorophyll deficiency, virescence, and mottled leaf appearance and dominant mutations controlling fruit ripening and growth habit. Genetic analysis was completed by evaluating self-fertilized R_2 progeny of selected R_1 plants and, in some cases, by crossing with known mutant lines. Extensive genetic analysis has been completed for several of the variants.

Genetic analysis was first completed for the tangerine-virescent (**tv-tcl**) character. This character is a single recessive allele that results in orange flowers and fruit and yellow virescent leaves. The R_1 segregation data observed in the field (30 red to 6 tangerine fruit) first suggested control of the trait by a single recessive allele. Fruit was collected from eight individual self-fertilized plants. Six of these R_1 plants had red fruit while two had tangerine fruit. The two tangerine fruits contained seed that bred true for the tangerine-virescent phenotype. Of the six red-fruited plants, three bred true for red fruit and three segregated for red vs tangerine-virescent. The pooled progeny of the three segregating plants fit a 3:1 ratio (58 red to 17 tangerine). In addition, among the segregating R_2 progeny, the flower, fruit, and leaf color defects cosegregated, suggesting control of two pigments, carotenoids (flower and fruit) and chlorophyll (leaf), by a single pleiotropic gene. Such pleiotropic genes controlling carotenoids and chlorophyll, two compounds both found in plastids, have been reported previously. A single mutant R_1 plant was crossed to a known fruit variant, tangerine, and all hybrid progeny had tangerine fruit. As these two mutants do not complement, it can be concluded that the new somaclone tangerine-virescent is a mutant for a new allele in a previously known gene at position 95 on the long arm of chromosome 10. This has been the most precise genetic characterization of a new somaclonal variant to date. In addition, by evaluating the self-fertilized progeny of the hybrids between the tv-tcl and the earlier tangerine mutant for the virescence character, it has been possible to conclude that the locus

contains two elements, one controlling chlorophyll synthesis and one controlling carotenoid synthesis, that mutate independently. The new **tv** allele recovered from somaclonal variation is recessive to the t allele for the virescence phenotype.

Jointless pedicel is a recessive mutant of tomato that is well characterized. Plants with this character are desirable for mechanical harvesting as the harvested fruit has no stem attached. Two mutants with jointless pedicel were identified in a somaclone. The first was as expected, i.e., the regenerated plant was normal, and the recessive mutant was detected in about 25% of the R_1 progeny. However, the original regenerated plant of the second variant **(j-tc2)** already expressed the mutant trait. This jointless R_0 plant bred true in both the R_1 and R_2 generations. Based on crosses with a known jointless mutant and with a normal jointed tomato, it has been ascertained that the original R_0 plant was homozygous recessive for the jointless mutant. Presumably this somaclone originated by mutation, followed by mitotic recombination and subsequent shoot regeneration. This new mutation complements the known **j** mutation so that it is not encoded in the same gene. However, it does not complement the known **j-2** mutant, and is, therefore, allelic with this somaclonal mutant and maps at position 0 on the short arm of chromosome 11. The mottled mutation **(m-tcl)** uncovered among somaclones is a chlorophyll-deficient mutant that was identified in the R_1 generation in the greenhouse. Selected mottled R_1 plants breed true for the mottle appearance. The variegation is somewhat similar to the previously reported plastome mutants. When chlorophyll-deficient sectors of plastome mutants are placed in vitro, it is possible to establish shoot cultures of pure chlorophyll-deficient tissue (Gleba 1979). However, the mottled phenotype of m-tcl is quite stable and is maintained in shoots regenerated from leaf explants of mottled plants. In addition, it is also possible to discern orange-red mottling on fruits of homozygous (m-tcl/m-tcl) plants. Once again, as with tv-tcl, the m-tcl mutant has an effect on both the chlorophyll and carotenoid pigments. This mutant appears to be distinct from any of the hundreds of previously reported tomato mutants.

Several other single gene mutations have been identified in R_1 progeny tests of regenerated tomato plants including a semidominant allele controlling an electrophoretic variant of alcohol dehydrogenase and a dominant allele conferring resistance to *Fusarium oxysporum* race 2 (Miller et al. 1985). The *Fusarium*-resistant mutant is allelic to the previously reported I-2 gene. In addition, several somaclones have been identified with agriculturally important traits that do not appear to be controlled by single genes. For example, new lines have been developed with higher pigment and solids.

Morphological variants have also been detected among regenerated plants of tomato that bred true in the R_1 generation. In many cases the altered somaclones have larger leaf size, darker leaf color, or reduced fruit set. In some cases, these R_1 plants have been evaluated, and preliminary evidence obtained, by restriction enzyme analysis of isolated chloroplast DNA, suggests that genetic changes have occurred in chloroplast DNA (Evans et al. 1984). Chloroplast genes are inherited maternally in tomato. Hence, one would expect the variant to breed true in the R_1 progeny. It is not surprising that chloroplast DNA variants are uncovered as the number of plastids in a developing shoot apex is much smaller than in a mature cell (Bendich and Gauriloff 1984). Hence, if a mutation occurs in chloroplast DNA, it is more likely to become the dominant plastid type during sorting out, if the mutant occurs in 1 of 10 plastids than in 1 of 100 plastids, for example.

In addition, several other tomato varieties have been regenerated using these procedures. Single gene mutations have been recovered in several of these varieties including several male sterile and chlorophyll-deficiency mutations. A recessive mutation for chlorophyll-deficiency was identified in one tomato breeding line. This new mutant appears distinct from other known chlorophyll-deficient mutations. New fruit color mutants have also been detected including a dominant orange mutation and a recessive yellow mutation. The orange mutation appears to be pleiotropic as it is associated with bushy foliage and altered flower color. The yellow mutation, on the other hand, affects only fruit color.

The following can be concluded regarding somaclonal variation in tomato:

1. Chromosome number variation can be recovered in regenerated plants.
2. Several single-gene mutations have been recovered in several different tomato varieties.
3. Somaclones include dominant, semidominant, and recessive nuclear mutations.
4. The frequency of single-gene mutation using our procedure is in the neighborhood of 1 mutant in every 20–25 regenerated plants.
5. Some evidence suggests that new single-gene mutants not previously reported using conven-

tional mutagenesis have been recovered using somaclonal variation.

6. The occurrence of 3:1 ratios for single-gene mutants in R_1 plants suggests that mutants are of clonal origin, and that the mutation occurred prior to shoot regeneration (i.e., no mosaics are detected).
7. Evidence suggests that mitotic recombination (reciprocal or nonreciprocal) may also account for some somaclonal variation.
8. Evidence suggests that mutations in chloroplast DNA (detected by both maternal inheritance and restriction enzyme analysis) can also be recovered.
9. Agriculturally useful variants leading to development of new breeding lines have been recovered via somaclonal variation.

Development of New Ornamentals

As demonstrated by the work on tomato, it is possible to use somaclonal variation to uncover new, useful genetic variants. In addition to those variants already described, many morphological variants, with novel leaf, flower, and fruit color were detected. These unique variants, though not useful for a commercial horticultural crop, are the type of variants often used to release new ornamental varieties. To evaluate the potential value of somaclonal variation in the development of new ornamental varieties, we regenerated plants of *Nicotiana alata* 'Nicki Red'.

This species has a diploid chromosome number of 18. It is self-incompatible. Young, fully expanded leaves of the cultivar 'Nicki Red' were surface-sterilized in 8% Clorox® for 8 min, then rinsed three times in sterile distilled water. Leaves were dried under sterile conditions and then cut with a sterile scalpel into 2.3 × 2.5 cm sections and placed onto agar-solidified media composed of Murashige-Skoog (MS) macro- and micronutrients and B5 vitamins. This modified Murashige and Skoog medium was supplemented with 0, 0.5, 1.0, 5.0, or 10.0 μMol of 6-benzyladenine (BA) to determine capacity for plant regeneration.

Leaf explants were cultured under 16 hours of light with a mean temperature of 29.4°C (85°F). When regenerated shoots had reached a height of 3 cm, they were excised and placed onto rooting medium: modified MS with 0.1 μMol 3-aminopyridine (Evans et al. 1980). Following root development, shoots were transplanted to Jiffy® pots and subsequently to 10-cm pots when secondary leaves developed. Plants were transferred to the greenhouse where they were raised to maturity.

Shoots were regenerated from explants cultured on either 1.0 μMol or 5.0 μMol BA within 28 days. After 56 days in culture, none of the explants on hormone-free medium had regenerated shoots. In the medium containing 0.5 μMol BA, a small amount of callus was formed from which only one small shoot was regenerated from the eight explants at this treatment. Large shoots with normal morphology were obtained using 1.0 μMol BA, but the frequency of shoot formation and number of shoots recovered was less than on the medium containing either 5.0 μMol or 10.0 μMol BA. Five μMol BA was the optimum as it resulted in the highest number of shoots in the shortest time period.

Some regenerated plants were phenotypically distinct from seed-derived plants. Seventy-two percent of the plants regenerated in the presence of 10 μMol BA exhibited first and second leaves that were wider than young leaves of seed-grown *N. alata*. However, subsequent leaves of these regenerated plants developed normally. Hence, this trait is epigenetic. On the other hand, a small percentage of plants regenerated on modified MS containing 5.0–10.0 μMol BA flowered as soon as they were transferred to 10-cm pots, at a height of only 7.5 cm. This type of dwarfness was sexually transmitted to the R_1 self-fertilized progeny of these plants (Table 2). Plant height behaved as a quantitatively inherited character as a range of intermediate heights were observed among the R_1 progeny. While the population size was insufficient to ascertain the mode of inheritance, it is nonetheless clear that this trait, expressed as mean plant height, is stably transmitted to the R_1 generation. Evaluation of the R_1 generation will permit a more detailed genetic analysis of this trait. Changes

Table 2. Statistical comparison of dwarf *Nicotiana alata* self-fertilized progeny for flowering heights. Leaf explants cultured on modified MS medium containing 5 or 10 μMol BA.

Plant material	Mean ht(cm)	t-Test	Probability
Control (seed-derived)	52.0	–	–
10 μMol BA Regenerate, 3	29.5	2.52	0.01
10 μMol BA Regenerate, 5	38.7	2.45	0.025
10 μMol BA Regenerate, 10	37.2	1.97	0.05
Control (seed derived)	42.3	–	–
5 μMol BA Regenerate, 21	28.7	2.53	0.01
5 μMol BA Regenerate, 23	20.6	4.19	0.01

in gross chromosome number can be excluded from consideration as all regenerated dwarf plants had 2n=18.

Chromosome number was ascertained for each plant regenerated in the presence of 5.0 and 10.0 μMol BA. At both of these cytokinin concentrations 25% tetraploidy was observed in the root tips of the regenerated plants. These tetraploid regenerates were much slower to flower than comparable diploid regenerates. After 6 months from the date of culture, all of the diploid regenerates, but less than 50% of the tetraploid regenerates, had flowered.

Some tetraploid regenerates were crossed with diploid regenerates. Tetraploid plants were used both as female and male. Crosses were completed using regenerates that had already been observed to self-pollinate and set seed. It was presumed that these plants would have a higher probability of successful crossing than regenerates that had been unable to set seed. Several of these crosses were successful; however, triploid progeny were recovered only when the tetraploid was used as the female parent.

Plants regenerated on modified MS containing 5.0 or 10.0 μMol BA exhibited decreased pollen viability compared with seed derived plants when stained with acetocarmine. Plants regenerated on 10 μMol BA were also observed to produce less pollen than normal *N. alata*. Pollen viability remained low even after plants were 7 months old. These plants eventually produced a small amount of self-pollinated seed after 10–12 months in the greenhouse.

A great deal of variation was observed in *N. alata* plants regenerated from leaf explants. This included variation for morphological traits (flower shape, leaf shape, plant height), pollen viability, and chromosome number.

Some of the variation induced may have ornamental value. Currently, one of the most undesirable features for using ornamental types of *Nicotiana*, as bedding plants, is that they flower at heights of 30 cm or taller. While *N. alata* may be commercially treated with growth retardants such as daminozide, it is desirable to produce dwarf plants that will reach maturity rapidly and flower at a short height. Dwarf plants were recovered among the population of plants regenerated from leaf and petal explants. This population of dwarf regenerated plants had concomitant decrease in pollen viability. Such reduction in pollen viability is a hinderance to integration of these somaclonal variants into a breeding program. However, for *N. alata*, an alternative approach for the production of dwarfs was uncovered. Some individual diploid and tetraploid regenerated plants that retained high pollen viability were used in crosses to obtain triploid progeny. Triploids flower at lower plant heights than do seed-derived controls and behaved in many respects as dwarfs. In addition to the dwarf characteristics, triploids have the added advantage of reduced seed-set.

Gametoclonal Variation

The types of genetic changes that are recovered in plants regenerated from cell culture are dependent upon the donor material that is used for plant regeneration. For tomato a homozygous, diploid inbred variety was used. Hence, most regenerated plants were heterozygous resulting in segregation of new mutations in the R_1 generation. Lorz and Scowcroft (1983) used heterozygous material so that mutations could be visually detected in regenerated plants. Among these somaclones, several plants had distorted segregation ratios in the R_1 generation. Since the generation of variations will ultimately be used for breeding and crop improvement, it is important to distinguish somatic-derived somaclones and gametic-derived gametoclones. The gametes are products of meiosis, governed by Mendel's laws of segregation and independent assortment. Three genetic differences should be pointed out as evidence that gametoclonal and somaclonal variation are distinct.

1. Both dominant and recessive mutants induced by gametoclonal variation will be expressed directly in haploid regenerated plants from diploid anthers, since only a single copy of each gene is present. Hence, regenerated gametoclones (R) can be analyzed directly to identify new variants.
2. Recombinational events that are recovered in gametoclones would be the result of meiotic crossing over, not mitotic crossing over. While not fully characterized, evidence from *Neurospora* suggests that these two phenomena do not occur at the same frequency along the gene map. For example, meiotic crossing over may be used to separate genes that are hard to separate by mitotic crossing over.
3. To use gametoclones, the chromosome number must be doubled. The most frequently used method to double chromosomes is treatment with colchicine. Colchicine is known to induce mutations (Franzke and Rose 1952). Hence, by the time gametoclones are genetically analyzed,

some of the observed variation may not be due to gametoclonal variation but may be due to mutagenic treatment of colchicine.

The value of gametoclonal variation is evident from the reports of new variety development by Chinese workers (Zeng 1983). For wheat and rice, anthers of F_1 hybrid plants were introduced into culture and the recombinant microspores produced new doubled-haploid lines that contained genetic information from both parents. Anther culture has been used for the recovery of recombinant plants of a F_1 hybrid between Xian nog 5675 (a variety with white glumes, top awn, clavate spike, and tall stalk) and Jili (a variety with red glumes, awn, fusiform spike, and tall stalk). Several doubled-haploid plants were recovered that expressed mixed characters of the two parents. Hence, gametoclonal variation is valuable for hybrid sorting, i.e., development of homozygous diploid recombinant lines from interspecies or intraspecies F_1 hybrids.

Since growth of cells in culture results in single-gene mutations (Evans and Sharp 1983), culture of microspores could result in recessive mutations that are visible in the R_0 generation. Hence, it is likely that gametoclonal variants can be detected in the R_0 generation, while evaluation of R_1 progeny must be completed to detect the full spectrum of somaclonal variants. It is likely that mutant cells do not regenerate as well as wild-type cells. Hence, the mutation spectrum and frequency obtained from regenerated haploids may be strikingly different from regenerated diploid tissue, in which recessive mutations in heterozygous condition may have no effect on regeneration capacity. Oono (1981) has used gametoclonal variation to recover several mutants in rice as homozygous diploid plants. These included variation for heading date, seed fertility, plant height, morphology, and chlorophyl content.

It is also possible that certain variants may not be recovered using conventional mutagenesis but may be recovered using gametoclonal variation. Using conventional mutagenesis or somaclonal variation, no new variants for the S locus governing self-incompatibility (S = self-sterility) in *Lycopersicon peruvianum* were detected (Sree Ramulu 1982). However, S allele changes were detected for 17 of 53 gametoclonal regenerants of two genotypes. These changes were heritable and one new type of S allele was detected. This result, though preliminary, suggests that the mutation spectrum obtained by gametoclonal variation may differ from that obtained by somaclonal variation.

Implications for Breeding

Based on the results presented for tomato and ornamental *Nicotiana*, an overall breeding strategy is evident for use of somaclonal and gametoclonal variation to develop new cultivars of crops and ornamentals.

As single-gene mutations and organelle gene mutations have been produced by somaclonal variation, an obvious strategy is to introduce the best available cultivars into cell culture to select for improvement of a specific character. Hence, somaclonal variation could be used to uncover new variants that retain all the favorable qualities of an existing cultivar while adding one additional trait, such as disease resistance or herbicide resistance. Work with sugarcane and tomato has already suggested that this approach is feasible. Once new R_1 variants are identified, these should be field-tested in replicated plots to ascertain genetic stability. Seed should be increased at the same time to permit rapid cultivar development of promising lines. Reciprocal crosses between desirable R_1s and seed-derived controls can be used to gain an understanding of the genetic basis of the somaclonal variants. New, promising breeding lines can be introduced into cell culture to add an additional character, or to improve agronomic performance, or ornamental appearance of a selected somaclonal variant. By using this approach, it is possible to produce new breeding lines with desirable traits in a short period of time. While tissue-culture cloning has found widespread application in commercial horticultural operations for micropropagation, somaclonal variation has not been so widely used. It is apparent from the unique variants that are generated, the ease of the technology, and the ease of integration into a conventional breeding program, that this biotechnology tool will have increasing usefulness in horticulture and crop improvement during the next several years. The value of somaclonal variation is that it can be exploited for both crops and ornamentals to produce incremental changes.

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Manipulation of Cell and Protoplast Culture in Rice and *Brassica* Species

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Abstract

In rice, a viable system was developed for haploid and diploid plant regeneration from protoplasts that were isolated from cell suspension of anther callus. The nitrogen source of the culture media was found to be the critical factor for the success of this system. The possibility for direct gene transfer into protoplasts is discussed. In Brassica *species, somatic hybrid plants of* "BRASSICOMORICANDIA" *have been obtained through protoplast fusion between* B. oleracea *and* Moricandia arvensis. *We also succeeded in selecting a universal hybridizer from the cell suspension of* Sinapis turgida, *and in regenerating plantlets from somatic hybrids between* B. oleracea *and* B. nigra. *Prospects for somatic hybridization in crop improvement are discussed.*

Introduction

Regeneration of plants from protoplasts permits the use of somatic hybridization and direct gene transfer for crop improvement. Extensive attempts have been made to regenerate plants from protoplasts of many plant species. Success, however, has been largely limited to solanaceous species (Vasil 1983, Harms 1985). Plant regeneration from cereal protoplasts has been achieved with rice, *Oryza sativa* (Fujimura et al. 1985, Yamada et al. 1986, Shimamoto et al. 1986, Toriyama et al. 1986, Abdullah et al. 1986). In addition to plant regeneration from rice protoplasts, we succeeded in producing intergeneric somatic hybrid plants involving *Brassica* species (Toriyama et al. 1987a,b). This paper describes the techniques adopted by us for protoplast culture in rice, and for protoplast fusion in *Brassica.*

Plant Regeneration from Protoplasts in Rice

Successful plant regeneration from rice protoplasts has been reported by different scientists (Table 1). Although the methods employed by scientists vary, protoplasts isolated from cell cultures are currently the only source of totipotent protoplasts. Sustained and consistently reproducible divisions have not yet been reported with mesophyll protoplasts.

Cell suspension cultures as a source of protoplasts

Establishment of a friable cell suspension consisting of small rapidly dividing cells seems to be a critical step in the protoplast culture of cereals (Vasil 1983, Vasil and Vasil 1984). Such a cell suspension for rice was obtained either by selecting special cell lines or by using special media. Yamada et al. (1986) selected a cell line with a good growth rate and dense cytoplasm, while Fujimura et al. (1985) stressed the necessity of selection for good protoplast releasing and plant regeneration properties. We found that finely dispersed cell suspensions can easily be established in an amino acid-based medium (AA medium). The friability of callus was found to vary depending on the nitrogen source in the culture medium. The AA medium promoted the release of protoplasts effectively (Toriyama and Hinata 1985a,b).

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Table 1. Conditions for protoplast culture in rice.

Cultivar (origin)	Medium for Cell suspension	Protoplast culture	Regeneration
Fujimura et al. 1985			
Nipponbare Sasanishiki (immature embryo)	R2 medium B5 vitamins 2,4-D:1 mg L^{-1} CH:0.3%	R2 medium B5 vitamins 2,4-D:2 mg L^{-1} sucrose:0.4 Mol	N6 medium hormone-free
Yamada et al. 1986			
Fujiminori (seed)	LS medium 2,4-D:20μMol	RY-2 medium 2,4-D:2 mg L^{-1} glucose:0.5 Mol	LS medium BA:4μMol sucrose:8%
Shimamoto et al. 1986			
Nipponbare Norin 14 (seed, immature embryo)	R2 medium B5 vitamins 2,4-D:1 mg L^{-1} CH:0.3%	KM8P medium 2,4-D:2 mg L^{-1} sucrose:0.4 Mol Agarose-beads method with nurse cells	N6 medium hormone-free
Toriyama et al. 1986			
Yamahoushi (anther)	AA medium[1] 2,4-D:1 mg L^{-1} KIN:0.2 mg L^{-1} GA3:0.1 mg L^{-1}	NO_3 medim[2] 2,4-D:1 mg L^{-1} KIN:0.2 mg L^{-1} GA3:0.1 mg L^{-1} glucose 5%	N6 medium IAA:0.2 mg L^{-1} KIN:1 mg L^{-1}
Abdullah et al. 1986			
Taipei 309 (young leaf and root)	AA medium[3] 2,4-D:2 mg L^{-1} KIN:0.2 mg L^{-1} GA3:0.1 mg L^{-1}	Kao macro B5 micro salts 2,4-D:0.5 mg L^{-1} NAA:1mg L^{-1} Zeating:0.5 mg L^{-1} Agarose:1.2%	N6 medium hormone-free sucrose:8%

1. Modified B5 medium without inorganic nitrogen and supplemented with 876 mg L^{-1} glutamine, 266 mg L^{-1} aspartic acid, 174 mg L^{-1} arginine, and 7.5 mg L^{-1} glycine.
2. Modified B5 medium without $(NH_4)_2SO_4$. It contains KNO_3 as the nitrogen source.
3. Modified MS medium without inorganic nitrogen and supplemented with 877 mg L^{-1} glutamine, 266 mg L^{-1} aspartic acid, 228 mg L^{-1} arginine and 75 mg L^{-1} glycine.

Abbreviations: LS medium: Linsmaier and Skoog 1965; R2 medium: Ohira et al. 1973; N6 medium: Chu et al. 1975; KM8P: Kao and Michayluk 1975; Kao 1977; B5: Gamborg et al. 1968; 2,4-D: 2,4-dichlorophenoxyacetic acid, IAA: indole-3-acetic acid; BA: 6-benzylaminopurine; KIN: Kinetin; GA3: gibberellic acid; CH: Casein hydrolysate.

Protoplast culture

Isolated protoplasts were successfully cultured in several well defined media (Table 1). For example, in the RY-2 medium, calf serum was added, glucose was used as the osmoticum, and the ammonium and ferric ions were decreased (Yamada et al. 1986). Additionally, agarose was sometimes added to the medium, as reported for other protoplasts (Shillito et al. 1983). Abdullah et al. (1986) reported a marked improvement in plating efficiency when the agarose concentration was increased from 0.6 to 1.2%. Shimamoto et al. (1986) imbedded protoplasts in agarose beads and used a nurse culture for colony formation.

The morphology of callus obtained from protoplasts varied with the nitrogen source of the culture medium. A compact callus was formed in the nitrate-based medium, while a friable one was formed in the AA medium. The compact callus was found to be superior for regeneration (Toriyama et al. 1986). Colonies were usually obtained from a small percentage of the protoplasts.

Plant regeneration

The success of regeneration from protoplasts depends upon the genotype and source of the cell suspension. In Japanese rice cultivars, the progenies of cultivar Asahi, such as Nipponbare and Yamahoushi, were reported to have a high regeneration capacity in anther culture (Sasaki 1986), and are often used for protoplast culture. In such cultivars, green plants were easily regenerated from protoplast-derived calli with a frequency of about 20%. Shimamoto et al. (1986) also succeeded in regenerating plants from the major Japanese commercial cultivars, Sasanishiki and Koshihikari.

Protoplasts isolated from callus derived from "young tissue" are considered to be the most suitable for regeneration (Vasil 1983, Vasil and Vasil 1984). Immature embryos, young leaves and roots, or anthers are generally used. Plant regeneration has also been achieved from protoplasts derived from mature seeds (Table 1).

The regenerated plants were successfully grown in soil and most of them set seeds normally. Haploid plants were also obtained when protoplasts were isolated from anther-derived callus (Toriyama et al. 1986).

Haploid and diploid plant regeneration from protoplasts of anther callus in rice

Regeneration of haploid and diploid plants was demonstrated from protoplasts that were isolated from cell suspensions of anther callus (Toriyama et al. 1986). The cell suspension in the AA medium that contained four amino acids as the sole nitrogen source was friable, finely dispersed, and readily released a large number of protoplasts. These protoplasts, subsequently cultured in NO_3 medium that contained nitrate as the sole nitrogen source, formed compact calli. The compact calli produced green plants with a frequency of about 20%. Out of 33 flowering plants, about 40% were diploids with normal fertility and about 30% were haploids, and the other 30% were showed sterile aneuploids. The selfed progenies of the diploid plants showed normal fertility and uniform morphology.

Prospects for genetic engineering in rice

Direct gene transfer by polyethylene glycol (PEG) treatment or electroporation is now available for cereal protoplasts (Lorz et al. 1985, Potrykus et al. 1985, Fromm et al. 1985, 1986). In rice, Uchimiya et al. (1986) succeeded in transferring chimaeric plasmids, containing a kanamycin-resistant gene, into protoplasts; stable expression of that gene has been obtained in callus, although regeneration from transformed callus has not yet been achieved. Genetically engineered rice plants can be produced in the near future, if the regeneration and transformation techniques are combined.

Production of Somatic Hybrid Plants in *Brassica* Species

Somatic hybridization by protoplast fusion is a powerful method for increasing genetic variation in higher plants. In the current fusion technique, there is an obvious need to select the fusion products from parent cells (Harms 1985). Somatic hybrid plants in *Brassica* were produced by using five methods of selection as shown in Table 2. Where hybrid cells show strong hybrid vigor, a selection procedure is not necessary.

Selection of a universal hybridizer in *Sinapsis turgida* and regeneration of plantlets from somatic hybrids with *Brassica* species

If a double-mutant can be established with markers that are both dominant and counter-selectable, it

Table 2. Selection methods and the somatic hybrid plants produced in Brassicaceae.

Mechanical isolation by micromanipulator
Arabidopsis thaliana + *B. oleracea* (Gleba and Hoffmann 1978)
B. campestris + *B. oleracea* (Sundberg and Glimelius 1986)
Automatic isolation by fluorescence-activated cell sorter
B. campestris + *B. oleracea* (Glimelius et al. 1986)
Eruca sativa + *B. napus* (Fahleson and Glimelius 1986)
Inactivation by iodoacetamide
B. campestris + *B. oleracea* (Terada et al. 1986)
Universal hybridizer
Sinapis turgida + *B. oleracea* (Toriyama et al. 1987a)
Sinapis turgida + *B. nigra* (Toriyama et al. 1987a)
Hybrid vigor
Moricandia arvensis + *B. oleracea* (Toriyama et al. 1987b)
B. campestris + *B. oleracea* (Schenck and Robbelen 1982)

can serve as a universal hybridizer in selecting somatic hybrids after protoplast fusion with any other wild-type partner (Toriyama et al. 1987a). Such a double mutant, that is unable to grow in a medium with NO_3 as the sole nitrogen source, and is resistant to 5-methyltryptophan (5MT), was selected from a cell suspension of *Sinapis turgida* by culturing the cells in AA medium supplemented with 50 mMol chlorate and 229 μMol 5MT. Protoplasts of this cell line were fused with mesophyll protoplasts of *Brassica oleracea* with dextran (Kameya 1984) and six somatic hybrids were selected initially by culture in the NO_3 medium and then by transfer to the NO_3 medium supplemented with 229 μMol 5MT. The somatic hybrids produced embryoids, leaves, and plantlets on a regeneration medium. The hybrid characters were confirmed by investigations of acid phosphatase and peroxidase isozymes, chromosome number, growth on NO_3 medium, 5MT resistance, and capacity to regenerate plants. Somatic hybrids between *Sinapsis turgida* and *Brassica nigra* were also obtained using this method.

Production of somatic hybrid plants, "BRASSICOMORICANDIA", through protoplast fusion between *Moricandia arvensis* and *Brassica oleracea*

Intergeneric hybrid plants were obtained through protoplast fusion between *Brassica oleracea* and *Moricandia arvensis*, without selecting hybrid protoplasts (Toriyama et al. 1987b). Protoplast fusion was carried out by the dextran method (Kameya 1984) using protoplasts isolated from hypocotyls of red cabbage and from suspended cells of *M. arvensis*. The fusion-treated protoplasts cultured in B5 medium (Gamborg et al. 1968) with 2 mg L^{-1} 2,4-D and 1 mg L^{-1} BAP produced abundant calli and, of these, eight calli produced hybrid plants. The regenerated plants exhibited combined morphological characteristics of both parental species. Analysis of leaf isozymes for acid phosphatase and aspartate aminotransferase also indicated their hybridity. Even though no selection was performed for hybrid cells, all the regenerated plants were hybrids. It is considered that the somatic hybrids exhibited hybrid vigor and readily produced shoots. Two somatic hybrid plants were also obtained between cauliflower and *M. arvensis*.

Successful regeneration of plants from protoplasts can probably be achieved in most cereals. Regeneration from protoplasts makes it possible to transfer alien genes into plants and to produce somatic hybrids. Cybrids are obtained in protoplast fusion (Harms 1985). These techniques can be widely applied to crop improvement.

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Tissue Culture Approaches to Pigeonpea Improvement

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Abstract

Pigeonpea anthers were cultured on different media in an effort to develop haploids. MS medium supplemented with 2 mg L^{-1} of 2,4 dichlorophenoxy acetic acid (2,4-D) supported the development of callus from Cajanus cajan *anthers, while potato starch extract medium promoted the best response from anthers of* Atylosia gradifolia *and* A. volubilis. *Such calli failed to differentiate on subculturing on media supplemented with combinations of hormones. Screening for chromosome elimination following intergeneric hybridizations also did not meet with any success in haploid formation. Culture conditions for plantlet regeneration from immature embryos of* Cajanus *have been standardized for further embryo rescue following distant hybridizations. An age-dependent embryo response was evident. Embryos older than 11 days developed into plants on MS or B5 media supplemented with 2,4-D (1 mg L^{-1}). The B5 medium was superior to MS for regeneration. Plantlets were also regenerated from explants of cotyledons from mature seeds, leaves and epicotyls of seedlings of* Cajanus *cultivars and* Atylosia *species in different media supplemented with hormones [2,4-D, naphthalene acetic acid (NAA), benzyl adenine (BA), kinetin and gibberelic acid (GA3)]. Whole cotyledons or their proximal segments were found to be suitable for regeneration.*

Introduction

Pigeonpea (*Cajanus cajan*) is an important pulse crop in the tropics, and ranks fifth, in area grown, among the edible legumes. Diverse gene pools have been the foundations for effective crop improvement programs. Among options open to plant breeders to widen the genetic base are exploitation of alien variation and somaclonal variation. There has been a rapid accumulation of literature on wide hybridization in crop plants (Collins and Grosser 1984). Pigeonpea has a wealth of related wild species in the genus *Atylosia*, with the same basic chromosome number as pigeonpea (x=11). Many of the *Atylosia* species possess desirable characters such as disease and insect resistance, high seed protein content, photoperiod insensitivity, drought and salt tolerance, and frost tolerance (Remanandan 1981).

Wide hybridization is often hampered by postfertilization barriers such as failure of endosperm development and subsequent degeneration of hybrid embryos. In such cases, viable hybrids have been produced using embryo rescue techniques (Raghavan 1977, Stewart 1981). Several studies have shown that certain *Atylosia* species hybridize successfully with *Cajanus* while others fail to cross (Table 1). Apart from being a novel source of variability, wide hybridizations in barley proved to be a potential means of haploid production (Kasha and Kao 1970) via selective chromosome elimination (Subrahmanyam and Kasha 1973). Pigeonpea is a long-duration, photoperiod-sensitive plant and can normally be grown once a year. Development of haploids would, therefore, have immense value in pigeonpea breeding in reducing the time required for developing inbred lines. Unfortunately, haploid induction

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Table 1. ***Atylosia*** **species successfully hybridized with pigeonpea.**

Species	Reference
A. albicans	Kumar et al. (1985a)
	Pundir and Singh (1983, 1985a,b)
A. acutifolia	Dundas (1984), Dundas et al. (1986)
A. cajanifolia	Tripathi et al. (1984), Kumar et al. (1985a,b), Pundir and Singh (1983, 1985a,b)
A. lanceolata	Kumar (1986)
A. latisepala	Kumar (1986)
A. lineata	Deodikar and Thakar (1956), Kumar et al. (1958), Kumar and Thombre (1958), Reddy (1981a), Reddy and De (1983)
A. pluriflora	Dundas (1984), Dundas et al. (1986)
A. reticulata	Dundas (1984), Dundas et al. (1986)
A. grandifolia	Kumar (1985)
A. sericea	Deodikar and Thakar (1956), Reddy (1981b), Kumar et al. (1985a)
A. scarabaeoides	Reddy (1981c), Pundir and Singh (1983, 1985a,b)
A. trinervia	Pundir and Singh (1983, 1985a,b)

techniques in legumes have not shown much promise (Bajaj et al. 1980). Tissue-culture techniques are emerging as a major supplement to conventional plant breeding procedures (Vasil et al. 1982) due to the increasing importance of clonal propagation (Murashige 1974), haploid production (Chu 1982), and production of disease-free plants (Gengenbach et al. 1977). Such studies are limited in pigeonpea (Kusumakanta and Padmanabhan 1964, Shamarao and Narayanaswamy 1975, Mehta and Mohanram 1981, Kumar et al. 1984). In the light of these considerations, investigations were undertaken:

1. to assess the crossability relationship between *Cajanus* and its wild relatives,
2. to develop methods to improve species crossability,
3. to screen for haploids following wide hybridizations, and
4. to develop in vitro regeneration techniques in pigeonpea.

Material and Methods

Emasculation, hybridization, and hormone treatments were done according to Kumar et al. (1985a). MS medium (Murashige and Skoog 1962), BP 5P medium (Gamborg et al. 1968), White's medium (White 1954) and potato starch extract medium (Anonymous 1976) were used as the basal media. Sucrose at 3% concentration was used as the carbon source, and pH was adjusted to 5.6–5.8. Media were solidified with 0.8% Difco® agar.

Seeds (for cotyledon and embryo cultures) and flower buds (for anther cultures) were surface-sterilized for 15 min in 10% chlorox (Chlorox®, USA), washed thrice with sterile water and retained in sterile water for 30 min, followed by drying prior to inoculation. For cotyledon cultures, the seed coat was removed and the seed was split so as to obtain two distal and two nodal halves of the cotyledons. All operations were conducted under asceptic conditions in a laminar flow hood. The cultures were maintained at a temperature of 25 ±2°C under cool flourescent light (6.8 W m^{-2}). Four *Cajanus* cultivars were used for standardizing the culture conditions for immature embryos. For anther cultures, in addition to *Cajanus, Atylosia albicans, A. grandifolia,* and *A. volubilis* were tested.

Results

Crossability

Eight species of *Atylosia* (*A. albicans, A. cajanifolia, A. grandifolia, A. lanceolata, A. latisepala, A. lineata, A. scarabaeoides* and *A. sericea*) hybridized with *Cajanus*, while four species (*A. mollis, A. platycarpa, A. rugosa, A. volubilis*) failed to cross with *Cajanus*. The crossability between *Cajanus cajan* and *Atylosia* species varied with both the cultivars and the species used (Table 2). Reciprocals were attempted of the 24 successful combinations, but success was very low (Table 2). In the crosses involving *A. mollis* and *A. volubilis* and *Cajanus* cultivar Pant A2, pod development was normal but the seeds from such pods were shrivelled and nonviable.

Table 2. Pod set (%) in crosses of *Atylosia* spp with different cultivars of *Cajanus cajan* (reciprocals in parenthesis).

Pollen parent	Cultivars			
	Pant A2	Baigani	ICP 7035	C 11
A. lineata	19.5	12.3	1.4	15.2
A. albicans	9.2(0.2)	7.2	1.1	5.1
A. sericea	2.4(0.2)	3.0	0.8	1.7
A. scarabaeoides	4.6	2.4	0.5	1.5
A. cajanifolia	2.7	3.1	0.6	2.1
A. grandifolia	1.7	-	-	3.1
A. latisepala	0.9	-	-	2.0
A. lanceolata	1.7	-	0	0.9

Effect of hormone treatments

Cajanus cajan cv Pant A2 was the female parent in the hormone studies. The successful combinations exhibited a uniform response to hormone treatments. GA3 was found to be superior irrespective of the *Atylosia* species used as pollen parent and irrespective of the cross combination at concentrations up to 50 ppm to controls (Table 3). For instance, when *A. grandifolia* was the pollen parent, pod set was increased from 2% in the control to 14% in treatments with 50 ppm of GA3. The optimum concentration was found to be 40-50 ppm. Higher concentrations were detrimental with complete failure at 80 ppm or above. Treatments with GA3 + kinetin mixture did not improve pod set, and were detrimental at higher concentrations in crosses of *C. cajan* with *A. albicans, A. cajanifolia* or *A. sericea*, as male parents (Table 3). Treatments showed that GA3 alone, or in combination with kinetin, increased the pod length at physiological maturity to 7 cm as compared with 4.5-5.5 cm in the controls. The number of seeds per pod increased from 1.6-2.2 in the controls, to 3.5-4.0 when GA3 or GA3 + kinetin treatments were given. Hormone treatments did not influence seed size. ,

Among unsuccessful combinations, bud drop commenced within 2 days after pollination. GA3 treatments prolonged ovary development and delayed bud drop for varying periods depending upon the cross combination and hormonal concentration. When either *A. platycarpa* or *A. volubilis* was the pollen parent, bud drop commenced 2 days after pollination in the control. GA3 prolonged this period to 5 days. When *A. mollis* was the pollen parent, bud drop was delayed by 3-4 days following GA3 treatment. Increase in ovule size was evident in cross combinations where bud drop was delayed. In all reciprocal crosses, there was variation in response to hormones. Crosses involving *A. sericea* and *A. grandifolia* did not respond to any of the treatments. In *A. cajanifolia*, GA3 delayed bud drop while a mixture of GA3 and kinetin or kinetin alone were ineffective. A similar trend was observed in unsuccessful crosses involving *Cajanus cajan* as female parent.

Screening for chromosome elimination

The F_1 hybrids in general tended to be intermediate in morphology between the parental species. *Atylosia* characters such as seed strophiole, seed mottling, pod hairiness, and persistance of petals were expressed in the F_1 generation. Leaf shape of hybrids between *Cajanus* and *A. albicans* was intermediate, with an obtuse tip in the initial stages of growth.

Table 3. Pod set (%) in *Cajanus cajan* cv Pant A2 × *Atylosia* spp crosses following hormone treatments.

Concentration (ppm)	Pollen parent											
	A. albicans			*A. cajanifolia*			*A. gradifolia*			*A. sericea*		
	GA3	KIN	GA3+KIN	GA3	KIN	GA3+KIN	GA3	KIN	GA3+KIN	GA3	KIN	GA3+KIN
Control	7	7	7	2	2	2	2	2	2	1	1	1
10	8	8	6	3	2	1	1	3	1	2	0	1
20	8	8	6	3	2	1	1	3	1	2	0	1
30	10	7	7	4	2	1	4	2	1	3	2	2
40	13	6	8	11	3	1	5	1	0	7	3	1
50	17	8	3	7	3	0	14	2	0	5	2	1
60	4	5	1	3	2	0	3	3	0	10	1	0
70	1	4	0	0	2	0	0	2	0	0	2	0
80	0	5	0	0	1	0	0	2	0	0	1	0

Leaves that developed after 100 days on some branches resembled those of the *Cajanus* parent (having an acute leaf tip). The texture and leaf surface revealed similarities between leaves of the *Cajanus* parent and those of the *Cajanus*-like leaves produced on the hybrid. Both exhibited long trichomes with uniform spread (Kumar 1985). Leaves of the *A. albicans* parent have a dense population of trichomes, whereas the initially developed, intermediate-type leaves had a sparse population of short trichomes. Floral initiation occurred only on branches that had developed *Cajanus*-like leaves. Meiotic investigations revealed the hybrid nature of these F_1 hybrids (Kumar et al. 1984).

Embryo Culture

Since most of the *Cajanus-Atylosia* crosses are successful only with *Cajanus* as the female parent, selfed embryos from *Cajanus* were used for standardizing the embryo culture technique. Response of embryos from four genotypes of pigeonpea was tested on MS and B5 media supplemented with 2,4-D (1 mg L^{-1}). An age-dependent response was evident (Table 4). Eleven to 14-day-old embryos developed callus from which plantlets were obtained at low frequency. When 15-19-day-old embryos were cultured, seedlings were recovered directly, with small amounts of callus at the base of each plantlet. Direct seedling recovery and occasional callus formation were obtained after culturing embryos older than 19 days. Embryos younger than 11 days failed to respond. Since post-pollination GA3 treatments delay bud/pod drop in intergeneric crosses, further refinements in the embryo culture techniques are likely to help in obtaining desirable *Cajanus* × *Atylosia* hybrids that have not yet been possible.

Anther Culture

Callus was obtained from the anthers of *C. cajan* and *A. albicans* on MS medium supplemented with 2 mg L^{-1} of 2,4-D. Potato starch extract medium promoted callus development from anthers of *A. grandifolia* and *A. volubilis*. Callusing was more profuse from anthers of *A. albicans* and *A. volubilis* than from the other species. Attempts to induce differentiation by subculturing the callus on basal media supplemented with various hormone combinations were not successful. On subculturing, the callus turned brown and degenerated. The use of activated charcoal (100 mg L^{-1}) and polyvinylpyrolidine (1g L^{-1}) in the medium did not improve callus survival.

Tissue Culture

Exploratory attempts to standardize culture conditions for regeneration of *Cajanus* and *Atylosia* plants from cotyledons, mature seeds and leaf, and epicotyl segments from 1-week-old seedlings, resulted in different degrees of success. Cotyledon was found to be the best explant for regeneration. Four cultivars of *Cajanus* and one accession each of *A. cajanifolia*, *A. albicans*, and *A. sericea* were used. Three basal media (modified MS, B5, White's) supplemented with various hormones [2,4-D, indole acetic acid (IAA), NAA, BA, kinetin (KIN), and GA3] either alone or in combination were tested. Preliminary experiments revealed superiority of modified MS medium. Regeneration potential of whole cotyledons, and nodal and distal halves of cotyledons was studied.

The modified MS medium, supplemented with 2,4-D (2 mg L^{-}) induced copious amounts of healthy callus irrespective of the region of the cotyledon

Table 4. Percentage plantlet recovery from embryo cultures of pigeonpea on MS and B5 media supplemented with 2,4-D (1 mg L^{-1}).

		Cajanus cajan cultivars			
Embryo age	Media	Pant A2	Prabhat	ICP 7035	C 11
<11 days	MS	0	0	0	0
	B5	0	0	0	0
11-14 days	MS	11	8	10	7
	B5	17	7	19	13
15-19 days	MS	73	69	81	67
	B5	74	88	93	84
>19 days	MS	57	66	71	55
	B5	87	90	89	84

used. Whole cotyledons and nodal halves of cotyledons on modified MS medium supplemented with 2,4-D (0.5 mg L^{-1}) and BA (2 mg L^{-1}) developed multiple shoots (37) in addition to small amounts of callus in 2–46% of the cultures depending upon the cultivars (Tables 5,6). Among the *Atylosia* species, multiple shoots developed from 21% of *A. cajanifolia* cultures, but were rare in the cultures of *A. albicans* and *A. sericea.* In cultures of the distal segments of the cotyledons, only shoot bud-initiation was observed at low frequencies after profuse callusing. From the explants with multiple shoots, rooting was obtained on MS medium supplemented with NAA (2 mg L^{-1}) and BA (0.5 mg L^{-1}). Basal medium supplemented with 2,4-D (0.5 mg L^{-1}) and NAA (1 mg L^{-1}) also induced multiple shoots from whole cotyledons and nodal cotyledonary segments. With this hormone combination, both shoots and roots developed from 2–14% of whole cotyledon explants of *C. cajan* and from about 2% of *A. cajanifolia* (Tables 5 and 6). A low frequency of plantlet regeneration was obtained from nodal halves of the *C. cajan* cotyledons. In the cultures of *A. albicans* and *A. sericea* only shoot regeneration was obtained. Of the four *Cajanus* cultivars tested, three showed varying levels of regeneration, while the fourth cultivar (GS4) failed to respond. The response of *A. cajanifolia* compared well with the response of *C. cajan* cultivars. In general, wherever there was multiple shoot formation, only one or two shoots developed fully, while the others remained suppressed.

The present study revealed that crossability with *Atylosia* is significantly influenced by the genotypes used as *Cajanus* parent, and that postpollination hormone applications improve crossability. In the unsuccessful combinations, postpollination hormone treatments delayed bud/pod drop. Anther culture studies were not successful beyond the stage of callus induction (Bajaj et al. 1980). Cultivar differences with respect to the percentage of regeneration indicate genotypic variation for this trait.

Table 6. Percentage regeneration from cotyledon cultures of different cultivars of *Cajanus cajan* and species of *Atylosia* on modified MS medium supplemented with 2,4-D (0.5 mg L^{-1}) and BA (2 mg L^{-1}).

Species/cultivars	Whole cotyledon	Cotyledon segments	
		Nodal	Distal
C. cajan cv ICP 4726	38	33	7
C. cajan cv ICP 7035	46	31	13
C. cajan cv Pant A2	27	29	6
C. cajan cv GS4	2	–	–
A. cajanifolia	21	14	10
A. albicans	19	16	–
A. sericea	11	10	–

Table 5. Percentage plantlet and shoot regeneration from cotyledon cultures of different *Cajanus cajan* cultivars and species of *Atylosia* on modified MS medium supplemented with 2,4-D (0.5 mg L^{-1}), BA(2 mg L^{-1}), and NAA (1 mg/L^{-1}).

Species/cultivar	Whole cotyledon		Cotyledon segments			
			Nodal		Distal	
	Shoots	Plantlets	Shoots	Plantlets	Shoots	Plantlets
C. cajan cv ICP 4726	32	8	31	3	17	–
C. cajan cv ICP 7035	39	14	22	2	13	–
C. cajan cv Pant A2	26	2	23	12	–	
C. cajan cv GS4	–	2	–	–	–	–
A. cajanifolia	29	2	25	1	8	–
A. albicans	21	–	9	–	–	–
A. sericea	14	–	11	–	–	–

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Experiments on Protoplast Fusion in *Trifolium*

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Abstract

Red clover (Trifolium pratense) *and alsike clover* (T. hybridum) *are both important fodder plants and their hybridization is of considerable agricultural interest. Because their hybridization by conventional means is difficult, hybrid production is being attempted by protoplast fusion. Green mesophyll protoplasts and colorless protoplasts from suspension cultures were fused using either polyethylene glycol (PEG) or electrofusion. Fusion products were visually identified and picked out with glass capillaries and then cultured in microdroplets, or by using the nurse-culture technique. Sustained divisions and callus formation have been achieved from both parental species, but shoots have been regenerated so far only from* T. hybridum. *Of the fusion methods tested, electrofusion seems the most promising. The fusion frequency is at least 10 times that of the PEG method. Isolated hybrid protoplasts have regenerated cell walls and some divisions and colony formation have been seen in microdrop cultures. Some small calli, which are supposed to be of hybrid origin, have been regenerated using a nurse-culture technique.*

Introduction

Clover was the dominant nitrogen-fixing plant in the meadows of northern Europe until 30 years ago. Low cost and abundant availability of nitrogenous fertilizers have relegated clover to a low level of importance in the farming systems of Europe. Most adapted cultivars disappeared as a consequence. When the interest in clovers was renewed a decade ago, due to a steep rise in the cost of nitrogenous fertilizers, it became necessary for breeders to start with the weed-like clovers from road sides. Red clover, *Trifolium pratense*, was the most promising sward component to be grown together with timothy (*Phleum pratense*) or meadow fescue (*Festuca pratensis*). However, it was not persistent, and often disappeared from the sward after 2 growing seasons. Introducing better persistence from its relative, alsike clover, *T. hybridum*, has thus been attempted using both the embryo-rescue and the protoplast-fusion techniques. The resulting alloploid will have to be bred further by conventional methods both for agronomic characters and adaptation to the sward community.

Very few natural hybrids between species are known, but species within sections can often be hybridized by applying the embryo-rescue technique (Merker 1984). Red clover (*T. pratense*) and alsike clover (*T. hybridum*) belong to two different sections within the genus, sections *Trifolium* and *Lotoidea* respectively (Table 1). Red clover has a

Table 1. Taxonomy and chromosome number of the most common clover species within the two sections relevant to the hybridization of red and alsike clovers.

Section	Species	Chromosome number (x)
Trifolium	*alpestre*	8
	diffusum	8
	medium	aneuploid
	pallidum	8
	pratense	7
	sarosiense	24
Lotoidea	*ambiguum*	8
	hybridum	8
	nigrescens	8
	repens	16

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basic chromosome number of × = 7 while that of alsike clover is × = 8, a more common basic number in the genus.

Interspecific isolating mechanisms may be spatial, physiologic or genetic (Stebbins 1950). Barriers between the clovers appear to be physiological and/or genetical. Both red and alsike clover have a one-locus multiple-allele incompatibility system that avoids selfing at the gametophytic level (Taylor and Smith 1979), and ensures cross-pollination in both species. However, this system is not involved in avoiding interspecific crossing. The interspecific barriers to hybridization, we believe, are due to failure of fertilization or, possibly, hybrid abortion at an early stage of embryo formation (Kivinen 1987).

We have attempted to overcome the hybrid barrier between the two clover species by

1. using the embryo-rescue method (Kivinen 1987), and
2. by applying the protoplast-fusion method (Honkanen et al. 1986).

In the embryo-rescue method we have faced considerable problems and, so far, have not been able to produce hybrid plants. At the moment the protoplast-fusion method looks the more promising, particularly with electrofusion.

Material and Methods

Plant material

Four diploid and three tetraploid alsike clover and six diploid and two tetraploid red clover cultivars served as the source of protoplasts that were isolated from leaves and from suspension cultures. The material was selected out of 28 alsike clover and 32 red clover cultivars (Honkanen et al. 1984, Honkanen et al. 1986).

Plants were maintained as aseptic shoot cultures on L2 medium (Phillips and Collins 1979) without hormones at 24 ± 2°C under a 16-hour photoperiod. Cell suspension cultures were first established on B5 medium (Gamborg et al. 1968) supplemented with 2 mg L^{-1} 2,4-D and 0.5 mg L^{-1} kinetin and then maintained on B5 or SL medium (Phillips and Collins 1980) with 0.06 mg L^{-1} 2,4-D and 0.1 mg L^{-1} BAP. Sucrose contents of all media were 20 gL^{-1} and pH was adjusted to 5.8. Suspensions were cultured at 130 rev. min^{-1} on a platform shaker and they were subcultured once a week. Cultures were screened for best-growing cell lines and only these were used as donor material for protoplast isolation.

Isolation and culture of protoplasts

Leaves were sliced into strips about 2 mm thick. These were incubated overnight in an enzyme solution consisting of 0.5% Macerozyme R-10 (Molar Co.), 1% Cellulase Onozuka R-10 (Molar Co.) and 1% Cellulysin (Calbiochem) in 0.5 Mol sucrose with 5 mMol $CaCl_2 \times 2H_2O$. The pH was adjusted to 5.6 and the solution was filter-sterilized. After enzyme treatment the released protoplasts were filtered through 100 μm stainless steel sieves and centrifuged for 10 min at 100 × G. The floating protoplast layer was further washed with 0.35 Mol sucrose or with W5 salts (154 mMol NaCl, 125 mMol $CaCl_2 \times H_2O$, 5 mMol KCl, 5 mMol glucose). Protoplasts were either used for fusion studies or they were diluted with culture medium to a density of 10^5 protoplasts mL^{-1} and used for regeneration studies.

Protoplasts were isolated from suspension cultures by incubating one volume of actively growing, packed-cell clusters with four volumes of enzyme solution. Enzyme solution, purification, and washing procedures were the same as with mesophyll protoplasts.

Protoplasts were cultured as liquid cultures, and by using the bead-type technique (Shillito et al. 1983) in which protoplasts were plated into an agarose (type VII, Sigma) containing culture medium, and, after gelling sections of agarose, were transferred into liquid medium. Culture media used were KM8P medium (Kao and Michayluk 1975) and B5 medium supplemented with 250 mg L^{-1} caseinhydrolysate, 250 mg L^{-1} NH_4NO_3, 630 mg L^{-1} $CaCl_2 \times 2H_2O$, 90 g L^{-1} glucose and 2 mg L^{-1} each of 2,4-D, NAA and kinetin and 90 g L^{-1} glucose. These media (pH 5.8) were filter-sterilized.

Cultures were kept in the dark at +24°C for the 1st week after isolation. Otherwise culture conditions were the same as for the mother plants. After protoplasts had started to divide, osmolarity and auxin concentration of the culture medium was reduced stepwise by adding fresh culture medium with lower osmolarity. When developing colonies had grown to small calli size, they were transferred onto various agar-solidified media for differentiation.

Chemical PEG-fusion

PEG solution used for fusions contained 40% PEG, 100 mg L^{-1} $CaCl_2$ in 0.3 Mol glucose. Two parts of mesophyll protoplasts and one part of suspension culture-derived protoplasts in W5 medium were

mixed. Fusions were made on plastic petri dishes by adding PEG fusion solution to the droplets of mixture so that the final PEG concentration was about 20%. Treatment time was 5–15 min after which PEG was removed by washing protoplasts carefully with W5 medium, or by gradually lowering PEG concentration by adding a solution with lower concentration of PEG. The final wash was made with culture medium.

Electrofusion

Fusions were made in 0.35 Mol sucrose using a high-voltage cell processor. Equal parts of mesophyll protoplasts and suspension culture-derived protoplasts were mixed so that the final protoplast densities were at least 2×10^6 protoplasts mL^{-1}. Volumes of 0.3–0.5 mL of protoplast mixture were placed in the fusion chamber with 3 mm spacing between the electrodes. An electric impulse of 200–600 V with pulse duration of 30 microseconds was given to the protoplasts after a 1 to 3 seconds RF-field (amplitude 100–200 VAC).

Selection and culture of fused protoplasts

Fusion products between mesophyll protoplasts and colorless protoplasts isolated from suspension cultures were visually identified by the presence of green chloroplasts originating from mesophyll protoplasts and cytoplasmic strands originating from suspension protoplasts. These heterokaryons were picked out into microcapillaries within 36 hours of fusion, by using a micromanipulator. Selected protoplasts were either cultured in Cuprak® dishes, and in multivial dishes or they were cultured on Millicell™ HA (Millipore) plates that were inserted in small petri dishes with 1.5 mL of *Trifolium* protoplast suspension, which served as nurse cells. Densities in microdrops were 1000–10 000 protoplasts mL^{-1} and the number of selected protoplasts was 100–200 per millicell HA plate.

Results and Discussion

Regeneration of *Trifolium* protoplasts

In our studies, the KM8P medium did not induce sustained divisions. The best protoplast growth of both parental species was obtained in B5 medium. First divisions of alsike clover were seen within 1 week (optimally 3 days) and developing colonies could be transferred into agar media within 1 to 2 months. The bead-type culture technique proved to be very suitable for *Trifolium* protoplasts. Sustained divisions and callus formation were achieved from all of the seven tested alsike clover cultivars. No differences in callus formation between diploid and tetraploid forms could be seen. With red clover, profuse budding was observed and it usually took over 2 weeks before first divisions could be seen. With red clover sustained divisions and callus formation were achieved from only three of the eight cultivars tested: all three were diploid.

Shoot development from protoplast-derived calli has been induced in two diploid alsike clover cultivars. Media that induced shoot formation were MS medium (Murashige and Skoog 1962) with 1 mg L^{-1} BAP and B5 media supplemented with 0.5 mg L^{-1} BAP and 0.2 mg L^{-1} IAA or 0.5 mg L^{-1} kinetin and 0.1 mg L^{-1} NAA. With red clover we have managed to produce roots on all protoplast-derived callus lines but shoot regeneration has so far failed. However, in previous experiments with callus cultures shoot formation was achieved. Red clover has a tendency to form embroids in suspension cultures. With red clover, SL medium proved to be better than B5 medium, for supporting cell growth in suspension cultures.

Regeneration of fusion products

Of the two fusion methods tested, electrofusion seems clearly to be more promising than PEG fusion, although we have not been able to produce callus from electric-field-treated protoplasts as we have done with PEG-treated protoplasts. In our preliminary studies with electrofusion, the fusion frequency has been at least 10 times that of the PEG method. With our chemical-fusion method the frequencies were usually below 1%, optimally about 5%. After electrofusion, protoplasts float freely in liquid and they are thus easier to pick out than protoplasts fused with PEG, which tend to adhere to the bottom of the fusion dish. Heterokaryons could still be easily identified 36 hours after fusion and they could be picked out into microcapillaries. Our rate of picking was about 50–100 hybrids per hour. Heterokaryon frequencies in fusion dishes were considerably higher (about 10%) at the time of picking than directly after fusion, due to excessive break-

down of the mesophyll protoplasts. Heterokaryons produced with either PEG or electrofusion and cultured in Cuprak® and multivial plates were observed usually to undergo divisions only up to the 20-cell state. Sustained divisions could not be achieved, mainly due to excessive phenol output of protoplasts. In preliminary experiments with a new nurse-culture technique, of Millicell HA inserts, these phenol problems have been slightly reduced, and heterokaryons fused with PEG have divided to macroscopically visible colonies and small calli.

Current Progress

Most interspecific protoplast-fusion work has been done within the families Solanaceae and Brassicaceae (Negrutiu et al. 1984, Glimelius et al. 1986). It has become clear through these experiments that organnelle 'cybrids' may a priori be of more immediate use to the plant breeder than fully fledged nuclear hybrids. The reasoning behind this prediction is that the large nuclear genome is the result of a very long genetic-adaptation process within the species and that the break-up of such coadapted complexes through nuclear fusion would result in unbalanced genomes. It would take a long time for the breeder to restore balance in such cases.

Organelle cybrids, however, may affect critical steps in anther development and pollen formation through mitochondrial DNA information, which can be utilized by the breeder in cytoplasmic male-sterility mechanisms. The genetic information in the chloroplast DNA may have direct applications for the breeder. Some of the important genes of the ribulose-1, 5-bisphosphate-carboxylase (RuBPC) enzyme, and of the photosystems I and II, which can be used in manipulating toxin or herbicide resistance, are located in chloroplast DNA. There are also genes affecting photorespiration rates connected with the functioning of the RuBPC enzyme. In some *Brassica* species it has been shown that probably the only site of de novo synthesis of the agriculturally important seed-fatty-acids is probably in the chloroplasts of the leaf tissue and the protoplastids of the embryo.

Following protoplast fusion, about 10–12% of the regenerated plants are nuclear hybrids and only about 1% are cybrids. We need efficient selection methods for the production of cybrids. The selection of fused protoplasts is done at present either directly, under the microscope by visual inspection and micromanipulation, or by using cell-flow sorting of heterokaryons. More elegant methods are to use biochemical auxotrophic mutants, chlorophyl mutants, or antibiotic resistance as marker genes to pick up complementation products directly on the petri dish. These refined biochemical methods may be very effective but they introduce mutations into the breeding material, and this may be harmful from a breeder's point-of-view. The much higher fusion rates that we obtained in the application of the electrofusion method may, to some extent, solve the problem of selecting large quantities of fused protoplasts. Indeed, this method may be refined to fuse protoplasts with organelles, or directly introduce DNA sequences through electroporation.

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Wide Hybridization in Legumes at ICRISAT

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Abstract

A major objective of the Legumes Program at ICRISAT is to overcome constraints to production of groundnut (Arachis hypogaea), *chickpea* (Cicer arietinum) *and pigeonpea* (Cajanus cajan). *Sources of resistance to some pests and diseases have been identified among wild relatives of these crops. Attempts have been made to cross* Cajanus cajan *with* Atylosia *species, and to investigate causes of, and develop techniques to overcome, intergeneric incompatibility. Pods formed following some interspecific hybridizations contained shriveled seeds, and hybrid embryos have been cultured. Seeds have been obtained from some interspecific crosses with chickpea. Hormone treatment at pollination has been used to produce hybrid embryos from crosses between* A. hypogaea *and distantly related tetraploid wild species. Ovule and embryo culture has been used to produce hybrid callus and shoots, but effective roots have not been induced. Hybrid shoots have been grafted onto groundnut seedlings. Compatible diploid wild species have been used as sources of resistance, and a number of high-yielding, disease-resistant lines have been produced following ploidy and genome manipulations.*

Introduction

Work on chickpea and pigeonpea started at Patancheru in 1972, when ICRISAT was founded, and, on groundnut in 1976 after groundnut became the fifth ICRISAT mandate crop.

The Legumes Program consists of breeders, cytogeneticists, pathologists, agronomists, physiologists, and entomologists with responsibility for one, or more, of the three crops. The objective of the program is to overcome constraints to production of the three crops. Breeders in cooperation with other scientists are producing new genotypes adapted to conditions in the semi-arid tropics, and with resistances to major pests and diseases.

There has been considerable success in all three crops. Groundnut varieties ICGS 11 and ICGS 44 have been released for cultivation in India. In pigeonpea, ICPL 87 (Pragati) has been released, and the chickpea breeders have produced varieties for release in both desi and kabuli seed types. The wild relatives of all three crops are sources of a few desirable genes, but their traits are seldom easy to transfer to cultivated taxa by conventional techniques.

The priority has been to transfer genes for resistances not presently available in the cultivated germplasm. Wherever resistance is available, the incorporation of genes from exotic species widens the breeders' options, and it can lead to more stable resistance. This paper reports the work that has been done at ICRISAT to make genes from wild species available to breeders.

Pigeonpea (*Cajanus cajan*)

Cajanus is a monotypic genus, but there are a number of compatible and incompatible species in *Atylosia*, a genus taxonomically very close to *Cajanus*. They are of interest to breeders, because of their potential as sources of resistance to pests and/or diseases, drought tolerance, early maturity, high protein content, and/or annuality (Table 1).

A number of *Cajanus* × *Atylosia* crosses have been

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Table 1. Sources of characteristics sought for pigeonpea improvement.

Character	Source of gene
1. Insect resistance (*Heliothis*)	*Atylosia scarabaeoides*
2. Disease resistance	
Wilt (*Fusarium*)	*A. platycarpa*
	A. volubilis
Blight (*Phytophthora*)	*A. platycarpa*
	A. sericea
Sterility mosaic virus	*A. volubilis*
3. Drought tolerance	*A. albicans*
	A. lineata
	A. scarabaeoides
	A. sericea
	A. volubilis
4. Early maturity, annuality	*A. platycarpa*
5. High protein	*A. albicans*
	A. lineata
	A. scarabaeoides
	A. sericea

attempted, but only *Cajanus cajan* × *A. acutifolia* and *C. cajan* × *A. reticulata* formed mature seeds. Neither of these wild species contain desirable traits, but they do cross with other species. Therefore one approach is to use these species as a bridge to gain access to the desired genes. This will further our understanding of the genomes involved as well as generating a range of interspecific derivatives probably crossable with *Cajanus cajan*.

Nineteen combinations were attempted at ICRISAT (Table 2). The bridge-cross most likely to succeed is *C. cajan* × *A. acutifolia* and *A. cajanifolia* × *A. acutifolia*, as both crosses produce mature seed, though *A. cajanifolia* is not a species with a desirable trait. *A. acutifolia* is a possible means of accessing *A. platycarpa* and *A. scarabaeoides*, but only if shriveled seeds can be grown, or young embryos rescued.

Studies of seed formation, from *A. platycarpa* × *A. scarabaeoides* crosses, showed that endosperm degeneration began about 7 days after pollination, when the embryo is too small to dissect and culture. The largest hybrid embryos grew to a maximum length of 1 mm about 19 days after pollination, compared with 5-6 mm in *C. cajan* at that age. Pigeonpea embryos have been successfully cultured (Kumar et al. 1985), so embryo culture was attempted in two combinations important as bridge-crosses *A. platycarpa* × *A. acutifolia*, and *A. platycarpa* × *A. scarabaeoides*. Embryos were excised 16–19 days after pollination, when they were 0.7–1.0 mm long, and had reached the heart-shaped stage. The medium used was Gamborg's B5 (Gamborg 1984) with 0.8% agar and 2.0% sucrose. When 1 mg L^{-1} 2,4 dichlorophenoxy acetic acid (2,4-D) was added, profuse callus was formed. When embryos were placed on the same basic medium but with 0.25 mg L^{-1} kinetin and 0.1 mg L^{-1} naphthalene acetic acid (NAA), less callus was formed than on the medium containing 2,4-D. Some shoots were formed, though not at a frequency high enough for a practical program on gene transfer.

Nurse culture

An investigation of nurse-culture techniques was undertaken with the objective of increasing the frequency of rescue of hybrid embryos. Embryos were placed on B5 medium, with selfed embryos or endosperm placed immediately adjacent to the hybrid embryo. Embryo development was impaired when maternal tissue was present, as necrosis spread from the ovular wall to the embryo, but when all maternal tissue was removed, hybrid embryos grew, and the frequency of root and shoot formation was increased (Table 3). Over all crosses, 48% of embryos produced callus or shoots and roots with nurse tissue. In another experiment, nurse endosperm gave 45% response, compared with 35% where the embryo was used as a nurse.

Dark treatment

Hybrid embryos from *A. platycarpa* × *A.* spp crosses were cultured on B5 medium and kept in the light, or in the dark, for 1 week after culture. Fifty-seven percent of embryos kept in the dark responded favorably compared with 30% kept in the light.

Chickpea (*Cicer arietinum*)

There are a number of wild *Cicer* species with characters that would be useful in chickpea improvement (Table 4). *C. reticulatum*, which has resistance to ascochyta blight (ICRISAT 1980) has been crossed with *C. arietinum* (Pundir and van der Maesen 1983). Four chickpea genotypes were used as female parents, and three crosses were successful, giving 9%, 8%, and 7% hybrid seeds. Hybrids produced were normal in morphology and fertility.

Table 2. Pod set (%) and number of pollinations made (in parentheses) in crosses between *Cajanus* and *Atylosia* spp.

Female / male	Prab	Baig	*A.acut*	*A.caj*	*A.plat*	*A.plur*	*A.retic*	*A.scar*	*A.ser*	*A.vol*
Prabhat	–	–	6.6[1] (30)	–	–	0 (32)	13[1] (31)	–	–	–
Baigani	–	–	–	–	0[3] (80)	–	–	–	–	–
A. acutifolia	–	–	–	0 (50)	0[3] (100)	–	–	–	–	–
A. cajanifolia	–	–	4.0[1] (100)	–	0 (80)	–	0 (50)	–	–	–
A. platycarpa	–	–	16.0[2] (25)	3.0[2] (100)	–	–	0 (37)	35.3[2] (232)	–	–
A. pluriflora	–	–	–	–	–	–	–	–	–	–
A. reticulata	–	–	–	0[3] (50)	0 (84)	–	–	–	–	–
A. scarabaeoides	–	–	–	1.25[2] (80)	–	–	–	–	–	–
A. sericea	–	–	–	–	–	–	–	–	–	–
A. volubilis	–	–	–	0 (20)	–	–	–	0 (62)	0 (40)	–

1. Pods contained mature seeds. 2. Pods contained shriveled, nonviable seeds. 3. Pods aborted.

Table 3. Frequencies for six types of response noted for immature embryos on artificial medium with and without nurse tissue. Embryos were produced following three interspecific crosses involving *Atylosia platycarpa* (female), *A. acutifolia*, *A. cajanifolia*, and *A. scarabaeoides* (males). (Numbers in parentheses refer to number of embryos for each treatment.)

	Without nurse tissue				With nurse tissue					
	Embryo response[1]				Embryo response					
	No change	Size increase	Callus	Shoot	No change	Size increase	Callus	Shoot	Root	Shoot + root
Male parent:										
A. acutifolia	–	–	0.25	0.75 (4)	0.55	0.10	0.05	–	–	0.30 (20)
A. cajanifolia	–	–	–	–	0.10	0.40	0.20	0.30	–	– (10)
A. scarabaeoides	0.64	0.27	0.09	– (11)	0.32	0.15	0.23	0.15	0.04	0.11 (47)
Means	0.47	0.2	0.13	0.2 (15)	0.35	0.17	0.18	0.13	0.03	0.14 (77)

1. None of these embryos developed roots or into plantlets.

Table 4. Characters in wild *Cicer* species useful for chickpea improvement.

Species	Character
C. judaicum	Resistant to fusarium wilt
	Resistant to gray mold
	Resistant to ascochyta blight
C. reticulatum	Promising against ascochyta blight
	Acceptable seed size (10 g 100^{-1} seeds)
C. pinnatifidum	Resistant to aschochyta blight
C. montbretii	Resistant to aschochyta blight, 3-7 seeds per pod
C. bijugum	Acceptable seed size (9 g 100^{-1} seeds)
C. cuneatum	Good vigor, 3 seeds per pod, resistant to fusarium wilt
C. microphyllum	Cold tolerance

Source: Adapted from van der Maesen and Pundir (1984).

C. arietinum × *C. cuneatum* was attempted with seven genotypes of chickpea, and the reciprocal with two genotypes. The only successful combination was *C. arietinum* 'G-130' × *C. cuneatum*, and that combination produced only one seed from 304 pollinations (Pundir and van der Maesen 1983).

All other crosses of cultivated chickpea with *C. pinnatifidum*, *C. bijugum*, *C. chorassanicum*, and *C. judaicum* were unsuccessful. However, a number of crosses between wild species were successful. These were *C. pinnatifidum* × *C. judaicum*, and the reciprocal, *C. pinnatifidum* × *C. bijugum*, and *C. judaicum* × *C. bijugum*.

The future emphasis in our chickpea research will be to repeat these crosses and attempt others, using mentor pollen, hormone treatment, and embryo rescue techniques.

Groundnut (*Arachis hypogaea*)

The genus *Arachis* has been divided into seven sections based on morphological and cross-compatibility studies, and sections subdivided into series (Gregory and Gregory 1979). There are 22 described species and possibly another 40 distinct species among recent collections (Smartt and Stalker 1982, Stalker 1985).

The cultivated groundnut, *A. hypogaea*, belongs to section *Arachis* and is a tetraploid, 2n=4x=40. It is readily crossable with a closely related tetraploid wild species, *A. monticola*, and produces fertile hybrids. A cultivar Spancross, has been developed from such a cross in the USA (Hammons 1970). *A. monticola* has been proposed as a wild subspecies of *A. hypogaea* (Singh and Moss 1982, 1984a, Smartt and Stalker 1982). Other closely related species in section *Arachis* are diploid, 2n=2x=20. The majority of these species have the 'A' genome, only one species, *A. batizocoi*, has the 'B' genome, and a new collection, *A. spinaclava* has the 'D' genome (Stalker 1985). The A genome species have been further subdivided on chromosome morphology using Mahalonobis D^2 analysis (Singh and Moss 1982). *A. hypogaea* has been crossed with both A and B genome species, and has been concluded to have AABB genomic formula. Hybrids have not been produced with *A. spinaclava*.

There are no confirmed reports of species in sections other than *Arachis* having been crossed with *A. hypogaea* by conventional means. A number of intersectional crosses involving diploid species in section *Arachis* have been successful (Gregory and Gregory 1979), but none has been used as a bridge-cross to transfer genes to *A. hypogaea*.

Many of these taxa are resistant to important pests and diseases that cause economic losses in many groundnut growing areas (Moss et al. 1987) (Table 5). The primary interest at ICRISAT was to transfer resistance to *Phaeoisariopsis personata* (Berk. and Curt.) v. Arx, late leaf spot, from species in section *Arachis*, and resistance to a number of viruses and insect pests from section *Rhizomatosae* (Moss 1985a,b).

Therefore there were two main thrusts. The first was centered on species, in section *Arachis* to transfer disease resistance and to explore the effects of genomes on gene transfer, as Smartt et al. (1978) had proposed that susceptibility in one genome could not be overcome by transferring a resistance gene into the other genome. The second thrust was to understand the barriers to hybridization, and to discover means of overcoming them, with the objective of utilizing taxa in section *Rhizomatosae* in the genetic improvement of cultivated groundnut.

Intersectional gene transfer

When *A. hypogaea* is pollinated by *A.* sp 276233 (*Rhizomatosae*) pollen germinates and penetrates the stigma. There are many abnormalities of the pollen tubes; tubes are irregular in shape, vary in width, often with swollen tips, and with callose plugs. However, some pollen tubes reach the base of the style, and reports of some pegs being formed indicated that they may be effective at fertilization.

Table 5. Immune (I), resistant (R), and tolerant (T) reactions of wild *Arachis* species to pests and pathogens (data from ICRISAT screening and various authors).

Section series/ Species	Pathogen								Pest			
	RUS	LLS	ELS	PSV	GRV	PMV	TSW	PCV	THR	APH	MIT	JAS
Arachis Annuae												
A. batizocoi	I											
A. duranensis	I			R					R			R
A. spegazzinii	I											
Arachis Perennes												
A. helodes								T				
A. villosa	I			R						R		R
A. correntina	I			I		R	R			R	R	R
A. cardenasii	I	I				R	R			.		R
A. chacoense	I	R*	R/I			R	R		R	R		
A. stenosperma	R	R	R									
Arachis spp				R[1]								
Ambinervosae												
Arachis spp												R[1]
Caulorhizae												
A. repens		R	I	R	R				R		R	
Extranervosae												
A. villosulicarpa	I	I	I*								R	
A. macedoi									R			
Arachis spp										R[1]		
Triseminalae												
A. pusilla	I					R	R		R			R
Erectoides Tetrafoliolatae												
A. benthamii				R								
A. paraguariensis	I	R							R			
Arachis spp				R[1]					R[4]			R[1]
Erectoides Procumbensae												
A. rigonii												R
A. appressipila	I	R										
Arachis spp		R[2]	R									
Rhizomatosae Eurhizomatosae												
A. glabrata	R	I/R	R	R*	R	R			R	R	R	
A. hagenbeckii	I	R	I						R[8]			R
Arachis		R/I										R[11]

RUS = Rust, *Puccinia arachidis*
LLS = Late leaf spot, *Phaeoisariopsis personata*
ELS = Early leaf spot, *Cercospora arachidicola*
PSV = Peanut stunt virus
GRV = Groundnut rosette virus
PMV = Peanut mottle virus
TSWV = Tomato spotted wilt virus
PCV = Peanut clump virus
THR = Thrips, *Scirtothrips dorsalis*
APH = Aphids, *Aphis craccivora*
MIT = Mites, *Tetranychus* sp
JAS = Jassids, *Empoasca* sp

* = Conflicting reports may be due to misidentification, or variation in the wild species, the pathogen, or the test conditions.
1,2,3, superscripts = number of species or unnamed accessions.

Mentor pollen or mentor pollen leachate was applied to styles to increase the frequency of fertilization (Sastri and Moss 1982, Moss and Sastri 1986). These treatments were combined with kinetin applied to the base of the hypanthium, and it was found that kinetin alone increased the number of pegs. Subsequently gibberellic acid (GA) was found most effective, up to 82% of pollinations with GA treatment producing pegs (Table 6). However, many of these pegs do not form pods, but application of auxin subsequently increases the rate of pod formation (Nalini and Sastri 1985a). The most effective treatment was indole acetic acid (IAA) at 50 or 100 ppm applied to the developing peg 15-25 days after pollination. This sequence of treatments produces up to 32 pods for every 100 pollinations, but if left on the plant the embryos do not develop fully, and pods contain shriveled seeds.

The timing of the second hormone application, and the nature of the hormone used, has an influence on the size of the ovules as well as on the number of pods produced. Ovules up to 4.8 mm long were excised from pods of *A. hypogaea* cv Robut 33-1 pollinated with *Arachis* sp 276233, after treatment with 87.5 ppm GA at pollination and 100 ppm IAA 20 days after pollination (Table 7).

A range of cultivars of *A. hypogaea* were used as female parents, and different accessions of rhizomatous species used as male parents. There was little difference in the frequency of peg formation after gibberellin treatment, but the frequency of pod formation, without further hormone treatment, varied depending on the genotypes used (Table 6). The percentage pod production per peg ranged from 0 to 42%, *A. hypogaea* MK 374 × *Arachis* sp 276233 being the best combination for pod production (Table 6) from a single hormone treatment with GA at pollination. There were also differences in the sizes of the ovules dissected from the pods. Ovule lengths ranged from 1.6 mm to 4.8 mm (Table 7). Ovules from the three crosses longer than 3 mm could be dissected, and the embryo excised and cultured, but ovules shorter than 3 mm were cultured intact (Nalini and Sastri 1985b).

Thus, although the hormone treatments to produce the highest numbers of pegs did not produce the largest ovules, there was a range of hormone treatments in most hybrid combinations that gave

Table 6. Peg and pod production after GA treatment in *A. hypogaea* × *Arachis* sp crosses.

Female parent	TMV 2			Robut 33-1			MK 374			Chico		
Male parent	a	b	c	a	b	c	a	b	c	a	b	c
A. sp 276233	408	77	27	491	82	18	648	68	42	58	66	9
A. sp 9649	11	73	0	82	44	6	26	42	15	26	73	19

a = Number of pollinations. b = pegs per pollination (%). c = pods per peg (%).

Table 7. Ovule length (mm) from pods obtained in three *A. hypogaea* cultivars crossed with *Arachis* sp 276233 with subsequent hormone treatments.

Hormone treatment[1] (ppm)	DAP[2]	MK 374	TMV 2	M 13	Robut 33-1
Nil		1.6			
GA		2.6	2.3	2.8	
GA; IAA (10)	10		3.8		2.8
GA; IAA (10)	15	3.1		2.8	2.1
GA; IAA (25)	10			2.5	2.4
GA; IAA (25)	15	2.0			2.2
GA; IAA (50)	15	2.4			2.1
GA; IAA (100)	15	2.8			2.1
GA; IAA (100)	20				4.8

1. GA = Gibberellic acid (87.5 ppm aqueous) applied to bases of flowers soon after incompatible pollinations.
   IAA = Indole acetic acid at different concentrations, in lanolin, applied to peg bases on different days after pollination.
2. DAP = days after pollination.

an acceptable frequency of ovules that could be cultured. The present technique is to pollinate all flowers on a plant over a period of about 20 days, treating them with GA at pollination and IAA or naphthalene acetic acid (NAA) about 20 days later, and then to harvest the plant. This technique has been applied to other wide crosses in *Arachis*, and pods have been produced from intersectional combinations where there has been no success previously. Pods have been produced in crosses of *A. hypogaea* with species in sections *Erectoides* and *Extranervosae* as well as *Rhizomatosae*. Ovules were excised and cultured on MS medium. When benzyl amino parine (BAP)-NAA were used, ovules survived longer and formed callus, but kinetin-IAA media stimulated embryo growth better than BAP-NAA. Embryos were dissected from ovules larger than 3 mm, but were often abnormal. Embryos were cultured on MS + 2.0 mg L^{-1} NAA and 0.5 BAP mg L^{-1}. This stimulated callus formation, and subsequently shoots were produced. Shoots were grafted onto *A. hypogaea* seedlings, where vegetative growth was good.

Gene transfer from wild diploids in section *Arachis*

There are a number of options for transferring genes from wild diploids to a cultivated tetraploid, but they can be broadly divided into direct hybridization followed by ploidy manipulations, and ploidy manipulations including hybridization of the wild species before crossing with the cultivated species (Singh 1986a).

Direct hybridization produces a triploid. At ICRISAT, the eight original species, including *A. cardenasii*, a species resistant to late leaf spot, were crossed with *A. hypogaea* (Singh and Moss 1984a). Triploids were treated with colchicine to produce hexaploids, which have been backcrossed to *A. hypogaea*. The chromosome number was reduced in successive backcross generations. The fertility was low in early backcross generations. Ten cytologically stable tetraploid progenies were produced (ICRISAT 1983).

Triploids, although previously reported to be sterile, were observed to form pods under certain conditions at ICRISAT (Singh and Moss 1984b). Progeny from selfed triploids were mostly hexaploids resulting from fusion of unreduced gametes, but plants with other ploidy levels were also produced, including tetraploids that were represented by about 8% of the progenies. They were the result of fusion between balanced gametes resulting from unequal segregation. Analysis of meiosis in triploids indicated that recombination had occurred between chromosomes from the wild and cultivated parents. Stable, tetraploid, disease-resistant plants have been selected from progenies of selfed triploids (ICRISAT 1983).

Ploidy manipulations of wild species, before crossing to *A. hypogaea*, include the production of autotetraploids and of amphidiploids.

Autotetraploids have been produced from A-genome species and the only B-genome species, *A. batizocoi* (ICRISAT 1983, Singh 1986b). These were crossed with *A. hypogaea*. The resultant hybrids were backcrossed to *A. hypogaea*. Fertility was low in the early generations. Nevertheless about seven pods per 100 backcross pollinations were produced and stable, fertile, disease-resistant plants were selected in subsequent backcross generations' progenies.

Amphidiploids have been produced by colchicine treatment after crossing A-genome species with *A. batizocoi*, and also after intercrossing A-genome species (Singh 1986c). The amphidiploids were crossed with *A. hypogaea*, and selected hybrids were backcrossed to the cultivated parent. In the amphidiploids, one or both genomes are homologous with the *A. hypogaea* genomes, as indicated by chromosome pairing that should facilitate gene transfer from the common genome. However, even in hybrids between *A. hypogaea* (AABB) and amplidiploids with the AABB genome combination, many univalents (I in Table 8) were observed.

The frequency of trivalents and quadrivalents in these hybrids indicates that intergenomic A-B pairing does occur. In successive backcrosses to *A. hypogaea*, fertile stable disease-resistant plants have been selected at frequencies of around 1%, indicating that amphidiploids are a practical means of transferring genes from wild species.

Agronomic characters of derivatives of *A. hypogaea* × wild species hybrids

The hybrids and backcross derivatives that are cytologically stable, fertile, and disease-resistant have been selected for a number of agronomic characters, including yield of pods, kernels, and of haulm, which is valuable as animal feed: and a number of advanced lines have been bred (ICRISAT 1985) (Table 9). These have been distributed to breeders in many countries, who now have access to genes from wild species in their breeding programs.

Table 8. Chromosome associations in amplidiploids and their F_1 hybrids from crosses with *A. hypogaea*.

Amphidiploid or hybrid	Genomic formula	Means of different associations				
		I	II	III	IV	VI or VII
A. villosa × *Arachis* sp HLK-410	AAAA	2.36	13.6	0.68	2.0	
A. hypogaea × (*A. villosa* × *Arachis* sp HLK-410)	AAAB	9.72	12.5	1.36	0.27	
Arachis sp HLK-410 + A sp 10038	AAAA	3.45	14.0	0.41	1.80	
A. hypogaea × (*Arachis* sp HLK-410 × *Arachis* sp 10038)	AAAB	10.04	12.0	0.8	0.88	
A. batizocoi × *A. correntina*	AABB	4.50	16.6	0.5	0.20	
A. hypogaea × (*A. batizocoi* × *A. correntina*)	AABB	7.90	13.3	1.45	0.27	
A. villosa × *A. batizocoi*	AABB	1.52	18.2	0.40	0.16	0.04
A. hypogaea × (*A. villosa* × *A. batizocoi*)	AABB	4.52	14.9	0.68	0.88	

Table 9. Agronomic and botanical features of cytogenetic entries currently in AICORPO Trials.

Identity	Pedigree	Duration (days)	Seed characteristics[1]					
			Disease score		100 seed mass (g)	Shelling (%)	Yield (kg ha^{-1})	
			R	LLS			Pod	Haulm
1. CS-2	(*A. batizocoi* × *A.* sp 10038) × *A. ogaea*	120	2	8	33	76	3230	5590
2. CS-39	*A. hypogaea* × *A. cardenasii*	120	6	3	27	64	3200	6760
3. CS-52	*A. hypogaea* × *A. cardenasii*	120	3	5	40	60	3260	6320
Control								
Robut 33-1	Selection from Kadiri 3	100	9	9	36	70	1700	-
SE				±0.4	±0.4	±0.8		±186
CV(%)				14	13			12
4. CS-48	*A. hypogaea* × *A. cardenasii*	120	3	3	39	71	3250	6389
5. 943	*A. hypogaea* × *A. cardenasii*	120	6	2	30	53	3611	5611
Control Robut 33-1								
SE				±0.33	±0.25	±1.9	±283	±674
CV (%)				19	14	10	18	18

1. Rust = rust; LLS = late leaf spot } in scale of 1 (low infection) to 9 (high infection).

Prospects

Many interspecific and intergeneric hybrids have been produced at ICRISAT, using hormone treatments and embryo rescue as necessary. Some of the hybrids have been fertile, and genes from the wild species have been transferred to derivatives, which are fertile and fully crossable with the cultivated species. Significant progress has been made in basic techniques of tissue culture, and in developing techniques to transfer genes from more distantly related taxa. The application of these techniques to selected hybrid combinations holds considerable promise for the production of new cultivars incorporating genes from wild species.

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Selecting Cultivars for Resistance to High and Low Temperatures

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Abstract

Genetic improvement of major crop species entails the modification of yield components, including those conferring resistance to environmental stresses. Progressively, advances in plant physiology and the new tools such as recombinant DNA and tissue and cell cultures are beginning to make an impact on plant breeding. The efficiency of selection for temperature stress resistance, based on a physiological approach, and methods that could be developed using the new biotechnologies are evaluated on the basis of the genetical theory by which the structure and the procedures of a breeding program are generally defined. Methods of selection based on cell cultures and gametophytic selection are discussed for their potentialities and limitations.

Introduction

The well documented genetic gains obtained in plant breeding are generally evaluated in terms of yield (Russell 1974, Duvick 1981, Borlaug 1983). Although relatively few studies (Sneep and Hendriksen 1979) attempted to partition the gains in yield for underlying physiological attributes, it is reasonable to assume that yield increase is the result of the modification of several morphophysiological components, including resistances to environmental stress. On the assumption that resistance to environmental stresses is at least in part not due to those genes that control yield potential, artificial selection theory indicates that breeding procedures based on yield often lead to the application of a very low intensity of selection to each of the several interacting components. It then follows that selection programs that are specifically designed for environmental stress resistances, ought to produce a higher rate of genetical progress for this character, than do those designed for advancing the yield along with tolerance to stresses.

However, in practice, this difference is difficult to demonstrate, due to the physiological and molecular complexity of stress tolerance and the difficulties in its evaluation. The genetic theory of artificial selection remains valid irrespective of the methodologies of breeding being used. According to this theory it has been assessed that the genetic gain per year (Δg) under selection is a function of:

1. the genetic variability (σ^2_g),
2. the heritability of the character ($h^2 = \sigma^2_g/\sigma^2_p$),
3. the intensity of selection (i),
4. a coefficient (c) specifying reproductive parental control,
5. the amount of phenotypical variation (σ^2_p), and
6. the number of years (y) per selection cycle: $\Delta g = (h^2 i c \sigma^2_p)/y$.

Genetical Basis of Resistance to Temperature Stresses

Resistance to low and high temperatures can result from a large spectrum of mechanisms acting at different levels in the organization of the plant and at different stages of its life cycle. According to the type of protection conferred to the individual plant, the mechanisms are classified into two categories (Levitt 1980): stress avoidance, and stress tolerance.

The relative importance of such mechanisms as confer either avoidance and/or tolerance, and their

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genetic control are the basic information needed for a critical evaluation of different breeding procedures and for the choice and assessment of selection criteria. Leaf morphology and number, leaf pubescence, epicuticular wax, and transpiration intensity are some of the mechanisms conferring avoidance of heat stress. Inhibition of extracellular ice nucleation, protection of inflorescence and vegetative apexes by leaf architecture and winding are examples of mechanisms for avoidance of damage due to frost (Levitt 1980, Blum 1985, Jones and Qualset 1984, Monti and Leone 1986).

The physiological and molecular control of temperature tolerance has been studied in great detail. However, the cause-effect relationships remain unclear. There exists a large body of circumstantial evidence implicating several molecular and metabolic components. Much of this information is derived from inter- and intraspecific differences in plants adapted to different climatic conditions (McDaniel 1982, Mascarenhas 1984, Petolino and Collins 1984). The components of resistance include differences in membrane structure, enzymes, structural proteins, ribosome thermostability, hormonal response and regulation, and gene-expression thermostability. The heat-shock proteins (HSP), proteins induced by temperature stress, are indicated to play an important role (Mascarenhas 1984, Kimpel and Key 1985) in conferring protection at the cellular level. It is reasonable to assume that a single component of resistance accounts for only a small portion of the total resistance, and that resistance results from a number of structural and regulatory genes. As a consequence, resistance is expected to conform to a quantitative pattern of inheritance due to the segregation of several genetical units.

The complexity of the genetical basis of temperature resistances is indicated by the variation in cold tolerance of sorghum (Clegg et al. 1983) and soybean (Unander et al. 1986) and in cowpea for heat stress tolerance (Warray and Hall 1983). In oats winter survival and freezing resistance of the progeny of crosses between winter and spring parents has shown a large spectrum of variation, and the heritability of individual traits are variable (Muehlbaur et al. 1970). Results that can be interpreted on the basis of a polygenic model have also been obtained in barley and alfalfa (Daday and Greenham 1960). The same conclusions can be reached by considering transgressive segregations found in cereal crops (Marshall 1982) and combining ability analysis (Auld et al. 1983). The model applies also to heat resistance in corn and in oats, and to membrane thermostability in soybean. A detailed study of heat sensitivity has been carried out in *Drosophila melanogaster* (Stephanou and Alohiotis 1983) where genetical analysis and response to selection revealed a very complex situation. The character is quantitative, transmitted through maternal cytoplasm, with nuclear genes modifying its phenotypical expression.

Conventional Methods of Selection

Conventional methods of selection are largely based on field testing, where selection for stress resistance is practiced under natural environments. Although this approach achieved some practical results, the progress for a specific stress resistance is far from satisfactory. Even in a very well designed experiment, the response to selection is vitiated by the experimental error and by the genotype-environment interaction, arising from the variability, the heterogeneity within each location, and the effects of other environmental factors that are difficult to control in a field experiment. The efficiency of selection can be improved by an appropriate choice of locations, by increasing the homogeneity of the environment in the field trial, and by using suitable selection procedures.

Marshall (1982) in a review of the results obtained under natural selection for increase of winter hardiness in a number of crop species (barley, oat, alfalfa) concluded that this approach can produce adequate selection response, provided that the population has a wide genetic variability and a uniform and consistent breeding test can be applied. Consequently, natural selection in a homogeneous selective environment, combined with random mating can be recommended for population improvement at the early stages of a breeding program.

Population improvement for cold tolerance has been achieved using highly selective environments. Positive results for cold tolerance at seedling emergence and vigor have been obtained in corn (McConnell and Gardner 1979, Hoard and Crosbie 1986) and in sorghum (Bacon et al. 1986) by means of recurrent-selection procedures.

Improvement of stress resistance can produce undesirable genetic changes in other important agronomical characters due to pleiotropy, gene linkage or genetic drift (Hoard and Crosbie 1986). To overcome this problem the breeding program should be based on random mating of the selected parents and on a large, selected-individual sample to break linkage groups and avoid genetic drift. Selection

indices for resistance can be obtained by considering a family testing in both high and low stress environments. The difference between the yields of each family in the two environments could be used as an index of stress resistance (Lorenzoni 1986), although such values are expected to have large experimental errors. When an efficient laboratory test is possible, such as the screening for cold tolerance in corn, fieldwork can be reduced and large populations can be tested.

Statistical analysis of yield and yield components measured in different environmental conditions have made it possible to distinguish between yield potential and yield stability. The most widely used approach is based on regression analysis, in which stability is represented by the linear regression of yield values across environmental index values (Ottaviano and Sari Gorla 1972, Lin et al. 1986). The main limitation of this procedure is that it can be used only for parental evaluation and in the final stages of a breeding program.

Selection Based on Physiological Tests

The use of yield as the main selection index, and the use of natural environments for selection, may not assure the desired intensity of selection for temperature resistance. Two main arguments support this view. First, yield under stress conditions shows poor heritability and poor repeatability (Blum 1985) and second, stress resistance is under the control of genes that are not necessarily involved in the determination of yield potential, and consequently the intensity of selection tends to be low. For these reasons a breeding program should be based on a large population of individuals, or families, and on suitable experimental designs that reduce genotype-environmental interaction and error.

A different approach consists in the evaluation of physiological characters implied by the resistance mechanisms. However, due to the fragmentary knowledge of the physiological and molecular basis of temperature stress resistance, this approach relies on the evaluation of physiological indices that show the greatest changes under stress conditions and a significant correlation with stress effects on yield. On the basis of information from several authors (Monti and Leone 1986, Petolino and Collins 1984, Marshall 1982, Gusta et al. 1983, Gerik and Eastin 1985, Camussi et al. 1987) a number of physiological indices have been proposed (Table 1).

The predictive value is central to the selection

Table 1. Physiological indices for high and low temperature resistance evaluation.

Physiological indices	High temperature	Low temperature
Germination and emergence	x	x
Pollen viability and development	x	x
Dark respiration response	x	x
Photosynthesis stability (CER, chlorophyl fluorescence)	x	x
Chlorophyl development		x
Stability of cellular membrane (electrolite leakage)	x	x
Crown and seedling freezing resistance		x
Tissue viability (staining test)	x	x
Lipid changes		x
Stress accumulated solutes (proline, ur acid)	x	x
Cell extract pH		x
Osmotic potential		x
Viscosity of cell extract		x
Buffering capacity of cell extract	x	
Epicuticolar wax	x	
Leaf angle and morphology	x	
Soluble protein and enzyme degradation	x	
Respiration depletion of substrates	x	
Canopy temperature	x	

based on physiological traits. Efficient and unbiased predictive values that are generally estimated as correlations between the index and yield or yield components are obtained when yield (or yield components) values of different genotypes are estimated in experiments where error and genotype-environmental interaction are minimized, and correlations between stress resistance and physiological characters are not the results of association due to linkage or previous selection and random drift effects. The physiological character should represent the phenotypical expression of basic mechanisms governing stress response. Random inbred lines have been used in maize (Camussi et al. 1987) to relate CO_2 exchange rate (CER) to grain yield. This approach permitted measurement of the significance of CER for maize cultivated under low temperature stresses during grain filling and maturation periods. The results obtained showed that this physiological character is a significant component of grain production.

Because of the genetical and physiological complexity of resistance to temperature stresses, the use of a single index is not expected to have a satisfactory predictive value. For this reason a good predictor can be provided by a linear combination of indices having complementary information and therefore showing a minimum correlation between them. Suitable statistical procedures have been developed for this purpose (Fowler et al. 1981). Very little information is available in terms of experimental results of selection programs based on physiological traits. For resistance to drought, an important genetic gain has been obtained on the basis of an index that includes canopy temperature under stress (King 1983). Progeny selected for cooler leaves revealed a higher yield under stress, probably due to greater root growth and soil-moisture extraction.

Selection in Cell and Tissue Cultures

Some of the limitations typical of conventional breeding and selection could be overcome by selection at cellular level using in vitro culture techniques. Large populations of cells, protoplasts or calli from different tissue explants can be handled, so that culture and stress treatments can be applied uniformly. The system thus allows selection of mutants that are normally difficult to detect at the plant level. On the other hand, since some major physiological processes (e.g., photosynthesis, translocation) are absent, selection would operate only on tolerance mechanisms expressed at a cellular level.

Experimental results have been reviewed by several authors (King 1983, Faraughi-Wehr et al. 1986). Most studies on temperature stress concern chilling and freezing. In *Nicotiana sylvestris* and *Capsicum annuum* (Dix and Street 1976), *Daucus carota* (Templeton-Sommer et al. 1981), sugarcane (Chen et al. 1982), *Euphorbia pulcherrima* (Waltern and Preil 1981) and *Chrysanthemum* (Preil and Engelhardt 1982), it has been shown that cold-resistent lines can be easily selected. However, while the character is stable through vegetative transmission, it is generally lost in the sexual progeny of regenerated plants.

To evaluate the potentials of in vitro culture selection, the nature of the genetic variability of cell and callus populations should be taken into account. Tissue culture induces genetical changes (Larkin and Sawcroft 1981) in regenerated plants. Somatic variability of the original explant and/or the mutations produced by the passage of the tissue through in vitro cultures are assumed to be the cause of this variation (King 1983). Cell selection has produced positive results for traits with a simple genetic base, such as pathotoxines (Hammershlag 1984) and herbicide resistances (Beversdorf et al. 1980), while for quantitative characters the variation seems to be associated mainly with deleterious genetic changes (Schnell and Wernsnam 1986). While it is very unlikely that the situation would be improved by artificial mutagenesis, a promising approach to the introduction of useful genetic variability could be offered by asymmetrical protoplast fusion (Cocking 1981), using as a donor parent a cultivar, or a related species, having a high level of temperature tolerance.

Androgenic double-haploid lines (DH) from anther cultures is a system that combines the positive aspects of in vitro selection and sexual reproduction. Populations of DH lines express both the somaclonal variation and that due to recombination and segregation. However, if selection was applied to the regenerated plants, the advantages offered by in vitro cultures would be lost. Consequently, this method is most efficient when the isolated microspores are cultured and selected during the early stages of the regeneration process (Faraughi-Wehr et al. 1986). The possibility of application of this method in plant breeding depends on the recalcitrance to regeneration of many crop plants, the expression and the phenotypical stability of the selected character in the regenerated plants, and the development of suitable selection systems and criteria.

Male Gemetophytic Selection

Selection acting on the male gametophytic generation (GS) plays an important role in the evolution of crop plants (Ottaviano and Mulcahy 1987), and, more generally, in the evolution of higher plants (Mulcahy 1979). Moreover, this phenomenon can be used as a tool to improve the efficiency of conventional breeding procedures (Ottaviano et al. 1980, Ottaviano 1983; Ottaviano and Sari-Gorla 1979, Mulcahy 1983, Zamir 1983, Ottaviano and Sari-Gorla 1983).

As a breeding method, GS shares some basic features with cell culture selection. The male gametophyte is a simple structure formed by three haploid cells and a large population can be easily handled. Its application is not conditioned by plant regeneration. Pollen viability and function are important traits of plant fitness (Clegg et al. 1978, Harding and Tucker

1969) and pollen resistance to temperature stresses is a major component of tolerance in crop plants (Haterlein et al. 1980, Hong-Qui and Croes 1982, Mackill et al. 1982, Schoper et al. 1987).

The interpretation of sporophytic and gametophytic effects of GS relies on the assumption that a large portion of the genome shows postmeiotic (gametophytic) gene expression and that a large portion of these genes are also expressed in the sporophytic phase (haplo-diploid gene expression). If these conditions are met, the efficiency of GS is very high for three reasons. First, the large size of a gametophytic population permits an application of a very high intensity of selection. Secondly, a particular complex allele combination has a higher probability in the gametophytic than in the sporophytic generation. Finally, the haploid state leaves the recessive alleles uncovered and consequently the rate of evolution is much higher under GS than under sporophytic selection (Mulcahy 1983).

The above biological assumptions are supported by a number of investigations. In tomato (Tanksley et al. 1981) and maize (Sari-Gorla et al. 1986) it is estimated that 58% and 73% of the genes coding for enzymatic proteins are expressed in both pollen and sporophyte. Comparable figures have been found in *Pinus radiata*. Similar results have been obtained in maize by analyzing monogenic mutants affecting endosperm development (Ottaviano et al. 1986). About 48% of these mutants are also detected in the gametophytic phase. Molecular analysis of transcription products has shown that in *Tradescantia paludosa* (Willing and Mascarenhas 1984) and in maize (Mascarenhas et al. 1986) up to 20 000 different mRNA sequences are present in mature pollen grains and that about 54% in *Tradescantia* and 60% in maize of these sequences are found also in sporophytic tissues. Moreover, active protein synthesis and a large number of mRNA's are detected in germinating pollen of various species (Mascarenhas and Bell 1969, Mascarenhas and Mermelstein 1981, Mascarenhas et al. 1984).

Although GS has been proposed only recently as a breeding technique, positive results have been obtained for a number of important characters such as plant vigor (Mulcahy and Mulcahy 1975), endosperm development (Ottaviano et al. 1982, Ottaviano and Sari-Gorla 1986), pathotoxin tolerance (Kedar et al. 1967), and tolerance to heavy metals and salinity (Searcy and Mulcahy 1985, Sacher et al. 1983). For pollen resistance to temperature stresses several studies, based on the evaluation of pollen viability, germination, and tube growth have revealed an enormous intraspecific variability (Mulcahy 1979, Haterlein et al. 1980, Mackill et al. 1982, Quin et al. 1986, Binelli et al. 1986). However, only a few experiments have established that temperature-stress tolerance is, at least in part, determined by genes expressed in the gametophytic phase. In tomato, the GS response was analyzed in an interspecific cross between *Lycopersion esculentum* and *L. hirsutum*. The latter is a species from the Peruvian Andes adapted to low temperatures (Zamir and Vallejos 1983, Zamir et al. 1981, 1982). When pollen of the two species is mixed and utilized to fertilize tomato at low temperature, the gametophyte from *L. hirsutum* shows higher fertilizing ability than that of tomato. When pollen from the F_1 interspecific cross is used, the progeny contained a higher portion of the *L. hirsutum* genome, indicating that temperature tolerance is controlled by genes expressed in the gametophytic phase. However, den Nijs et al. (1986) reported different results in a similar experiment with an intervarietal hybrid of tomato. The discrepency is perhaps due to low intensity of selection and inability to discriminate between pre- and postpollination events.

There is considerable evidence to show that gene expression changes, during pollen-tube development and function (Stinson and Mascarenhas 1985, Frova et al. 1986). Most significant as to mechanisms conferring temperature stress tolerance, are the results obtained with regard to heat-shock proteins. In *Petunia hybrida* and *Lilium longiflorum* (Schrauwen et al. 1986), and in *Tradescantia* (Mascarenhas and Altschuler 1983), typical heat-shock proteins were not detected in germinating pollen, although a significant level of thermotolerance was induced by a previous heat shock. However, the analysis of developing microspores in maize has shown that, in this stage, the gametophyte responds to heat shock by synthetizing a set of proteins, some of which are specific for this tissue (Frova et al. 1986).

Conclusions

Resistance to temperature stresses is a complex character, controlled by a large number of genes, and is quantitatively inherited. The information concerning the molecular and physiological basis of the character is far from complete, and many mechanisms are perhaps involved. Because direct yield evaluation is largely affected by genotype-environmental interaction and is subjected to large experimental errors, a good predictor based on a physio-

logical index should serve to increase selection responses for stress tolerance. Androgenic double haploid lines from anther culture is the most promising in vitro culture technique. However, because of the predominance of negative genetical variability produced by in vitro culture, genotypes so produced should be considered as raw material for breeding. Gametophytic selection makes it possible to increase the intensity of selection and detect rare positive allelic combinations. Since only some mechanisms controlling stress tolerance would be affected by these two methodologies, both should be considered within the framework of a comprehensive breeding program to improve the efficiency of conventional approaches.

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Products and Uses

Bioenergetic Considerations in the Genetic Improvement of Crop Plants

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Abstract

The bioenergetic constraints to crop productivity originate from intrinsic thermodynamic considerations on the production and utilization of basic assimilates. The bioenergetic 'costs' for enhancing crop productivity, increasing seed protein, altering amino-acid and fatty-acid compositions of seeds, and conferring resistance to insects and pathogens have been examined. The biosynthetic cost of the end product is estimated in terms of glucose that is required to provide the carbon skeletons and also the energy for synthetic pathways. It is revealed that many of the desired goals in genetic improvement of crop plants entail an additional bioenergetic cost to the plants. It is therefore argued that genetic alterations of quality and resistance to stresses will invariably lead to a reduction in yield unless the availability of assimilates is simultaneously enhanced.

Introduction

Cropping systems aim to maximize the conversion of solar energy, a free resource, into food, feed or fiber within the constraints of temperature, water, plant nutrients, and biotic and abiotic stresses. The energy acquisition depends upon the photosynthetic rate, and the amount of solar radiation intercepted and utilized in photosynthesis during the cropping period. Human selection has not improved the photosynthetic rate; in cultivated wheat and barley it is lower than their wild relatives' (Dunstone et al. 1973, Austin et al. 1986). Human selection and breeding over the years have elevated the yield potential by an improved partitioning in favor of seed, or fruit, mass at the cost of vegetative growth. Improved farming practices such as irrigation, fertilizers, pesticides, and management amplify the fixation of solar energy and enlarge the overall bioenergy resource.

Carbon, hydrogen, oxygen, nitrogen, and sulfur are all incorporated in organic molecules via light-dependent reactions (Radmer and Kok 1977). These are utilized for construction, maintenance, and turnover of different macromolecules and of plant organs. The new organs help in further acquisition of carbon, nitrogen, water, and nutrients. In seeds and other storage organs these resources are invested for the sustenance of the future seedling.

The underlying assumption for the bioenergetic constraints is that crop productivity is limited by fixation of solar energy as chemical-bond energy, the bioenergy resource. These constraints are due to intrinsic thermodynamic limitations on the utilization of the resource. Their better appreciation can therefore help in the identification of breeding objectives within given sets of constraints. It may perhaps be possible in future to design crop plants based on precise estimates of carbon fixation, with defined inputs, constraints, and utilization of the energy as is already done for fermentors, chemical plants, and nuclear reactors.

Conversion of Substrate to Biomass

Conversion efficiency of substrate to biomass has been of interest to microbiologists and animal scientists for a long time. However, such estimates are relatively recent for higher plants (Rudolf 1971, Mooney 1972, Penning de Vries et al. 1974, Mc-

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Dermitt and Loomis 1981). The quantitative relations between the substrate (glucose) and end-product were estimated by Penning de Vries et al. (1974). These values were subsequently used by Sinclair and de Wit (1975) to estimate the seed biomass productivity (g of seed biomass per g photosynthate). Bhatia and Rabson (1976) and Mitra et al. (1979) examined the bioenergetic costs of increasing the grain protein and altering the amino-acid composition in cereal grains. Subsequently, these estimates were extended to other breeding goals and crops (Rabson et al. 1978, Mitra and Bhatia 1979, Bhatia et al. 1981, Mitra and Bhatia 1982, Bhatia 1983, Bhatia and Mitra 1983). The bioenergetic implications for genetic improvement of grain yield, resistance to biotic and abiotic stresses, enhancing symbiotic nitrogen fixation in grain legumes, and end-use qualities of grain and negative associations are considered here. Implications of horizontal gene transfers using recombinant DNA techniques are briefly examined. A glossary of bioenergetic terms used in this paper is presented in Table 1, and the abbreviations therein are used throughout in the text and tables.

The Bioenergetic Constraints for Grain Yield

The two major factors that contributed to an increase in cereal grain productivity are nitrogenous fertilizers, and the new 'high-yielding' cultivars. Other agronomic practices, such as higher density planting and improved water management and plant protection, have also synergistically contributed to higher grain yields.

The energy content of grain and crop residue is dependent upon their chemical composition. The chemical composition and consequently the energy content of grain is subject to considerable variation while that of crop residues is reasonably constant for a given species. On a dry-mass basis, the mean energy content in cereal grains is about 10% higher than that of the corresponding crop residue. This difference is greater for grain-legume and oilseed crops than for cereals (Table 2). Hence an improvement in the harvest index (HI) (defined in Table 3) of crops, in itself, elevates the demand on energy as well as for nitrogen and phosphorus (Bhatia et al. 1981). As stated by Donald and Hamblin (1976) there exist

Table 1. The terms used in bioenergetic computations.

Name	Symbol	Definition	Unit
Heat of combustion	ΔH^0_c	The enthalpy change for the reaction of combusting the chemical compound into constituent gaseous states, for example, amino acid at constant pressure at 298°K to yield CO_2, H_2O, N_2 (gas) and sulfur as H_2SO_4 (when applicable)	KJ mol^{-1}
Production value	PV	$\frac{\text{Weight of the end product}}{\text{Weight of substrate required for C-skeletons and energy production}}$ (based on biochemical pathways)	g g^{-1}
Inverse of production value	$\frac{1}{PV}$	Gram glucose required to make 1 g end product	g g^{-1}
Glucose equivalent	GE	Number of moles of glucose required to supply C-skeletons and electrons to build 1 mole product (based on proximate analysis or elemental composition)	mol mol^{-1}
Glucose value	GV	$GV = \frac{(GE \times \text{mol. wt of glucose})}{\text{mol. wt of compound}}$	g g^{-1}
Conversion of GV into PV	-	$PV = GV \times 0.88$	g g^{-1}

Oxygen requirement factor (ORF), carbon dioxide production factor (CPF), hydrogen requirement factor (HRF) and energy requirement factor (ERF), respectively, are the other measures used for oxygen consumed, CO_2 produced, $NADH_2$, and ATP requirement.

Based on Penning de Vries et al. (1974) and McDermitt and Loomis (1981).

the following four options to increase the grain yield (GY) of cereals.

A. Increasing GY and HI leaving biological yield unaffected.
B. Enhancing both biological yield and GY with an unchanged HI.
C. Simultaneous increases in biological yield, GY, and HI.
D. Increasing the biological yield and GY, but reducing the HI.

Table 2. Energy content of grain and crop residue.

Crop	Energy content ($MJ\ kg^{-1}$) Residue (1)	Grain (2)	% difference [(2)/(1) × 100]−100
Cereals			
Wheat	15.9	17.1	7.5
Barley	15.2	16.9	11.1
Grain legumes			
Chickpea	15.1	18.0	19.2
Pigeonpea	15.6	18.6	19.2
Oil seeds			
Mustard	13.9	25.7	84.9

These values are based on Sinha et al. (1982). Other estimates based on chemical compostion show similar trends though the values may differ.

Increasing the HI (A and C) demands the least increment in photosynthates and nutrient inputs over the baseline, whereas other alternatives necessitate a steep rise in demand for photosynthates and nutrients: figures are given in Table 3. The fertilizer requirements are also higher for the other options. This clearly shows that there exists no way to increase the productivity of cereals without additional inputs of fertilizer.

The large difference in the energy content of the residue and seed in oilseed crops further increases the requirement for additional photosynthates associated with improved HI (Sinha et al. 1982). This is perhaps one of the main reasons why substantial progress in increasing the HI could not yet be made in oilseed crops.

Biotic and Abiotic Stresses

The priority objective, next to yield, in most crop breeding programs is resistance to diseases and pests. Broadly, the mechanisms of resistance may be either constitutive, or pathogen-induced. Besides the various morphological and anatomical features that are reported to confer race-nonspecific resistance, different secondary plant products such as phenolics, glycosides, and alkaloids have been implicated in plant-pest relationships. Such chemicals are synthesized and maintained in adequate amounts in plants

Table 3. Alternatives for increasing grain yield.

	Biological yield ($kg\ ha^{-1}$) (1)	Grain yield ($kg\ ha^{-1}$) (2)	Harvest index (%)	N-requirement ($kg\ ha^{-1}$) (3)	Energy harvest ($MJ\ ha^{-1}$) (4)	% increase in energy harvest over baseline (5)
Baseline	8 000	2400	30	93.4	135 128	
Alternative						
A	8 000	4000	50	120.0	137 960	2.1
B	13 333	4000	30	155.7	225 208	66.6
C	10 000	4000	40	133.4	170 680	26.3
D	16 000	4000	25	173.6	268 840	98.9

A = Biological yield remains constant, harvest index and grain yield increase.
B = Harvest index remains constant, biological yield and grain yield increase.
C = Biological yield, grain yield and harvest index increase.
D = Biological yield and grain yield increase, harvest index decreases.
(4) = (2) × 0.0233 + [(1)−(2)] × 0.0067
(5) = (2) × 18.13 $MJ\ kg^{-1}$ + [(1)−(2)] × 16.36 $MJ\ kg^{-1}$

$$\text{The Harvest Index} = \text{HI}\ \% = \frac{\text{Grain yield}}{\text{Biological yield}} \times 100$$

These estimates are based on energy values for wheat grain and straw. The values for similar changes in other crops can be estimated (Bhatia et al. 1981).

so as to confer some protection from insects, in particular, and in some cases also against pathogens.

Induced or active resistance is due to activation of defence mechanisms in the host in response to infection. The host response involves synthesis and accumulation of pathogenesis-related proteins (Gianinazzi 1984, van Loon 1985) and also the low-molecular-mass chemicals, such as phytoalexins. It is thus obvious that the host plant expends some energy resources to repel or restrict the pathogen (Smedegaard-Petersen and Tolstrup 1985). This is further supported by a number of reports on increased respiration following incompatible host-pathogen interactions.

Smedegaard-Petersen and Stolen (1991) subjected barley plants to both a virulent and an avirulent race of mildew, *Erysiphe graminis,* and compared the respiration of infected plants with those of the non-inoculated checks. They observed that resistant plants that did not express any symptoms of disease showed a loss—in grain yield of 7%, in kernel mass of 4%, grain protein yield of 11%, and straw yield of 3%. Smedergaard-Petersen and Tolstrup (1985) concluded that the resistant plants expend a part of the host energy in defence mechanisms and that the 'energy cost' of the latter is responsible for the loss in yield. Similar reductions in yield and quality of tobacco lines, resistant to different diseases compared with corresponding disease-susceptible lines, was reported by Chaplin (1970). Plants resistant to tobacco mosaic virus (TMV) showed a yield reduction of 5.6% while those resistant to fusarium wilt (FW) showed a yield reduction of 6.5%. Plants resistant to both TMV and FW suffered a yield loss of 9%.

Mitra and Bhatia (1982) estimated production values (PV) (see Table 1) for some major chemicals implicated as phytoalexins in combating fungal and bacterial infections and for allelochemicals that are responsible either for nonpreference or antibiosis in interactions between the host plant and insects (Table 4). Compounds such as rishitin, ipomeamarone, pinosylvine, phaseollin, and fungitoxins such as medicarpin, make demands on the energy pool of host plants. The low concentration of these chemicals in tissues of resistant plants should not lead to the underestimation of the total quantities produced by the plant on a field scale and the consequent demand for bioenergy. Common abiotic stresses that affect productivity of crop plants are drought, high and low temperatures, salinity, flooding, and toxicity of heavy metals. Plant breeders face the challenge of incorporating genetic resistance to these stresses into crop plants (Boyer 1982). Host plants respond to abiotic stresses, by the production of a wide range of biochemicals and by altering physiological reactions (Key et al. 1981, Bewley et al. 1983, Downton 1984, Czarnecka et al. 1984, Heikkila et al. 1984, Morgan 1984, Blum 1985, and Guy et al. 1985). Osmotic stress results in an accumulation of small organic molecules such as glycine betaine, pro-

Table 4. Values characterizing the conversion process of glucose into individual chemical compounds implicated as phytoalexins in combating fungal or bacterial infection or allelochemicals known to be responsible for nonpreference or antibiosis in insect plant interactions.

Group/name	PV[1]	1/PV (gram glucose) required to synthesize 1 g end-product)
Phenolics		
Orchinol	0.40	2.50
Coumaric acid	0.46	2.17
Cinnamic acid	0.41	2.44
Duroquinone	0.36	2.78
Coumarin	0.41	2.44
Pinosylvine	0.39	2.56
Alkaloids and nitrogen compounds		
Hordatine A	0.42	2.38
Berberine	0.47	2.13
Isoflavonoids		
Luteone	0.49	2.04
Pisatin	0.58	1.72
Phaseollin	0.43	2.33
Terpenoids		
Rishitin	0.35	2.86
Ipomeamarone	0.35	2.86
Fungitoxins		
Medicarpin	0.38	2.63
Resveratinol	0.42	2.38
Allelochemicals		
DOPA	0.60	1.67
Resorcinol	0.31	3.23
Nicotine	0.34	2.94
Canavanine	0.51	1.96
Juglone	0.48	2.08
Gossypol	0.48	2.08
Solanidine	0.34	2.94

Source: Mitra and Bhatia (1982). 1. For PV, see Table 1.

line betaine, proline, and others in plant and bacterial cells (Le Rudulier et al. 1984). The production of these osmoprotective molecules entails the withdrawal of carbon and nitrogen from the metabolic pool.

Since photosynthesis and transpiration are closely linked to the onset of drought stress, stomatal closure has its impact on reduction of photosynthesis (Hanks and Rasmussen 1982). Growth as well as maintenance respiration increase under heat and water stresses invoking additional demand for photosynthates (Penning de Vries et al. 1979). Thus, under stress conditions, the acquisition of energy is curtailed and part of the energy resources are diverted to fight the stress at molecular, cellular, physiological, and organ levels. The present knowledge of the relationships between the molecular events and stress resistance, does not permit even a gross estimate to be made of the energy lost to the plants as a result of recurrent alternating spells of stress and favorable environment.

Symbiotic Nitrogen Fixation in Grain Legumes

Next to water, the most limiting factor in crop productivity is the availability of nitrogen in utilizable form. It is widely recognized that symbiotic nitrogen fixation entails a substantial cost to the host plant by way of supplying energy to nitrogen-fixing microbes. However, the estimates of relative bioenergetic cost of dinitrogen fixation, and nitrate assimilation and reduction, differ widely. In respect of energy budgets, some of these considerations favor nitrate reduction, and others favor diazotrophy. They influence the competitive effectiveness of diazotrophy versus nitrate reduction differently according to organism (or symbiotic system) and environment. Generally speaking, nitrate reduction turns out to be an economy, both for plants and microbes. It is generally accepted that an increase in nitrogen fixation by grain legumes may be possible either by enhancing the supply of carbon assimilates to the nodule, or by a more efficient use of carbon compounds, within the nodule (Larue et al. 1984).

The available evidence suggests that, even in nitrogen-fixing grain legumes, both fixation and grain yield are limited by the supply of carbon assimilates (Sinclair and de Wit 1975). Due to higher protein concentration, the nitrogen requirement of developing seeds in grain legumes is higher than in cereals (Sinclair and de Wit 1975, Bhatia 1983). In annual crops such as soybean and mung bean, the nitrogen demand is met by mobilization of nitrogen from foliage which results in premature senescence and abcission of leaves. Sinclair and de Wit (1975) call this a self-destructive process. The inability of legume foliage to cope with the demand for photosynthates by both the developing grain and nodule bacteria, and the inability of the latter to cope with the peak demand for nitrogen at the grain-filling time, are the reasons for the low yield of grain legumes. The loss of functional leaf area, and consequent reduction in the supply of assimilates, intensify the competition between root nodules and developing seeds.

End-use Quality of Grain

The quality of the harvested grain depends upon the concentration of carbohydrates, proteins, and lipids in the grain. Their relative proportions determine the energy density and quality of grain when used as animal feed. Increased grain protein or improvement in the limiting amino acids enhances the nutritional value of feed grains. Like the PV for a chemical end-product, seed-biomass productivity can be estimated, based on its chemical composition (Sinclair and de Wit 1975). The seed-biomass productivity values are highest for cereals, a little lower for grain legumes, and the least for oilseeds (Table 5).

Increasing the Concentration of Grain Protein

Bhatia and Rabson (1976) observed that the increase in protein content of the grain is accompanied by a corresponding increase in energy and nitrogen requirement of the plants. One percent increase of nitrogen in cereals requires an additional 6–11% nitrogen for grain protein stoichiometry alone, depending upon the crop variety and initial protein concentration. In grain legumes, the additional nitrogen requirement for 1% increase in grain protein was computed to be about 3.5% (Bhatia 1983).

Altering the Composition of Amino Acids

The PVs for the 18 amino acids and the two amides normally found in grain protein are listed in decreasing order of their PV in Table 6. The production of histidine, arginine, lysine, and tryptophan requires

Table 5. Seed-biomass productivity of cereals, grain legumes, and oilseed crops.

Crop	Biomass productivity seed g g⁻¹	(1/PV) × 100 g glucose required for the production of 100 g seed biomass
Cereals		
Rice	0.75	133.3
Wheat	0.71	140.8
Maize	0.71	140.8
Barley	0.75	133.3
Sorghum	0.70	142.8
Oats	0.70	142.8
Grain legumes		
Chickpea	0.64	156.2
Lentil	0.65	153.8
Pea	0.65	153.8
Mung bean	0.66	151.5
Pigeonpea	0.66	151.5
Oilseeds		
Sunflower	0.48	206.4
Safflower	0.48	207.8
Soybean	0.47	208.3
Groundnut	0.40	248.1
Sesame	0.39	254.1

Seed composition and biomass productivity values for cereals and legumes are from Sinclair and de Wit (1975); for oil seeds: Mitra and Bhatia (1979).

nearly double the amount of glucose that the production of glutamic acid (which is the predominant amino acid in cereals and grain legumes) uses.

In the diet of humans and monogastric animals, proteins are the sole source of the eight essential amino acids. The distinction between indispensable and dispensable amino acids is related to the diet. Histidine and arginine may also be indispensable (Visek 1984) in some diets. Lysine, tryptophan, and threonine are the three amino acids that are deficient in cereal grains, while methionine and cysteine are deficient in most grain legumes. If the amount of glucose required for the synthesis of one unit of glutamic acid is 100% then it is increased for other amino acids, e.g., tryptophan—182%, lysine—178%, methionine—164%, cysteine—144%, and threonine—124%. The energy requirements for the synthesis of essential amino acid is higher than that for glutamic acid, the predominant amino acid in grain proteins. The average PV of those eight amino acids originally considered indispensable is 0.4477, compared with 0.7054 for glutamic acid and 0.5054 for glutamine. In other words, 2.23, 1.42, and 1.98 g of glucose, respectively, would be required for production of 1 g of the indispensible amino acids, glutamic acid, and glutamine.

Increasing the Oil Content

The major constituents of the grain of oilseed crops are carbohydrates, protein, and oil. An increase in oil content of these grains must obviously be at the expense of either carbohydrate or protein, the former being preferable. But the energy requirement is greater when the oil content is increased at the expense of carbohydrate rather than of protein (Mitra and Bhatia 1979). An increase of five percent points of oil in the grain entails an enhancement in photosynthate requirement by 4.3%.

Changing the Composition of Fatty Acids

The conversion values of glucose into common fatty acids found in vegetable oils are given in Table 7. The energy requirement increases with the increase in chain length of fatty acids. In general, the unsaturated fatty acids require more glucose for their biosynthesis than do saturated fatty acids of similar chain length as shown by the carbon atoms : double bond on Table 7. Further, it was estimated that reduction of erucic-acid content in rapeseed oil had no additional energetic cost (Mitra and Bhatia 1979).

Negative Associations

Various negative correlations are observed in different crops between:

1. number and mass of grains;
2. grain yield and grain protein concentration;
3. protein and oil concentrations in seed;
4. oil concentration and yield.

It is difficult to visualize a genetic basis for such associations. In fact, they represent the competition for the same limited resource. If large numbers of grains are to be filled, grain mass must be lower. When the grain mass is high, the available resource would not be sufficient for all the fertilized embryos to develop. Negative correlation between grain yield

Table 6. Production costs, energy values, and nitrogen content of the individual amino acids and amides.

Amino acids and amides	PV	Heat of combustion ΔH_c^0 (KJ mol^{-1})	% glucose requirement to synthesize 1 g of amino acid instead of 1 g of glutamic acid	% N
Aspartic acid	0.8024	1604	88	10.52
Glutamic acid	0.7054	2247	100	9.52
Serine	0.6928	1457	102	13.33
Glycine	0.6091	975	116	18.66
Threonine[1]	0.5711	2104	124	11.76
Alanine	0.5527	1623	128	15.72
Asparagine	0.5259	1932	134	21.20
Glutamine	0.5054	2575	140	19.17
Cystine	0.5018	4258	141	11.66
Cysteine	0.4913	2265	144	11.56
Valine[1]	0.4750	2924	148	11.96
Proline	0.4669	2732	151	12.17
Leucine[1]	0.4534	3588	156	10.69
Tyrosine	0.4443	4436	159	7.73
Methionine[1]	0.4309	3392	164	9.39
Phenylalanine[1]	0.4248	4652	166	8.48
Isoleucine[1]	0.4190	3589	168	10.68
Lysine[1]	0.3952	3688	178	19.16
Tryotophan[1]	0.3880	5634	182	13.72
Arginine	0.3601	3745	196	32.16
Histidine	0.3499	..	202	27.08

1. Amino acids widely considered indispensable for humans and monogastric animals.

Source: Mitra et al. (1979).

Table 7. Production cost and energy values of individual fatty acids excluding the cost of glucose uptake.

Fatty acids Name	No. of carbon atoms : double bond	PV	ΔH_c^0 (KJ mol^{-1})	Percent glucose requirement relative to palmitic acid to synthesize 1 g product
Saturated				
Lauric acid	12:0	0.361	7423	94
Myristic acid	14:0	0.349	8740	97
Palmitic acid	16:0	0.340	10049	100
Stearic acid	18:0	0.333	11362	102
Eicosanoic acid	20:0	0.328		104
Behenic acid	22:0	0.323	13988	105
Lignoceric acid	24:0	0.320	11133	106
Unsaturated				
Palmitoleic acid	16:1	0.327		104
Ricinoleic acid	18:1	0.333		102
Oleic acid	18:1	0.323	11208	105
Linoleic acid	18:2	0.311	10988	109
Linolenic acid	18:3	0.300	10770	113
Eicosenoic acid	20:1	0.318		107
Erucic acid	22:1	0.313		109
Nervonic acid	24:1	0.309		110

Source: Mitra and Bhatia (1979).

and grain protein concentration in cereals at current levels of productivity has been attributed to limited nitrogen availability (Frey 1979). We attribute it to limitation of both energy and nitrogen resources.

Gene Transfers using Recombinant DNA Techniques

Rapid developments in genetic transformation of plant cells have been achieved in the past few years. Integrative transfer and expression of genes from bacteria, other plant species, and animal cells have been demonstrated (Fraley et al. 1986, Bhatia et al. 1986). The transferred genes are expressed by the production of protein product using the resources and cell machinery of the host cell. It is to be expected that the genetic gains made by using the recombinant DNA techniques will also be subject to compensation and intrinsic bioenergetic, input, or environmental constraints.

Validity of the Bioenergetic Constraints

Precise experimental evidence is difficult to obtain for the ideas presented here. The bioenergetic constraints in the improvement of grain protein and in the alteration of amino acid composition of grain were pointed out and are accepted (Bhatia and Rabson 1976). However, the observed grain yield reductions in the genotypes with high protein and lysine are still greater than expected on the basis of bioenergetic computations. Perhaps there are other factors that contribute to yield reduction. Cultivars of maize with high lysine and of wheat and barley with high protein are available. However, these high-quality cultivars have never matched the productivity of cultivars with normal protein content and amino acid compositions. Ryan and Asokan (1977) express agreement with Bhatia and Rabson (1976) on the bioenergetic constraints in breeding for quality, and consider that high productivity rather than improved quality will benefit both the producers and consumers of food crops. There is a de-emphasis on breeding for high-protein content and high-lysine content in cereals and renewed emphasis on increasing the productivity.

The Overall Implications

The bioenergetic constraints pointed out should not be construed to imply that a simultaneous improvement of two or more parameters competing for energy resources in the plants is not possible. This would be true only when the available energy remains limited. Researches in breeding and agronomy are directed to enhance the energy flow through cropping systems. Gains in overall energy resource have been made in the past, and will continue to be made in future. Plant breeders can exercise the options within the limits of available energy to elevate productivity, to combat stresses, or to improve the quality of produce. Unless there is a simultaneous increase in the availability of energy, any genetic improvements in stress resistance and in quality can be expected to result in a lowering of yields.

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Progress and Prospects of Biotechnological Applications to Improving the Quality of Cereals and Legumes

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Abstract

A new repertoire of plant breeding techniques is available for genetic improvement of crops. The present position, limitations, and possible future applications of foreign gene transfer and direct DNA transfer to improve the quality of cereals and legumes via Agrobacterium *methods is reviewed. The fluorescence method lends itself to use by plant breeders in the classification of genotypes in segregating populations. Its use is illustrated using the protein improvement of faba beans as an example. In the short term, techniques that increase the speed and efficiency of conventional plant-breeding methods show the greatest promise, but in the longer term, genetic-engineering techniques present an exciting prospect for overcoming many of the constraints encountered in conventional plant-breeding procedures, and for bridging sterility barriers between distantly related crop plants.*

Introduction

The productivity of several major crops has registered a steep rise, over the past few decades, particularly in developed countries. Conventional breeding techniques and improved crop-husbandry practices have been implicated with an equal share of contribution (Bingham 1984) in bringing about this change. Some novel techniques e.g., tissue culture, have made inroads into crop improvement, with varying degrees of impact. More recently, there has been a revolutionary change in the conceptual framework of our understanding of biology and in the potential power of new techniques available to agricultural scientists. Plant biotechnology is the term coined to encompass any laboratory-based manipulation of the genetic makeup of higher plants and its application in crop improvement. The potential of biotechnological applications in plant breeding unit operations are listed in Table 1. In many cases, field-testing of plants derived from such applications is still in progress.

Biotechnology in relation to the food industry may be defined as the application of biological systems to food manufacture. Much of the food that is consumed is processed in one way or another, and thus biotechnology has a potential impact in the food industry in both production and processing. While the application of biotechnology in plant breeding is relatively recent, its application to food processing, such as fermentation (a biotechnology), is well established. In the food industry, mechanical (e.g., grinding), physical (e.g., membrane separation, cooking), and chemical (e.g., hydrolysis, salting) methods are contrasted with biological (biotechnological) methods, such as fermentation and enzymatic treatment.

Biotechnology in the agri-seeds industry and in food processing have a common ground since the starting materials of the food industry are often crop plants. It is convenient, therefore, to separate the impact of biotechnology in agriculture into **agronomic**, i.e., advantages originating from increased yields, extended growth range, and environments from which principally farmers benefit; and **non-agronomic**, i.e., improvement of plants and microorganisms in order to benefit the food processor, retailer, or the consumer. Just as biotechnology will

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Table 1. How biotechnology will impinge on plant breeding at all unit operations.

Unit operations involved in plant breeding			Potential biotechnological impact
Define trait			Increased understanding of complex characters
Variation exists	-	*No*	Create variation by in vitro or in vivo mutation using gene technology
Yes			
Sexual cross	-	*No*	Gene transfer (protoplast fusion), directly (micro-injection) or by vectors (*Agrobacterium*)
Yes			
Amount of unwanted characteristics carried along with desired trait	-	*High*	Speed up introgression time (haploids, somaclonal variation, markers)
Low			
Screening	-	*Difficult*	New screening methods, e.g., cell culture, new screening probes, e.g., RFLPs (restricted fragment length polymorphism)
Simple			
Action			Increased knowledge so enabling better registration and protection, etc., e.g., isoenzymes as phenotypic markers

impinge at various levels in plant breeding, so it will in the food chain (Fig. 1).

This paper briefly reviews the current state of gene transfer techniques, i.e., genetic engineering of higher plants and the present knowledge of those genes which, if transferred and stably expressed, might lead to an improvement in the nutritional quality of legumes and cereals.

Technical Considerations

Cloning

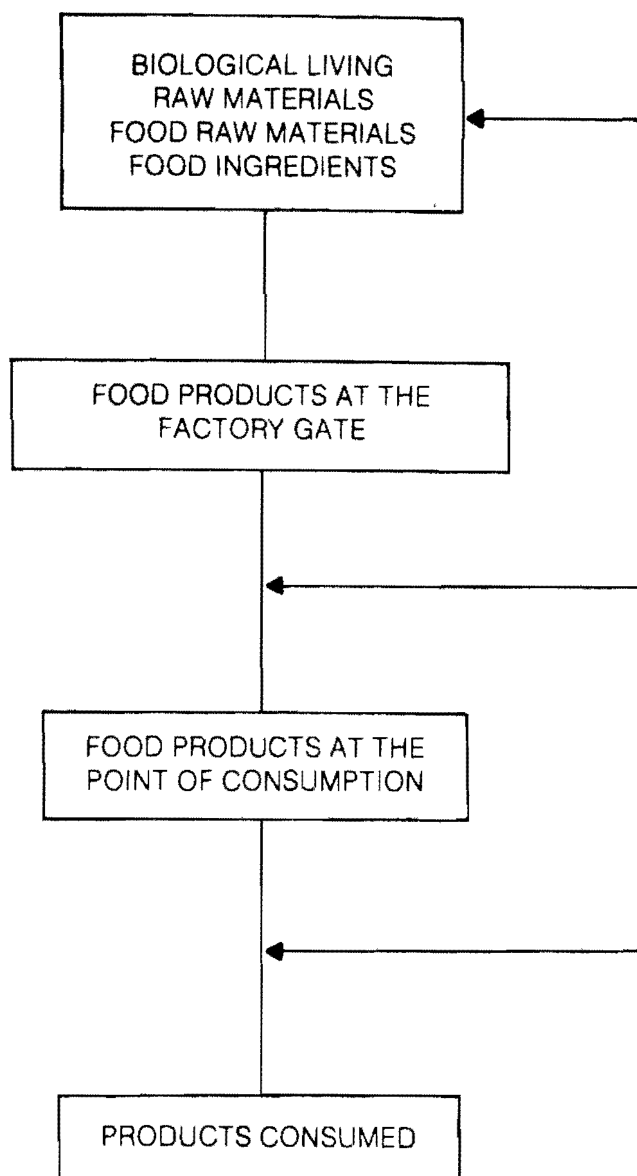
The complete repertoire of basic recombinant DNA methods (Maniatis et al. 1982) has been successfully applied to cereal grain and legume seed. Seed messenger RNAs have been purified from a variety of crops, and cDNA banks have been prepared and screened. In the same way genomic DNAs have been purified, and genebanks constructed and screened. Several seed-specific genes of potential nutritional interest have been isolated and sequenced from both cereals and legumes and transferred into model systems (EMBO 1986). Putative promoter sequences that control the expression of genes have been identified, as well as the sequences that may target gene products to cell organelles such as chloroplasts and protein bodies (Croy and Gatehouse 1985).

Transformation and plant regeneration

Foreign gene transfer to *Nicotiana* spp and *Petunia*, by using *Agrobacterium tumefaciens* Ti plasmids, has proved to be successful with high transformation frequencies and plant regeneration (Potrykus et al. 1986). More than 50 different genes have been transferred. The transformed lines were selected, using marker genes, and the transferred genes were shown to be expressed in the recipient genotypes. Expression is usually very poor, but varies greatly with different transformation events. Storage protein genes from both legumes and cereals have been transferred and expressed in *Nicotiana* and *Petunia* (EMBO 1986). In many cases transferred genes have been stably expressed through sexual crosses for

Food chain

Potential biotechnological impact



Agronomic: increase yield, extend geographic and environmental range, all-year growing.
Nonagronomic: increase benefit to processor by lowering the costs of manufacturing operations, stay fresh longer, improve texture and taste, phytoproduction of flavors, colors, and other more natural additives using tissue culture, single-cell protein.

Improve distribution and product quality by inhibiting physical, chemical, and microbiological deterioration, introducing less harsh processes and new preservation regimes.

By ensuring products meet the consumers' expectations of texture, flavor, nutrition, preservation, wholesomeness, and being more 'natural'.

Figure 1. How biotechnology will impinge at all levels of the food chain.

several generations, thereby establishing the veracity of transformations. The *Agrobacterium* system is now being developed successfully with other solanaceous species, such as tomato and potato, and also with forage legumes and *Brassica*.

Many important dicotyledonous crops, such as soya and faba beans, are less amenable to transformation with *Agrobacterium*. Also, plant regeneration is often poor for these species. However, rapid progress is being made in several laboratories worldwide, and it appears probable that the *Agrobacterium* system will be adapted to work effectively with a wider range of dicotyledonous crops in the next decade.

Equally impressive progress has been made with direct gene transfer to protoplasts that are then regenerated into plants (Potrykus et al. 1986). Efficiencies of 1-2% are now being obtained and here the host range of the recipient DNA is almost unlimited. The major limitation in employing this technique is the ability to regenerate whole plants from protoplasts. In general, the results of expression, inheritance, and stability, using this method, have been similar to those obtained with the *Agrobacterium* system.

However, a major problem remaining is the transformation of cereals, a field in which progress has been made only recently. We are at present entering a very interesting phase of field-testing and evaluation of transformed plants.

Nutritional Quality

Changes in seed composition

Much of the protein of human food is obtained either directly or indirectly from the grain proteins of cereals and legumes. The leaves, roots, and tubers of other crops contribute smaller amounts of protein to human diets. Seed proteins may provide most of the protein consumed in the diets of people in developing countries (Croy and Gatehouse 1985). Numerous animal, and some human, nutritional experiments have established the need for a balanced, essential amino-acid composition in dietary protein, as exemplified in nutritionally excellent proteins such as egg ovalbumin (WHO 1973). The legume and cereal grains fall below this standard as sources of protein, because of their amino-acid composition, lower digestibility, and the presence of antinutritional factors.

The nutritional balance of essential amino acids in the grain legumes can be improved largely by increasing the sulfur amino acids and, to a lesser extent, increasing tryptophan or valine, depending on the legume in question. Similarly, the nutritional balance of essential amino acids in cereal grains is improved with the increase in lysine, tryptophan, or isoleucine, (FAO 1970, Payne and Rhodes 1982, Croy and Gatehouse 1985). An additional improvement would be expected by the removal of a variety of toxic or antimetabolic constituents, many of which are proteinaceous, e.g., lectins, enzyme inhibitors, etc. In a few instances, the identity of an antinutritional factor has been unequivocally established, e.g., the lectin proteins of *Phaseolus vulgaris*; but this is usually not the case (Gatehouse 1984). The need for removal or inactivation of these compounds has to be considered on a case-by-case basis, since their levels vary widely in different species and cultivars, as well as their effects on different geographic human populations and animals. Furthermore, protein antimetabolites are heat-denaturable and, therefore, are likely to be inactivated by cooking, although it is still open to question whether their presence as a source of protein is desirable at all. The problem is further complicated by the fact that, although in most cases their biological function is not known, in some cases they have been implicated as components of the insect- and disease-resistant mechanisms (Gatehouse 1984). In such instances, their removal or inactivation would be clearly undesirable unless alternative protective measures were available. Similar arguments are advanced in respect of the removal of nonheat denatured antimetabolites, such as condensed tannins.

While this review concentrates on aspects of improving nutritional attributes of cereal and legume proteins, particularly with reference to developing countries, mention should be made, at least in passing, to the increasing role of seed proteins as food additives to provide functional properties, and of properties such as bread-making quality, which are conferred to doughs by particular proteins of wheat and rye (Payne 1986).

Also, cereals and legumes supply various components, other than protein, in the diet, (e.g., carbohydrates, lipids, fiber, minerals, and vitamins) and undoubtedly improvements are also possible in respect of these compounds.

Appropriateness of biotechnology

It is unlikely that biotechnology will significantly shorten the time-span for the development of a new variety (Austin et al. 1986). As a consequence, biotechnological applications are more likely to be chosen where the conventional breeding programs have failed, for one reason or another, to bring about the desired level of improvement. Plant breeders have not met with an anticipated measure of success in the nutritional improvement of cereals and legumes. For example, the programmes for breeding high-protein wheat and rice, high-lysine wheat, barley, and maize, higher level of sulfur-containing amino acids in peas, soybeans, and faba beans have, so far, been unsuccessful with conventional breeding. Thus, the application of biotechnological methods to nutritional improvement of cereals and legumes is highly appropriate and appears feasible. However, the lack of knowledge about the genes that might regulate the amounts of compounds that reduce palatability, digestibility, etc., precludes the immediate application of biotechnology in this area.

Seed-storage proteins of special position

Seeds contain different kinds of proteins; and these are classified as metabolic, structural, or storage proteins (Boulter and Parthier 1982). Metabolic proteins are diverse. Storage proteins, on the other hand, consist of only a few different types that quantitatively predominate, and thereby principally determine the overall seed protein composition and qual-

ity. Thus, a single storage protein may, in some instances, represent over 50% of the seed protein, e.g., vicilin in *Phaseolus vulgaris*. The seed-storage proteins and the genes that encode them have been extensively studied and comprehensive reviews exist (Croy and Gatehouse 1985).

A single storage-protein type, such as prolamin in maize or vicilin in peas, is composed of polypeptides that are encoded by a small gene family. The size of this family varies in different crops, usually being of the order of 10–20 for the two main legume storage proteins, vicilin and legumin, and perhaps 100+ in the prolamin family of some cereals such as maize. This has important implications for genetic engineering.

The extent to which different genes of a storage gene family are expressed has been determined only for a few gene families and, even in those, for relatively few of the genes of the family. For example, in the pea legumin-gene family, consisting of about 10 genes, we know that leg A is expressed to a greater extent than any of the others. While posttranscriptional and translational controls have been shown to operate in the control of the expression of at least some seed-protein genes (Boulter et al. 1987), current information suggests that the seed-protein gene expression is primarily controlled at the levels of transcription and that high levels of gene product, e.g., storage protein, are correlated with high messenger RNA levels, which in turn depend upon high transcription rates. Thus, storage protein genes contain control sequences which, in conjunction with cellular transacting proteins, lead to very high levels of expression.

The manipulation of storage proteins has therefore the following advantages:

1. high heritability;
2. genes already cloned and sequenced, with in vitro mutagenesis possible; and
3. products will be produced in large amounts so affecting the overall seed-protein composition.

Strategies for nutritional improvements

There are several potential biotechnological strategies to improve the essential amino-acid composition of seed proteins. These are given below.

1. Increase the proportion of one (or a few) high-quality proteins. Several nutritionally high-quality proteins have been identified in the seeds of many crop plants, but these occur in only small amounts (Casey and Short 1981). If their low concentrations were due to the poor expression of their encoding gene(s) and not to some other factor such as proteolysis, then it might be possible to substitute their regulatory sequences with those of a highly expressed gene, e.g., a storage-protein gene, leaving the coding sequence unchanged and under the latter's control. Alternatively, additional gene copies might be added to the genome.

   Yet another approach would be to increase protein yield by increasing the overall yield of a crop. Thus, Gates et al. (1983) screened the fababean germplasm using fluorescence microscopy to select types with independent vascular supply to each flower, in order to stabilize and improve yield, a strategy that is applicable generally to other legumes.
2. Change the subunit composition of a major protein. Usually some subunits of a storage protein, are for example, 'nutritionally' better than others (Nielsen 1984). Since there is already good evidence that different subunit compositions of a storage protein are biologically acceptable, this would appear a viable strategy (Croy and Gatehouse 1985).
3. Improve the amino-acid composition of a major protein e.g., a storage protein, either by adding desirable amino-acid codons, or by changing the reading frame in order to improve the amino acids encoded, or by adding parts of a high-quality protein from any available source. Information on codon usage, if available, should guide the choice of different possible isoaccepting codons (Lycett et al. 1983).

All three strategies suffer from the disadvantage that the endogenous genes giving the overall poor amino acid composition of the seed protein remain active. Thus, the added gene will be required to be greatly 'enriched', suggesting that strategy 3 might be the best. All three strategies require that directed change is possible in vitro. Generally speaking, this should prove to be the case since normally in vitro mutagenesis techniques, using either unique restriction enzyme sites, trimming, or oligonucleotide synthesis and M13 cloning, provide a range of possibilities for the directed changes that are required. All three strategies also require the ability to transfer the changed 'foreign' gene, but this technology is available in model systems and it is hoped will be generally available soon. At present, expression levels of transferred genes are low, emphasizing again the requirement for strong enrichment. Deletion of

endogenous homologous genes, for example, by homologous recombinatorial insertion, are at present unavailable in higher plants and transposon inactivation techniques are limited and undeveloped.

Accepting that one of these strategies is to be followed, the appropriate genetic engineering to be carried out would depend partly on whether or not there are any constraints to the changes that one could make to a storage protein. Such constraints to change could be of three types:

1. changes leading to loss of biological function;
2. changes giving reduced yield due to energetic considerations; and
3. changes in which the metabolic machinery is unable to accommodate the improved protein information, e.g., supply sufficient charged lysyl tRNAs, etc., in the case of a cereal, or sulfur amino-acid +tRNAs in the case of a legume. Thus, feedback-insensitive mutants (Rhodes and Jenkins 1978) which increase specific free amino-acids might, for example, be concomitantly required.

Other Considerations

Although we now have a good knowledge of the structure of seed proteins, we have little information on how this structure relates to their biological properties with regard to seed viability, vigor, storage, etc. Thus there will be constraints to the possible changes that can be made by protein engineering, although in this regard storage protein genes have probably a greater potential for change than do metabolically active proteins, such as enzymes. However, in spite of the function of storage proteins being only to supply amino acids to the germinating seedling, constraints to change exist for the maintenance of the correct overall amino-acid composition for their synthesis, and for the maintenance of the sequence and structure necessary for:

1. correct posttranslational transport to the protein bodies,
2. deposition (packing) in the protein bodies,
3. correct utilization (proteolysis) of the storage protein on germination.

The amino-acid sequences required for these functions, as well as those required for covalent association of other molecules, e.g., carbohydrates in those seed-storage proteins that are glycoproteins, are not all fully known or understood. However, sufficient information is known about the amino-acid sequence of several storage proteins, either directly or more usually from cDNA sequences, to be able to speculate upon what sequence regions could be manipulated, even in the absence of X-ray crystallographic data. For example, Croy and Gatehouse (1985) have considered four types of sequence regions for these purposes.

1. Hydrophilic regions on, or near, the surface of the protein.
2. Repeat sequences, since these may vary in length and type, suggesting that modification might be possible without serious disruption of function.
3. Regions linking repeat sequences.
4. N- or C-terminal regions to which extra amino-acid sequences might be linked.

While the metabolic-energy cost, for an increase in the percentage protein in seeds, normally results in a yield penalty, improved cereal and legume amino-acid profiles, along the lines indicated above, would not give serious yield penalties based on calculations similar to those of Penning de Vries et al. (1974), although these contain many assumptions.

Conclusion

Plant biotechnology is advancing rapidly, and it is already clear that it offers new opportunities for directed changes that will complement conventional methods of crop improvement. The next decade should reveal the extent of its usefulness in crop improvement, and this objective will be facilitated by the targeting of biologically feasible and economically desirable objectives. Whatever the outcome, biotechnological methods will greatly increase our basic understanding of crop plants. The fact that much of the research and development required will be industrially funded, underscores the importance of developing countries being involved in plant biotechnology.

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Overview of Sorghum and Pearl Millet Quality, Utilization, and Scope for Alternative Uses

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Abstract

The meals or flours ground from sorghum and pearl millet are coarse to the feel, unlike the soft flours of wheat or rye. Sorghum and pearl millet are therefore classified as coarse grains. Neither sorghum nor pearl millet contains gluten, and so their flours lack the properties that make good dough. It is therefore necessary to rely on the small particle size of the flour and the cohesive properties of water to produce an acceptable dough. The products made from sorghum and millet flours tend to taste gritty. Traditional foods made from these flours are processed so as to avoid this gritty taste. Both sorghum and pearl millet further present problems from the standpoint of animal nutrition, and these must be addressed if the full potential of the two grains is to be realized. Sorghum has limited digestibility, particularly with young animals. Pearl millet has been implicated in causing goiter. A greater understanding of means to alleviate these problems is essential before coarse grains can be considered as substitutes for maize in animal feed.

Introduction

When the term quality is applied to such cereal grains as pearl millet and sorghum, one generally understands it to mean the ability of the grain to produce the food product that is desired. Also inferred is that the grain would be suitable for human consumption. Although usually not mentioned, and often not even considered, the assumption is that the grain is also nutritious. The term nutritious implies high digestibility and the absence of major adversely nutritious factors.

A good-quality sorghum cultivar will produce a variety of good food products, be free of insect infestation, molds, and other undesirable contaminants, and be nutritious. It is, of course, doubtful if any one cultivar would meet all of those criteria. In wheat, where grain quality has been intensively studied and selected, it is clear that certain cultivars of wheat will produce good pasta but relatively poor bread and vice versa. The food quality of neither sorghum nor pearl millet has been studied sufficiently for one to say with any degree of certainty whether different cultivars are required to produce different products. It is also not clear whether cultivars that give good product quality are also of good nutritional quality. Also using the example of wheat, its baking quality is modified when the amino-acid composition of the protein is modified.

Sorghum and Pearl Millet Quality

There have been several reviews of the food uses of sorghum and pearl millet (Hoseney et al. 1981, Rooney et al. 1986, Hoseney et al. 1987). In addition, a fairly detailed book about sorghum and millet has been published (Hulse et al. 1980).

The average sorghum grain kernel weighs about 30 mg and is generally spherical in shape. The pearl millet kernel is much smaller, with an average mass of 9 mg, and is generally more tear-shaped. Of course, there is much variation in both crops. Sorghum grain varies in color from white to dark brown. The most common colors are white, bronze, and brown. Pearl millet also varies widely from yellow to a dark brown. However, most cultivars have a characteristic slate-gray color.

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Grain Morphology

The structure of pearl millet and sorghum is, in general, similar to that of other cereals (Zeleznak and Varriano-Marston 1982, Earp et al. 1983). The caryopsis has as its cover a pericarp. Inside the pericarp is the endosperm and a germ. The pericarp is a protective layer that also contains the cross and tube cells. Sorghum is unique among the cereals: (1) Its pericarp also contains starch. Those small starch granules are found in the mesocarp. (2) Certain cultivars have an intact inner integument. This is the site of the condensed tannins found in sorghum. Among the cereals only sorghum and barley contain condensed tannins.

The endosperm is the storage part of the caryopsis. It contains starch and proteins. The outer layer of cells of the endosperm is the aleurone. The aleurone cells do not contain starch and do not contain the storage protein found in the remainder of the endosperm. The contents of the aleurone cells are high in protein and ash. It also appears to be the site of enzyme synthesis in the grain.

The embryo is the living part of the seed and respires even when the seed is relatively dry. Sorghum and pearl millet differ in the size of their embryos relative to the overall seed size. Sorghum has an embryo that is about 10% of the seed while the pearl millet embryo accounts for 15-18% of the seed.

Endosperms of sorghum and pearl millet are alike in that they both contain vitreous and opaque parts. The vitreous endosperm is tightly packed, whereas the opaque endosperm is more loosely packed with many air spaces. The air spaces diffract light and make the endosperm appear opaque.

Grain Composition

The breakdown of the anatomical parts of the sorghum kernel and the composition of the parts is given in Table 1. The endosperm is by far the largest fraction, being slightly more than 80%. The germ, at about 10%, is slightly more than the bran (8%). In general the bran is high in fiber (cellulose and hemicellulose) and low in protein and ash. The germ is high in ash, protein, and oil but essentially free of starch. The endosperm is high in starch, moderately high in protein, and relatively low in ash and oil. In general terms this applies in all cereals. The composition of the various fractions of pearl millet grain is similar to that of sorghum. The most striking difference is the relatively high protein content of the bran of pearl millet (Table 2). Also noteworthy is the higher fat content of the whole grain. This appears to be because of the size of germ, its relatively high level of fat and also the high fat level of the bran.

Pearl millet and sorghum have similar protein contents, generally in the 9-13% range, the actual amount varying with the conditions under which the crop is grown. In general, high yields are associated with lower protein contents. Because pearl millet is often grown on poor soils, it often has a higher protein content than sorghum. As with other cereals, proteins from both grains are low in the amino acid lysine. Sorghum has a higher leucine to isoleucine ratio than pearl millet. A high leucine to isoleucine ratio has been implicated in causing the disease pellagra (Srikantia 1978).

Table 2. Composition of pearl millet fractions and whole grain (%).

Anatomical part	% of total	Ash	Protein	Oil
Whole grain	100	1.7	13.3	6.3
Endosperm	75	0.32	10.9	0.53
Germ	17	7.2	24.5	32.2
Bran	8	3.2	17.1	5.0

Data from Abdelrahman and Hoseney 1984.

Table 1. Composition of sorghum fractions and whole grain (%).

Fraction	Whole kernel	Ash	Protein	Oil	Starch
Whole grain	100	1.65	12.3	3.9	78.3
Endosperm	82.3	0.37	12.3	0.6	82.5
Germ	9.8	10.36	18.9	28.1	13.4
Bran	7.9	2.02	6.7	4.9	34.6

1. Adapted from Hubbard et al. 1950.

Food Uses of Sorghum and Pearl Millet

Pearl millet and sorghum, together with maize, are classified as coarse cereals. By this is meant that the ground grain has a coarse feel to it. It does not have the soft feel of wheat or rye flours. This coarse feeling is caused by the grains being very hard and the particles of flour remaining sharp and angular after grinding. Cooking does not overcome this gritty texture. The traditional food products made from the coarse grains employ techniques that mitigate the gritty character.

Food Products

The food products made from sorghum and millet are many and varied. However, they can be grouped into two general groups. One is the flat (unleavened) breads, and the other is cooked or fermented products. These are, for instance, the *roti* or *chapatti* of India. Neither sorghum nor millet contains gluten, as found in wheat. Olewnik et al. (1984) have suggested that the cohesive force in such doughs is related to the water in the system. When the interparticle distances are small, the surface tension of the water is sufficient to give a cohesive dough. The flour then acts as essentially inert particles. The key to the cohesive force is thus small particle size (in that the particles can get close together) and the correct amount of water (Fig. 1).

A bread-type product made in Sudan (*kisra*) is substantially different from unleavened bread. The meal undergoes a long fermentation with the naturally occurring bacteria. Fermentation lowers the pH and also tends to break down the hard particles. The fermented product is then heated rapidly at a high temperature so as to vaporize the water and

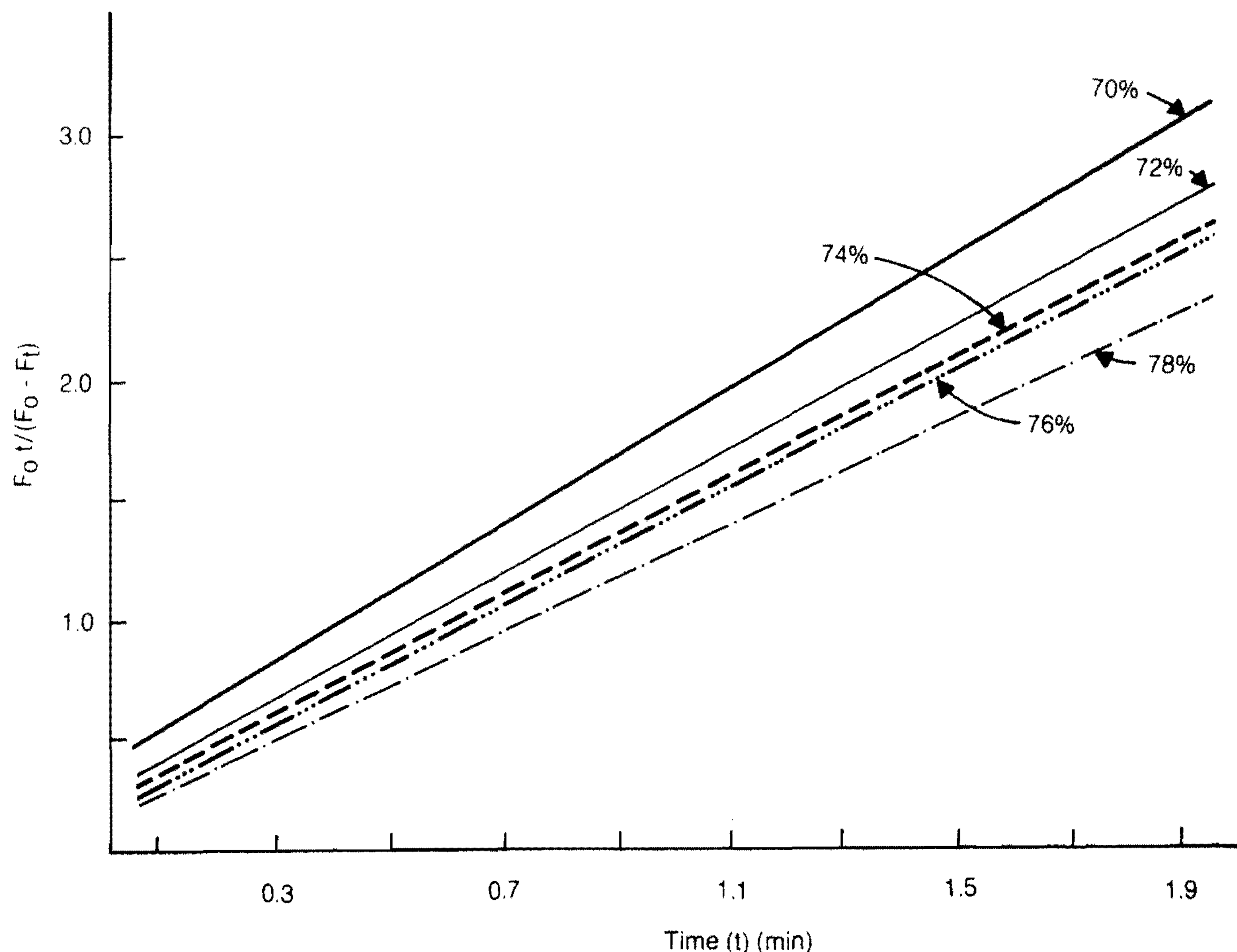

Figure 1. Normalized stress-relaxation curves for *roti* dough produced from one sample containing various percentages of water. (F_0 is the initial force and F_t is the force at time t.)

puff the product. The grittiness is nearly eliminated by the manner in which *kisra* is made.

Most of the other products made with sorghum or millet can be classified as some type of a gruel. Some are thick and others are thin. Some are acid while others are alkaline. The techniques used to make the pastes vary widely (Rooney and McDonough 1987). It is not clear what determines a good-quality flour or meal for these products. There is a thin gruel that is made in large quantities in many parts of Africa. This is home-made sorghum beer. It is better described as a gruel than as a beer, since it includes significant quantity of solids.

Nutritional Problems

Two major nutritional problems have been identified with sorghum. These are the presence of condensed tannins and the very low digestibility of sorghum in young animals and humans. Tannins combine with protein and make the protein nondigestible. Further, they react with the animals' digestive enzymes and decrease their activity. The chemistry of the condensed tannins is complicated (Butler and Rogler 1985). It has recently become clear that they are not responsible for all the problems associated with the digestibility of sorghum. The condensed tannins are under genetic control and so can easily be bred out of any line. In fact, relatively few of the known cultivars grown around the world contain condensed tannins.

MacLean et al. (1981) have shown that sorghum is not digested very well by young children. The reason for the reduced digestibility is not clear. The particles of sorghum do not break apart in the digestive system as other grains do. Therefore they do not present as large a surface area for enzymatic attack. Evidence has also been presented that sorghum particles are held together by a different mechanism than are pearl millet or maize particles (Abdelrahman and Hoseney 1984).

Pearl millet is more digestible and generally more nutritious than sorghum. However, it is not without problems. Reports from Sudan (Osman 1981, Osman and Fatah 1981, Osman et al. 1983) have implicated pearl millet in causing goiter. Goiter is generally caused by a deficiency in iodine. However, it is known that goiter can be caused by other factors. When rats were fed on diets based primarily on pearl millet, their thyroid glands became altered, as found with goiter (Klopfenstein et al. 1983, 1985). Recent work has shown that the C-glycosylflavones identified in pearl millet by Reichert (1979) may be involved in the problem.

Another major problem with pearl millet is the odor that emanates rapidly after the grain is ground. This is not strictly a nutritional problem but can limit the consumption of the product. Recent work has shown that it is not due to oxidation of lipids (Kaced et al. 1984), as was generally assumed, but is due to an enzyme working on the millet pigments (Reddy et al. 1986). The problem can be avoided by storing the grain at low moisture contents (below about 10% moisture).

Alternative Uses

Within the foreseeable future the major use of these cereals will continue to be as human food. It appears unlikely that the food prepared from these cereals will change much. The dishes now being prepared from these grains have been selected to take advantage of the unique characteristics of the grains. Even with a better understanding of the basic chemistry and physics involved, it appears unlikely that large changes can occur.

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Grain Quality and Utilization of Sorghum and Pearl Millet

R. Jambunathan and V. Subramanian[1]

Abstract

Sorghum (Sorghum bicolor) *and pearl millet* (P. glaucum) *are staple cereals in several parts of the semi-arid tropical regions and supply necessary calories, proteins, and other nutrients. The chemical constituents, including the amino-acid composition and the distribution of protein fractions in these cereals are discussed. Sorghum and pearl millet are processed in various ways for making acceptable foods, such as* roti *and* tô. *The processing and food quality attributes of the grain, including consumer acceptance and the role of physicochemical characteristics of these grains and their influence on various aspects of food quality, are also discussed. Prospects for industrial utilization of these coarse grains are briefly described.*

Introduction

Sorghum and pearl millet are staple foods and constitute a major supply of energy and protein to a large segment of the population living in the semi-arid tropical regions (Hulse et al. 1980). Since many of the people living in these areas suffer from chronic malnutrition, improving the yield potential and nutritive qualities of these grains is highly desirable. The components of grain-and-food quality are visual quality, milling and processing characteristics, digestibility, bioavailability of nutrients, keeping quality of foods, consumer acceptability, and storage stability. This paper deals with the progress that has been made in understanding some components of grain and food quality of sorghum and pearl millet.

Grain Characters

The cereal grain consists of a pericarp, germ, and endosperm. The color of sorghum grains may be white, yellow, red, or brown, and of pearl millet from gray-white to yellow. Sorghum grains are generally spherical to elliptic in shape and their 1000-grain mass ranges from 13.0 to 57.0 g. Pearl millet grains are oval to pear-shaped, with a mass of 4.8–10.1 g (1000 grains)$^{-1}$. The testa in sorghum is the seed coat joined to the outer edge of the inner integument. Some genotypes lack a testa or some have a broken testa (Rooney and Sullins 1977). Pearl millet in general does not have an inner integument. Protein bodies are the major sources of storage protein in sorghum, whereas in wheat, rye, and triticale, they coalesce to form a matrix protein as the seed matures (Adams and Liebenberg 1975). Most of the lipids are present in the aleurone layer, germ, and pericarp in pearl millet (Lai and Varriano-Marston 1980). The endosperm has floury and corneous portions. In the floury part, the spherical starch granules have small particles of protein adhering to their surfaces or are loosely associated with protein.

Chemical Composition

Carbohydrates

Starch is the major component of sorghum and pearl millet grains, and accounts for 55.6–75.2% of sorghum and 62.8–70.5% of pearl millet grains (Table 1). In sorghum the amylose content varies from 21.1 to 30.2%, and in pearl millet from 21.9 to 28.8%. The gelatinization temperature of starch is affected by

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Table 1. Chemical composition of sorghum and pearl millet grains.[1]

Constituent	Sorghum No. of genotypes tested	Sorghum Range	Sorghum Mean	Pearl millet No. of genotypes tested	Pearl millet Range	Pearl millet Mean	SE ±[3]
Protein (%)	10479	4.4-21.1	11.4	20704	5.8-20.9	10.6	0.10
Lysine [g (100 g)$^{-1}$ protein][2]	412	1.06-3.64	2.09	280	1.59-3.80	2.84	0.09
Starch (%)	160	55.6-75.2	69.5	44	62.8-70.5	66.7	2.36
Amylose (%)	80	21.2-30.2	26.9	44	21.9-28.8	25.9	0.88
Soluble sugars (%)	160	0.7-4.2	1.2	36	1.4-2.6	2.1	0.03
Reducing sugars (%)	80	0.05-0.53	0.12	16	0.10-0.26	0.17	0.01
Crude fiber (%)	100	1.0-3.4	1.9	36	1.1-1.8	1.3	0.03
Fat (%)	160	2.1-7.6	3.3	36	4.1-6.4	5.1	0.06
Ash (%)	160	1.3-3.3	1.9	36	1.1-2.5	1.9	0.01

1. Includes germplasm accessions and breeding materials.
2. Ion-exchange chromatography.
3. Standard error of estimation.

All values except protein are expressed on a dry mass basis.

the proportion of amylose to amylopectin (Hoseney et al. 1981). The gelatinization temperature of starch for 12 genotypes of sorghum studied varies from 66.0 to 70.5°C (Subramanian et al. 1982), and for pearl millet from 67.0 to 69.0°C. Relatively few data are available on the nature and amount of free sugars in sorghum and pearl millet grains. Sucrose is the predominant sugar in sorghum and pearl millet grains, and the presence of stachyose, raffinose, sucrose, glucose, and fructose has been reported (Subramanian et al. 1980, 1981).

Fat, crude fiber, and ash

The fat, crude fiber, and ash contents in sorghum and pearl millet showed wide variation (Table 1). Nutritional studies show evidence for deficiency in calcium, iron, and zinc (Hulse et al. 1980). A wide range for minerals and trace elements composition exists in these cereals (Table 2).

Proteins

The grain protein content of sorghum varies from 4.4 to 21.1%, with a mean of 11.4%. The protein content of pearl millet ranges from 5.8 to 20.9%, with a mean of 10.6% (Table 1). Frey (1977) reported that a negative correlation exists between yield and protein in several cereals, including sorghum.

The amino-acid composition of some selected cultivars of sorghum and pearl millet are given in Table 3. In general, sorghum has higher levels of glutamic acid, leucine, alanine, proline, and aspartic acid than other amino acids. In pearl millet the leucine : iso-

Table 2. Minerals and trace elements composition [mg (100 g)$^{-1}$] of sorghum and pearl millet grains.

Elements	Sorghum (n = 99) Range	Sorghum (n = 99) Mean	Pearl millet (n = 27) Range	Pearl millet (n = 27) Mean
Phosphorus	388-756	526	185-363	260
Magnesium	167-325	212	46-128	106
Potassium	363-901	537	294-460	379
Calcium	6-53	26	13-52	38
Iron	4.7-14.1	8.5	4.0-58.1	16.9
Copper	0.4-1.6	0.9	0.6-21.2	7.9
Zinc	2.5-6.8	3.9	1.0-6.6	4.0
Manganese	0.7-3.0	1.8	0.2-1.8	1.5

Table 3. Amino acid composition of sorghum and pearl millet grains [g (100 g)$^{-1}$ protein][1].

Amino acid	Sorghum			Pearl millet		
	M 35-1	CSH 8	SPV 351	BJ 104	MBH 110	WC-C75
Lysine	2.64	2.44	2.76	2.46	2.77	2.77
Histidine	2.28	2.33	2.55	1.95	2.20	1.98
Arginine	4.06	3.57	3.40	5.07	4.54	4.39
Aspartic acid	7.63	6.38	7.07	8.19	8.31	7.59
Threonine	3.26	2.95	3.18	3.28	3.24	3.23
Serine	4.12	3.79	3.86	3.94	4.13	3.56
Glutamic acid	20.97	19.25	19.46	24.66	24.38	20.64
Proline	7.93	6.80	7.90	6.01	7.00	5.58
Glycine	4.00	4.03	4.03	3.54	3.59	3.85
Alanine	11.15	9.84	9.95	8.31	9.51	7.70
Cystine	0.90	0.92	0.99	0.84	0.99	1.03
Valine	5.40	4.46	4.67	6.63	6.04	5.41
Methionine	1.67	1.59	1.65	1.45	1.32	1.32
Isoleucine	4.35	3.95	3.52	4.39	4.25	3.53
Leucine	13.12	12.14	12.00	11.51	11.58	10.07
Tyrosine	3.90	2.99	3.11	3.20	3.18	2.65
Phenylalanine	5.33	4.01	4.38	4.30	5.37	3.94

1. Ion exchange chromatography; protein : N × 6.25.

leucine ratio is lower than in sorghum. Lysine, threonine, and sulfur-containing amino acids are lower in sorghum and pearl millet than in other cereals. Attempts to increase the lysine content in sorghum indicated that derivatives of high lysine sorghums produced only a marginal increase in lysine content in grain and that these selections had lower kernel mass and floury endosperm.

Protein quality is associated with distribution of various protein fractions in the grain. Compared with legumes, the cereal proteins contain a high proportion of alcohol-soluble prolamines that are deficient in lysine and other nutritionally important amino acids. Fractionation studies of proteins carried out on selected cultivars are shown in Table 4. The levels of albumins and globulins are higher in pearl millet than in sorghum, while sorghum contains higher levels of cross-linked prolamines than pearl millet. Glutelin contents of sorghum are higher than in pearl millet. In high lysine sorghums (IS 11167 and IS 11758), the proportion of prolamine is lower, and albumin and globulin fraction is higher, than in normal sorghums (Jambunathan et al. 1975). In pearl millet cultivars containing more than 15% protein, the prolamine fraction is increased, resulting in lower proportions of glutelin, albumin, and globulin fractions.

Pigments and polyphenols

Sorghum contains polyphenols that are generally associated with grain pigmentation. The presence of condensed tannins in sorghum has been reported to

Table 4. Nitrogen distribution in whole kernels of sorghum and pearl millet expressed as a percentage of total nitrogen.

Fraction	Sorghum		Pearl millet	
	M 35-1	SPV 351	WC-C75	PHB 14
I (albumin and globulin)	14.2	18.0	26.2	20.9
II (prolamine)	15.4	12.9	23.7	35.0
III (cross-linked prolamine)	18.2	12.0	3.7	4.0
IV (glutelin-like)	3.6	4.7	4.9	5.0
V (glutelin)	38.9	41.8	17.6	17.1
VI (residue)	4.5	7.5	5.1	4.0
(% protein in grain)	(9.3)	(11.5)	(11.2)	(13.4)

affect adversely the utilization of sorghum proteins (Jambunathan and Mertz 1973, Butler 1982). Brown sorghums with high tannin are resistant to bird depredation (Tipton et al. 1970), and have reduced preharvest germination (Harris and Burns 1970) and grain molding (Harris and Burns 1973). Sorghum cultivars with low tannin, but which are reported to be bird-resistant, have been identified (Subramanian et al. 1983). The common color of pearl millet grains is slate-gray, which is due to β-glycoflavanoids. Colored pearl millet grains do not contain tannins.

Food Quality and Consumer Acceptance

Several food products are made from sorghum and pearl millet (Vogel and Graham 1979, Subramanian and Jambunathan 1980). *Roti*, an unleavened bread, is the common product in India, while porridges are common in African countries. Based on information available, these food products can be divided into eight different groups (Rooney and Murty 1982):

1. unleavened bread (*roti, tortilla*);
2. leavened product (*injera, kisra, dosai*);
3. thick porridge (*tô, ugali, bogobe, sankati*);
4. thin porridge (*oji, edi, ambali*);
5. boiled product (*soru, nifro*);
6. steamed product (*couscous*);
7. snacks (popped sorghum, shallow and deep-fat fried products); and
8. beverages (*burkuto, busa, obushera*).

Processing of grains

Sorghum and pearl millet grains are processed by dehulling, dry milling of whole or dehulled-grain into flour and grits, soaking and fermentation of grain and flour, or roasting and puffing. Regional variation exists in the methods of processing and preparation of products. In most parts of Africa, the grains are dehulled before use by the traditional method of pounding the grain with a mortar and pestle, and the outer layers are separated from the endosperm by winnowing. The dehulled grain is further pounded into grits or flour before use in food preparation. Sorghum kernels with a highly corneous endosperm and a thick pericarp were easier to dehull by hand-pounding than those with a thin pericarp and a soft endosperm (Murty et al. 1984).

Pearl millet grain is difficult to dehull. Mechanical dehulling using laboratory mills was superior to traditional dehulling methods. The recovery was lower in traditional process and varied from 57 to 77% for the nine cultivars tested (Table 5). Seed-size variation within the same cultivar influences dehulling. Milling quality of grain and consequently flour qual-

Table 5. Dehulling quality of pearl millet grains.

			Dehulled grain recovery (%)								
			Hand-pounding			Barley pearler			TADD[2]		
	1000-seed	Grain hardness[1]	Dehulled			Dehulled			Dehulled		
Cultivar	mass (g)	(kg)	Grain	Brokens	Total	Grain	Brokens	Total	Grain	Brokens	Total
Mossi Local	9.9	3.6	77.2	10.3	87.5	86.0	1.6	87.6	87.2	1.2	88.4
WC-C75	7.8	3.4	75.3	11.9	87.2	86.8	0.7	87.5	86.2	1.4	87.6
SAD 448	8.5	3.0	72.0	12.0	84.0	86.6	0.7	87.3	85.5	0.6	86.1
CIVT II	9.6	3.4	71.7	14.3	86.0	89.5	0.6	90.1	88.3	0.6	88.9
DSA 74	13.6	3.8	70.3	12.7	83.0	83.2	1.5	84.7	85.8	2.5	88.3
Nigerian Composite	8.6	3.3	69.5	13.2	82.7	86.9	0.5	87.4	86.6	0.4	87.0
Fakiayabad	11.3	2.6	68.8	15.6	84.4	76.5	7.1	83.6	77.1	2.1	79.2
Togo	11.0	3.6	66.7	14.7	81.4	84.8	1.9	86.7	85.2	1.6	86.8
Souna	7.7	3.8	56.9	19.2	76.1	89.1	0.7	89.8	86.6	1.2	87.8
SE	±1.06	±0.13	±1.94	±0.87	±1.16	±1.30	±0.70	±0.70	±1.08	±0.23	±0.98

1. Grain hardness was measured as the kg-force required to break the grain using a Kiya hardness tester.
2. TADD: Tangential Abrasive Dehulling Device.

Table 6. Physicochemical characteristics and roti qualities of sorghum and pearl millet.

	Sorghum (n = 45)		Pearl millet (n = 20)		
	Range	Mean	Range	Mean	SE±[1]
Physical characteristics of flour					
Swelling capacity	5.4–8.0	6.5	1.5–6.5	3.0	0.19
Water-soluble flour fraction [mg (100 g)$^{-1}$]	19.4–35.4	26.4	23.7–63.6	39.0	0.86
Chemical characteristics of flour (%)					
Total protein	8.0–14.1	10.6	8.6–15.6	12.2	0.10
Water-soluble protein	0.3–0.9	0.6	0.7–1.3	1.0	0.01
Starch	62.6–73.3	68.7	62.8–70.2	66.6	2.36
Amylose	21.2–30.2	27.2	23.6–28.8	26.4	0.88
Water-soluble amylose	4.8–12.7	8.5	1.7–4.2	2.8	0.20
Total sugars	0.7–1.6	1.0	1.7–2.2	2.0	0.03
Fat	2.3–4.7	3.3	3.9–5.5	4.6	0.06
Taste panel evaluation		(*Roti* score 1 = poor, 4 = excellent)			
Color and appearance	1.6–3.8	2.5	1.7–4.0	2.9	
Texture	1.2–3.8	2.5	2.5–3.3	2.9	
Flavor	1.7–3.4	2.6	2.1–3.6	2.8	
Taste	1.0–3.3	2.6	1.8–3.6	2.7	
General acceptability	1.5–3.5	2.5	1.8–3.7	2.6	

1. SE – Standard error of estimation.

ity are affected by the structure and moisture content of the grain as well as the milling equipment and grinding technique that are employed.

Relationship beween food quality and physicochemical properties of the grain

Roti, *tô*, and *soru* were prepared in the laboratory from selected sorghum and millet cultivars under identical conditions using standard laboratory procedures. Protein, water-soluble protein, starch, amylose, and water-soluble amylose contents in flour showed wide variation (Table 6). The relationship between the physicochemical characters and *roti* quality indicated that the quantity of water-soluble flour fraction, water-soluble protein, amylose, and sugars jointly influence the *roti* quality of sorghum (Subramanian and Jambunathan 1982). *Tô* quality was also positively correlated with water-soluble amylose, and negatively with the swelling power of starch. Water-soluble fraction in wheat has been reported to play a role in producing a normal loaf of bread (Hoseney et al. 1969).

Grains having soft endosperm fail to produce an acceptable boiled-food product, *soru*. Swelling power of starch has shown significant relationship with *soru* quality. In sorghum, the swelling power of starch at 60°C showed a significant and positive relationship with *soru* quality and a negative relationship with gruel solids content. This may be due to the association of starch with such factors as protein in the grain. In pearl millet, the swelling power of starch at 70°C was significantly and negatively correlated with *soru* quality.

Digestibility of Sorghum

Whole-grain flour of sorghum, when cooked and fed to children, exhibited poor digestibility (Nicol and Phillips 1978, MacLean et al. 1981). However, the digestibility was improved considerably when sorghum was fed after processing into *nasha*, a thin, fermented baby-food from Sudan (Graham et al. 1986).

Industrial Uses

Sorghum and pearl millet may find increasing use in industrial processing into flour, grits, and other pro-

ducts that can be utilized for the production of various foods, using blends of maize, wheat, and other commodities (Rooney et al. 1980, Miche et al. 1977). In Sudan, an industrial process is being tested for pearling sorghum (Badi et al. 1980). In Mexico, significant quantities of sorghum are used in the brewing industry (Pyler and Thomas 1986). Sorghum beer is prepared on a large scale, using modern industrial methods in southern Africa. Although the use of sorghum in industry is secondary, the interest and potential in commercial utilization are increasing. This will increase the demand for a variety of industrially desirable, biotechnologically induced qualities of sorghums.

Sorghum stems have been used for the production of syrup. In Brazil alcohol is produced from sorghum plants and is mixed with petrol to the extent of 10-15%, thereby supplementing the energy source (Schaffert and Gourley 1982).

Conclusions

In the absence of alternative cereals, sorghum and pearl millet will continue to be the staple food grains in the semi-arid tropical regions. Efforts are under way to improve and stabilize the yields of these two crops in various countries. However, it is very important to pay attention to the nutritional, processing, and food-quality attributes while developing new and improved cultivars. Very little work has been done in the processing and food-quality areas as compared with that for wheat and rice. There is an immediate need to develop a concerted action plan, to carry out basic research in these areas so that any new knowledge gained may be put into practical use. Improvement of protein nutritional quality is possible by the application of biotechnological methods. Grain characteristics may be altered, or may even be introduced by biotechnology to improve the processing and utilization of grains. There is considered to be good scope to discover avenues of alternative uses of these two cereals for improved utilization. Good interaction among plant breeders, food technologists, biochemists, biotechnologists, socioeconomists, and entrepreneurs would prove very beneficial in achieving rapid progress in this area.

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Food Products Prepared in Africa from ICRISAT Mandate Crops and Scope for Improved Utilization

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Abstract

The ICRISAT mandate crops, except chickpea, are widely grown and enter into a wide range of traditional food products in Africa. The most common use of grain legumes is in stews, sauces, or thick soups that accompany starchy (cereal and root crop) dishes. Sometimes, either the immature pods are boiled and eaten or the mature seed is boiled along with cereals and other vegetables. They are also soaked and ground into a paste that is either deep-fried in oil or is steam-cooked. Groundnut is eaten raw, boiled, roasted or pounded into butter. The preparation of sorghum-and millet-based products is varied. The products are either alcoholic beverages using malted grain, or numerous nonalcoholic products prepared as thick pastes or thin porridges. Cereal preparations are either lactic-fermented or plain. Some lactic-fermented porridges are baked into thin unleavened bread. There exists an enormous scope for improved utilization of sorghum and millet in place of maize and also for standardization in processing. Composite flours from cereals and legumes can find increased uses in baked and fermented products.

Introduction

The extent of cultivation and production of ICRISAT mandate crops in Africa, like many other crops, is very poorly documented. Their contribution to food supply in Africa is enormous, however; but their full potential for alleviating food shortage and famine conditions in Africa is not being realized. The yields of cereals and pulses in Africa are much lower than those of other regions (Table 1). This may mean that Africa is lagging behind other regions in crop improvement and/or management or that the national policies are not promoting indigenous production.

Sorghum and Millet Production

The extent of cultivation of sorghum and millets is variable in different countries of Africa. A majority

Table 1. Production and yields of cereals, pulses, groundnut, and soybean in Africa, Asia, and the World[1].

	Africa		Asia		World	
Crop	Production[2]	Yield (kg ha^{-1})	Production[2]	Yield (kg ha^{-1})	Production[2]	Yield (kg ha^{-1})
Cereals	66 980	918	629 984	1 831	1 553 076	2 041
Pulses	5 103	433	31 057	710	51 873	715
Groundnut	5 522	891	10 407	932	19 228	1 016
Soybean	241	840	15 047	915	94 206	1 660

1. Source: Kay 1979.
2. '000 tonnes.

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of countries produce variable amounts of these cereals as subsistence crops (May and McLellan 1970, Kay 1979). In countries such as Sierra Leone, Tanzania, Nigeria, and Sudan, sorghum and/or millets constitute the major, if not the main, cereals (May and McLellan 1970, pp 225-376). In a number of other countries, such as Malawi, Zambia, Zimbabwe, and Kenya, maize has replaced sorghum and millets as the main cereal staples (May and McLellan 1970, pp 225-376). The domestic requirements of the main cereals in 1983 and 1989 in Kenya, and the rates of production growth necessary to achieve self-sufficiency, are shown in Table 2. An annual growth rate of 9% in cereals is needed to attain self-sufficiency by 1989, but it is unrealistic to rely exclusively on maize. Such rates were attainable during early periods of introduction and adaption of hybrid maize. Also the anticipated rates of 14.8-16.4% for wheat and rice would be difficult to achieve. The only hope for self-sufficiency then lies with sorghum and millets, which must progressively replace and augment the production of maize, rice, and wheat.

Groundnut, Pigeonpea, and Chickpea Production

Groundnut is widely produced in Africa and especially in the humid West African countries of Guinea, Gambia, Liberia, Ghana, and Nigeria. In Gambia and a few other countries, it is grown as a cash crop for oil production and to a lesser extent as a food crop. The production/yield of groundnut in Africa compares favorably with those in other regions (Table 1).

Production statistics of pigeonpea for various countries in Africa are scanty. Table 3 shows the major producing countries of pigeonpea as documented by Kay (1979). A more recent review has indicated that Kenya is the world's second-largest pigeonpea-growing country after India, with about 115 000 ha of pigeonpea, mainly as a field crop (Kimani 1986).

Very little chickpea is grown in sub-Saharan Africa. Most of that grown in the continent is found in Egypt, Sudan, Ethiopia, and Morocco (Kay 1979). It has been successfully grown, however, in parts of Kenya on an experimental basis (Kay 1979).

Table 3. Major pigeonpea-producing countries: average annual production (tonnes).[1]

Country	1965-69	1970-74	1975
India	662 000	740 000[2]	818 000[2]
Uganda	26 800	38 800	40 000
Dominican Republic	21 600	27 800	29 000
Burma	22 600	24 800	24 000

1. Source: Aykroyd and Doughty 1982.
2. Unofficial data.

Table 2. Domestic requirements of the main cereals in 1983 and 1989 and the rates of growth of production necessary to achieve self-sufficiency in Kenya.[1]

	Estimated production[2]	Estimated domestic requirement[2]		Annual production growth % required for self-sufficiency	
	1980	1983[3]	1989	1980-83	1980-89
Maize					
a. 1980 production as base	1 620	2 777	3 514	19.7	9.0
b. 1976 production as base	2 264[4]	2 777	3 514	7.0	4.9
c. Mean 1976 and 1980 as base	1 942[4]	2 777	3 514	12.7	6.8
Wheat flour	142	292	493	27.2	14.8
Sorghum/millets	369	445	563	6.4	4.8
Rice	23	66	90	42.1	16.4

1. Source: Corine and Dendy 1984.
2. '000 tonnes.
3. Figures exclude production required to rebuild strategic reserve.
4. Hypothetical level of production.

Food Products from Sorghum and Millets

The starchy diet staples in most African countries are prepared from cereals, roots, tubers, or plantains. The products through which sorghum and millets are utilized are fairly similar, although they are referred to by different local names. These products can generally be classified into four groups, namely porridges, dumplings, baked products, and alcoholic beverages. The preparation of these products is described below, and often it is prolonged and complex.

Porridges

A list of porridges prepared in some African countries is given in Table 4. These products have more than 90% water; the majority are sour, and are prepared by either wet- or dry-milling processes (Muller 1980, p.541). In most of these products, the major ingredient is maize, perhaps due to its ready availability. Sorghum and millets are, however, used to replace maize in the production of these acidic gruels (Mbugua et al. 1983, pp 1-23). Sorghum and millets are considered nutritionally better than maize (Latham 1979). Millet flour is particularly preferred in the preparation of porridges for weaning children. A smooth texture in all the products is considered essential. Thus for *ogi*, wet-sieving is vital to remove coarse materials. For *uji*, the use of a very fine flour to reproduce the fine texture associated with the traditionally produced *uji* is essential (Mbugua 1985). Traditional *uji* was prepared by wet-milling plus the removal of over-tails through decantation. Fermentation is usually spontaneous and basically involves *Lactobacillus* sp and yeasts (Fields et al. 1981, Mbugua et al. 1983, pp 1-23). Such fermentation has been shown to improve the nutritive value of the product and to increase the availability of lysine (Hamad and Fields 1979). Not all porridges are fermented, however.

Dumplings

Kenkey in Ghana is fermented dough, cooked into a thick porridge, moulded into balls and wrapped in dried maize husks or plantain leaves and then steamed. It is mainly made from maize, although sorghum and millets are just as suitable (Odunfa 1985, p.155; Steinkraus 1983, pp 220-226; 1985; Amoa 1985).

Baked products

The two very important acid-fermented baked breads or pancakes from either sorghum or millets are *kisra* in Sudan and *injera* in Ethiopia. These constitute staple diets in these countries. Teff, cornflour, barley, millet, and sorghum are used for *injera* preparation. The process involves mixing flours with water and starter (*irsho*), incubation at room temperature for 17–25 or 48–72 hours, decanting the batter, and steam-baking in a thin layer for 2–3 min. *Kisra* is prepared from sorghum flour. It is estimated that 18 000 27 000 t of sorghum are consumed in the form of *kisra* in Sudan, and individuals consume about 200–500 g of *kisra* per meal (Steinkraus 1985). A refreshing sour drink resembling Coca-Cola® called *hulu mur* is prepared by soaking *kisra* in water (Odunfa 1985).

Alcoholic beverages

In Africa, sorghum and millets are widely utilized for the production of alcoholic beverages and, in

Table 4. List of porridges prepared in some African countries.

Product name	Country	Substrate	Microorganism involved
Koko	Ghana	Maize, sorghum or millets	*Lactobacillus* sp and yeasts
Uji (fermented)	Eastern Africa	"	"
Mahewu (*magou*)	Southern Africa	"	*L. delbrueckii* *L. bulgaricus* and yeasts
Ogi	Nigeria, Benin	"	*Lactobacillus* sp and yeasts
Ogi-baba	West Africa	Sorghum	*Lactobacillus* sp *Leuconostoc* sp and yeasts

Table 5. List of some fermented alcoholic beverages in Africa.

Product	Substrate	Culture	Country
Sorghum beer	Kaffir corn or sorghum	*Lactobacillus* and yeast	South Africa
Burukutu	Guinea corn or sorghum	*Lactobacillus*, molds and yeasts	Nigeria
Pito	Sorghum and millets	*Lactobacillus*, molds and yeasts	Nigeria and Ghana
Busaa	Maize, millets, and sorghum	*Lactobacillus*and yeasts	Eastern Africa
Merissa	Sorghum	*Lactobacillus* acetic acid bacteria and yeast	Sudan
Bouza	Wheat or maize	Unknown	Egypt
Talla	Sorghum	Unknown	Ethiopia
Togwa	Sorghum	Unknown	Tanzania
Chang'aa (distillate)	Busaa	(As *busaa* above)	Kenya
Kachasu (distillate)	Sorghum and millets	Unknown	Malawi
Chibuku	Maize, sorghum, and millets	Yeasts	Eastern Africa

particular, opaque sour beers. Table 5 gives a list of fermented alcoholic beverages of Africa. The preparation of these beverages involves lactic fermentation, followed by alcoholic fermentation (Nout 1980). Mbugua (1985) has shown that these products are more nutritious than the European-type lager beers.

Food Products from Groundnut, Pigeonpea, and Chickpea

Processing of legume-based products for consumption is simpler than for cereals. In general it involves boiling or steaming the whole pods or grains with, or without, prior soaking, and then direct consumption, or after conversion into a sauce or puree. In some countries, some grain legumes are eaten in the form of green vegetables (leaves or green pods). In others, the legume grain is soaked, pounded, and fried, or boiled, and eaten as a puree.

Groundnut

Groundnut is mainly produced for oil in the major producing countries. The most common form of utilization of groundnut is as a snack after roasting. In Liberia, press cake has been used in the preparation of weaning food through government-sponsored mother-and-child health centers (May and McLellan 1970). In Gambia, it is used for making a sauce to be eaten with rice. In other countries, eating of raw groundnut has been reported (May and McLellan 1970, pp 225-376). In Nigeria, groundnut flour is made into paste, or peanut butter, for soup or stew preparations (Aykroyd and Doughty 1982).

Pigeonpea

The utilization of pigeonpea in Africa is similar to that for common beans (*Phaseolus* sp). The grain is soaked, pounded, and fried, or boiled to make puree or sauce (Kay 1979). In Kenya, it is reportedly ground into flour and made into sauces that are consumed along with starchy dishes (May and McLellan 1970, pp 225-376). It can also be boiled whole together with corn, to prepare such dishes as *makande* in Tanzania and *githeri* in Kenya (May and McLellan 1970, Hoorweg and Niemeyer 1980). The green pods of pigeonpea are also used as a vegetable (Kimani 1986).

Chickpea

The utilization of chickpea is similar to that of other grain legumes. In India, 75% of chickpea is consumed as *dhal* (Kay 1979). Elsewhere, including in Africa, it is soaked and boiled, as well as parched in hot pans and eaten as a snack, like groundnut (Kay 1979).

Scope for Improved Utilization

Sorghum and millets

Sorghum and millets have a great potential to alleviate food shortages in Africa. This can happen only after they have entered the mainstream of the monetary economy rather than remaining outside it as subsistence crops, as they now are in most parts of Africa. Emphasis in research must shift from maize, rice, and wheat to sorghum and millets; and also the national policies must be supportive of indigenous coarse-grain production and processing.

Improved utilization of sorghum and millet can occur when they replace wheat, rice, and maize in the food products prepared from the latter. Improved processing techniques, such as milling, will enable sorghum and millets to replace maize, wheat, or rice. Dehulled sorghum has been introduced in Kenya to be prepared and eaten as rice. The use of composite flours, which include sorghum and millets in the production of bread, cakes, and biscuits, is certainly an area that needs to be critically examined. The majority of other cereal products mentioned (such as *kenkey* in Ghana, *ogi* in Nigeria, *kocho* bread and *injera* in Ethiopia, *ugali* in eastern Africa, *agidi* in Ghana, and many others), which are mainly based on maize, need to be investigated, and the possibilities of replacing maize with sorghum or millets examined. In any case, these products are occasionally made from sorghum or millets. The production of beers from sorghum and millets should be encouraged, and research intensified on standardization and improvement of the products.

Groundnut, pigeonpea, and chickpea

The only extensive preparation method for legumes is the preparation of *iru* in Nigeria as a condiment from the African locust bean (*Parkia biglobosa*) (Odunfa 1982, Steinkraus 1985). There exists a wide option among processing techniques such as dehulling, soaking, germination and sprouting, canning and cooking, roasting and frying, and fermentation, which could open up improved legume-based products and menus based on groundnut, pigeonpea, or chickpea. Fermentation products, such as *tempe* and *ontjom*, are known to be meat analogues and would most likely be acceptable to African consumers in the semi-arid and arid regions (Steinkraus 1983, pp 220-226). *Ontjom* is prepared from groundnut press cake by fermentation using *Neurospora* sp and *Rhizopus* sp (Steinkraus 1983, 1985). Acceptable *tempe* has been prepared from chickpea, pigeonpea, and common bean (Odunfa 1985, p.155, Robinson and Kao 1977, Gomez and Kothary 1979). Preparation of acceptable food condiments, using especially groundnut in place of the African locust bean, can be assessed. Fermentation of legumes is known to improve digestibility and nutritive value and eliminate antinutritional factors, apart from improving flavor and reducing the cooking time.

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The Role of Polyphenols in the Utilization of ICRISAT-mandated Grain Crops and Applications of Biotechnology for Improved Utilization

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Abstract

The productivity of sorghum is to some extent dependent upon chemicals in the plant tissue that confer defense mechanisms against herbivores, fungal pathogens, and parasitic weeds. However, these defense chemicals limit the utilization of sorghum as human food. Cultivars that lack these defense mechanisms are often too vulnerable to yield losses for their widespread cultivation. An important group of defense chemicals in sorghum is the polyphenols, particularly flavonoids and their oligomers, the condensed tannins. Out of the rich variety of polyphenols produced by sorghum, individual components responsible for particular types of resistance are being identified. It is now possible to screen sorghums for optimum polyphenol composition and to improve the nutritional value without losing pest resistance. Methods are being developed to generate and propagate, in tissue culture, polyphenol-rich cultivars, and variants in respect of polyphenol metabolism that are suitable for wider cultivation.

Introduction

Polyphenols are compounds having two or more phenolic hydroxyl groups substituted on aromatic ring structures. Animals cannot synthesize polyphenols from nonaromatic precursors, but plants synthesize a wide variety of polyphenols. The major groups include flavonoids, tannins, and lignin. Phenolic acids are usually considered with the polyphenols although several of them have only a single phenolic hydroxyl group.

Plant polyphenols are not directly involved in metabolic pathways for growth and reproduction, and are therefore considered to be 'secondary' metabolites. Most polyphenols have no well-established function, but many are considered to play a role in defending the plant against the onslaught of herbivores, pathogens, and competitors. At least partly because of their lack of a clearly defined metabolic role, as well as the difficulty of isolating and characterizing them, relatively little is known about plant polyphenols, compared with other major components of plants.

Sorghum is capable of producing larger amounts of polyphenols than most other plants (Butler, in press). In addition to the condensed tannins for which its seeds are well known, sorghum has the capacity to produce a rich variety of flavonoids, some quite unusual and most not identified (Butler, in press). Millet would seem to be similarly dependent on chemical defenses, but millet has received less attention than sorghum in this respect. Even less is known about the polyphenols of the other crops of ICRISAT's mandate. The methodology developed and the results obtained from studies of sorghum polyphenols should be applicable to the other crops of ICRISAT's mandate.

Owing to the harmful effects that at least some polyphenols can produce when eaten, the capacity to synthesize large amounts of polyphenols tends to be eliminated during the domestication of the plant (Harborne 1982). Their absence improves the food

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value, but may render the plant vulnerable to herbivores, pathogens, or competitors to which its undomesticated ancestors were resistant. In the case of sorghum, cultivars containing low levels of polyphenols have been developed. They have desirable characteristics for use as food or feed, but they cannot be reliably cultivated in some areas because of vulnerability to various pests. Fortunately, even after long domestication, sorghum cultivars that retain considerable polyphenol-synthesizing defensive capability are still available. These cultivars are grown in areas where the incidence of pests is severe.

It is the task of the plant biotechnologist to devise appropriate methodologies, by selective genetic modification of the plant or, by innovative processing, to minimize or eliminate the antinutritional features of the polyphenols of crop plants, including those in ICRISAT's mandate, without compromising the resistance of the plants. Success in this endeavor would greatly expand the areas of Africa where the high-yielding and nutritionally acceptable sorghum could be produced. Similar benefits should be possible with other crops.

Agronomic Effects of Plant Polyphenols

Production constraints of sorghum for which polyphenols contribute to resistance include grain losses due to predatory birds (McMillian et al. 1972), grain molding (Harris and Burns 1973), preharvest seed germination (Harris and Burns 1970), insect feeding on seedlings (Woodhead and Cooper-Driver 1979, Dreyer et al. 1981), and infection of seedlings by fungal pathogens and nonpathogens (Nicholson et al., in press).

We have recently found that the parasitic weed *Striga*, which is one of the major production constraints of sorghum and other crops in parts of Africa, utilizes a unique polyphenol exuded from sorghum roots as a germination signal. This polyphenol is the dihydroquinone of sorgoleone, a hydrophobic quinone that we have identified as the major component of the oily root exudate of sorghum root hairs (Chang et al. 1986). This chemical is implicated in signaling germination of *Striga* seeds. It has very low solubility in water and is chemically very unstable. Both these characteristics ensure the germination of any *Striga* seeds in close proximity to the host, and leave others dormant for subsequent seasons. Another agronomically beneficial effect of sorgoleone is inhibition of root elongation of certain plant competitors (Netzly and Butler 1986). The parasitic *Striga* apparently has adapted to the allelopathic defense chemical, utilizing its unstable precursor as a host-specific recognition signal. Strategies for controlling *Striga* by utilizing this polyphenol and other newly discovered chemical signals between *Striga* and its host are being developed.

Utilization Constraints for which Polyphenols are Responsible

In some cultivars of sorghum (Scheuring et al. 1982) and millet (Reichert et al. 1980, Reddy et al. 1986), polyphenols contribute colors and/or tastes that are generally perceived to diminish palatability. With respect to nutritional value, condensed tannins of sorghum seed (absent in millet) are often associated with diminished weight gains and feed efficiencies of animals on experimental diets (Butler et al. 1986). It is usually assumed that the antinutritional effects associated with high-tannin sorghums are due to inhibition of digestion by the dietary tannin, but there is little solid evidence for this mechanism. Evidence is accumulating that the antinutritional effects may largely be due to inhibition of metabolic utilization of digested and absorbed foodstuffs (Mehansho et al. 1987). Some of the antinutritional effects of high-tannin sorghums do not appear to be due to the polymeric tannin molecules but to associated low molecular weight flavonoids more readily absorbed from the intestine (Mehansho et al. 1987).

From our recent work it is clear that polyphenols in the diet would have much more severe antinutritional effects if it were not for specific proline-rich, tannin-binding proteins present in the saliva of most animals that consume polyphenol-containing foods (Mehansho et al. 1987). These specialized proteins form strong complexes with dietary polyphenols as soon as they enter the digestive tract, diminishing but not eliminating their antinutritional effects.

Applications of Biotechnology to these Constraints

The problem of antinutritional polyphenols in sorghum cannot be solved solely by developing cultivars low in total polyphenols. Such cultivars are available, but are too vulnerable to pests to be produced in some areas. In our laboratory we are attempting to identify specific polyphenols that are responsible for, or contribute to, particular agro-

nomic advantages or nutritional disadvantages. We would also like to determine the biochemical mechanisms of these polyphenol effects, and learn as much as possible about the biosynthesis of these specific polyphenols and how it is regulated. It may then be possible to use modern biotechnological methods to control or regulate the production of individual polyphenols, or groups of polyphenols, that are especially beneficial to the plant or especially harmful in the diet.

This approach can be successful only if different polyphenols are responsible for the beneficial agronomic effects and the harmful nutritional effects. Our early results suggest that this is the case, that the polyphenols responsible for antinutritional effects might be eliminated without leaving the crop too vulnerable. For example, the polymeric condensed tannins characteristic of the seed of 'bird-resistant' sorghums are virtually absent from the seed at the milk stage, when birds tend to do most of their damage. The 'bird resistance' seems to be due to shorter, lower-molecular-weight precursors of the polymers found in mature seed (Butler 1982). If these are the polymers that are largely responsible for the antinutritional effects of high-tannin sorghums, then inhibition or elimination of the enzyme that polymerizes the precursors should improve the nutritional value of the grain without leaving it vulnerable to birds. We have recently identified polyphenol oxidase as being at least partially responsible for the polymerization and we are seeking means of controlling its activity. Identification of a particularly crucial enzyme, especially one that is already available so that antibodies could be generated and used to identify in vitro translation products, open obvious opportunities to apply molecular biology to the problem.

Other examples of the association of specific polyphenols with particular agronomic benefits are the flavan-4-ols. These are monomeric flavonoids that are extremely rare. Sorghum is the only cereal (actually the only monocot) from which they have so far been positively identified. One of them, luteoforol, was reported from sorghum seeds by Bate-Smith (1969). We found that apiforol, which has one less hydroxyl than luteoforol, is relatively abundant and is present in leaf tissue as well as seed (Watterson and Butler 1983). Flavan-4-ols, which are inherited independently of condensed tannins (Watterson and Butler 1983), have no known antinutritional effects. Although our early work suggested a relationship between flavan-4-ol content and bird resistance (Subramanian et al. 1983), it was recently found that resistance to grain molding, rather than to birds, is associated with high flavan-4-ol content (Jambunathan et al. 1986). Even more recent studies, some with ICRISAT collaborators, have revealed that it is probably not the flavan-4-ols themselves that are responsibile for resistance to grain molds, but rather a series of flavanoids that are metabolically and chemically closely related to the flavan-4-ols. These components may be flavanones. In addition to polyphenols associated with agronomic benefits, we are attempting to identify polyphenol components responsible for antinutritional effects. The low-molecular-weight flavonoid fraction from quebracho was even more effective as an antinutritional agent in rat diets than was the high-molecular-weight polymer fraction (Mehansho et al. 1987). This experiment has not yet been done with sorghum, but preliminary observations suggest profound effects of high-tannin sorghums on metabolic parameters such as urinary volume. Reports of significant effects on phenolic detoxification enzymes (Sell and Rogler 1983) suggest that phenolic components of sorghum are absorbed from the digestive tract and strongly affect metabolism.

Our approach to applying biotechnology to the improvement of sorghum utilization begins with characterizing as completely as possible its individual polyphenol components with respect to their beneficial or harmful roles. This is presently one of our major emphases. Once particularly significant polyphenol components have been identified, we develop convenient assays for them so that breeders can utilize these results in plant-breeding programs. We are just beginning to be able to survey sorghum cultivars for the optimum complement of polyphenol components.

Our current approach to active manipulation of sorghum in order to optimize its polyphenol composition involves tissue culture of high-tannin genotypes. Most high-tannin sorghums grown as callus tissue produce massive amounts of polyphenols in intracellular membrane-bound inclusion bodies (Oberthur et al. 1983). Our observations suggest that these inclusion bodies eventually rupture and spill out their polyphenolic contents, which appear to be toxic to the cells that produced them. Out of the dying culture we rescue cells that can be cultured further, presumably because they have a diminished capacity to produce toxic polyphenols, or because they are more resistant to them. These cultures are of considerable interest with respect to their possible resistance to birds, molds, and other pests and the nutritional value. In 1986 we have learned how to

regenerate these calli into plantlets (Cai et al. 1987). The most difficult step is not regeneration but development of a viable root system. We are now evaluating, in the field, plants produced from the seed of hundreds of regenerated plants. This tissue-culture system appears to have advantages for isolation of variants that may have useful characteristics with respect to polyphenol production.

We are also interested in the possibility of modifying sorghum's complement of polyphenols by direct manipulation of its genome. Flavonoid metabolism in sorghum appears to differ considerably from other plants in that the unusual hydroxylation at the carbon 4, instead of carbon 3, seems to predominate in the monomeric flavonoids but not in the polymeric ones. A great deal of characterization of the enzymes of flavonoid biosynthesis must be carried out before attempting to manipulate the genome directly.

Processing Technology for Polyphenol-rich Sorghums

Instead of modifying the sorghum plant so that it does not produce antinutritional polyphenols, considerable effort has gone into modifying the polyphenol-rich sorghum grain to eliminate or inactivate the polyphenols. The widely practiced process of decortication by pounding the grain in a crude mortar and pestle removes tannin rather effectively but nutrients are also lost (Chibber et al. 1978). Seeking a less labor-intensive and nutrient-depleting method for improving the nutritional value of high-tannin sorghum, we found that moistening the grain with aqueous alkalies several hours before utilization effectively eliminates the antinutritional effects of tannin. The treatment has no effect on the nutritional value of tannin-free sorghum (Price et al. 1979). Ammonia is the most effective alkali, probably because it penetrates the grain more rapidly than other alkalies. Nothing is removed from the grain by the treatment; presumably the polyphenols undergo oxidative polymerization into nutritionally inactive forms in the alkaline environment. The detoxification process requires water, for anhydrous ammonia is far less effective than dilute aqueous ammonia. The treatment is much more effective on whole grain than on ground grain. Apparently the deleterious interaction of the polyphenols with other components of the seed is enhanced by grinding the grain. The alkaline treatment darkens the color of most sorghum cultivars. Wild birds tend to choose ammoniated high-tannin sorghum over the untreated form of the same cultivar. These results have been summarized (Butler, in press) elsewhere.

Imbibition of high-tannin sorghum grain with aqueous alkalies is an important step in the traditional processing of high-tannin sorghums of eastern Africa. In Rwanda, Burundi, and some districts of Uganda most sorghum produced is very high in tannin. The predominant sorghum product of this region is a beer that contains all the grain. This product is fed to children before it ferments into beer. An early step in the production of traditional beer involves mixing the high-tannin grain with wood ashes, wetting the mixture and leaving it overnight. We found that this processing step with aqueous alkalies extracted from the ashes very effectively detoxifies the antinutritional effects of the high-tannin sorghum. Clearly, our newly discovered detoxification technology was merely the chemical rationalization of a traditional methodology.

Musalac®, a popular nontraditional food developed in Burundi for mothers and infants, contains 35% sorghum, in addition to maize, soy protein, dry milk solids, and sugar. The sorghum utilized is the locally produced type, high in tannin and not treated with wood ash in the traditional manner as is done for making beer. Our analyses indicated that the level of tannin in the final product was sufficiently high to produce significant antinutritional effects. This is particularly unfortunate in a food specially recommended for nutritionally vulnerable segments of the population. Following my recommendation, Musalac® has been prepared, on an experimental basis, from sorghum detoxified by the traditional wood-ash treatment. Tests are under way to determine if the anticipated improvement in its nutritional quality warrants the extra processing step.

I hesitated to include this alkali-detoxification story because it does not involve the use of advanced biotechnological methods, but rather the rational application of time-honored traditional methodology. I decided to include it because it contains an important message for the biotechnologist seeking to improve food quality and enhance utilization of traditional crops. We would do well to carefully examine traditional production and utilization methods. Although the traditional farmer or consumer may not be able to articulate a scientifically valid rationalization of why he does what he does, I have come to believe from this, and several other examples, that we have much to learn from methods maintained through many generations of sorghum production and utilization. Combining modern bio-

technology with traditional methodology may prove to be a powerful means of improving sorghum.

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