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BIOTECHNOLOGY AND ITS APPLICATION IN AGRICULTURE

[1]J. E. IRVINE and [2]P. H. MOORE

[1]*Texas Agricultural Experiment Station, Texas A&M University, Weslaco, Texas, USA*
[2]*Agricultural Research Service, USDA, HSPA Experiment Station, Aiea, Hawaii*

Abstract

The man to first tame an animal and the woman to first plant a seed were the biotechnologists of past millennia. Recent rapid advances have dimmed the lustre of older achievements and have led to some excesses in expectations. Nevertheless, new techniques grouped under the banner of biotechnology do promise increases in accuracy, efficiency and productivity. Toxins are detected at new low levels, cryptic diseases can be identified, mammals can be genetically duplicated, animal genes can be expressed in plants, and we are mapping the human genome. Sugarcane can remain in pre-Mendelian times or, with effort and investment, emerge into the 21st century in step with the rest of agriculture.

Introduction

The term 'biotechnology' has been used so often and with such diverse meanings that a strong negative reaction is sometimes encountered, especially in the traditional reaches of agriculture. This reaction and its opposite, unbounded enthusiasm, tend to obscure a basic issue. Biotechnology is not new. The man to first tame an animal and the woman to first plant a seed were the biotechnologists of past millennia. Van Helmont and Robert Boyle, plant physiologists before the term was coined, were of the same ilk and their work leading to the discovery of fertilizer was surely biotechnology. More recently, Gregor Mendel's work with the genes of the garden pea gave the world a highly simplified introduction to the mechanism of heredity. It seems only a moment ago that Watson and Crick identified the structure of deoxyribonucleic acid (DNA) and opened the door to the biotechnical era, which, if reclassification is really needed, should more appropriately be called the 'era of molecular biology'.

Since the introduction of molecular biology less than 40 years ago, progress continues on an even more rapid scale. In the '60s, we learned that the fundamental function of genes is the production of peptides and proteins, frequently enzymes; one gene, one enzyme. In the '70s, genes were being transferred from one simple organism to another, by the '80s genes were being transferred in higher organisms and being made to work. USDA scientists co-mingled sunflower and bean genes in callus culture producing tissue that expressed characteristics of both. The 'sunbean' never developed into a plant, however. Others transferred the gene for the firefly enzyme, luciferase, into a petunia which actually glowed a little in the dark.

Predictably, work with animals progressed more rapidly, driven largely by the availability of grant funds, venture capital and the potential benefits for human health. Animal (including human) genes were transferred to micro-organisms to produce marketable commodities like insulin, interferons, erythropoietin, monoclonal antibodies, growth hormones and colony stimulating factors. Enormous progress was made in understanding the mechanisms of gene function and expression. An early discovery was the gene for sickle-cell anemia, a hereditary disease whose mechanism was identified in the '50s, detailed in the '60s and whose gene was identified in the late '70s. By the end of the '80s, the medical profession was offering diagnostic tests based on DNA that gave 99% accuracy in the identification of the gene.

The early success with sickle-cell anemia set the stage for rapid advances in medical molecular biology. Animals, and to some extent plants, serve as experimental organisms for medical science, providing both practical and intellectual benefits to agriculture. Much of the large investment in medical technology will result in techniques that improve efficiency in all biology and will therefore directly benefit agriculture.

Animal and plant cells have long been subjected to artificial culture. Veterinary science now has the ability to duplicate or quadruplicate animals on a routine basis. A fertilized egg from a highly productive cow mated with a prize bull can be allowed to progress in culture to the four-cell stage, divided, and one cell returned to the mother or a surrogate, and the remaining three held in storage until the first is proven in performance. No more appreciated by the public than artificial insemination, this technique is now used all over the world to improve herds.

The idea of mapping genes is as old as the realisation that genes reside in chromosomes. For the first half of this century, genes were recognised in cases of mutations where phenotypic expression was visible. Non-mutant genes were left to speculation. Cytogenetic mapping was possible only where chromosomes were few and morphologically distinct. Genetic mapping was possible only for haploids or diploids and only for traits encoded by single genes. *Neurospora*, fruit flies and maize are favorites. In 1968, molecular biologists discovered that DNA could be cut into pieces with various types of restriction enzymes. One enzyme would cut the DNA only at one specific sequence of base pairs, and others at other sequences. The fragments of DNA could be subjected to electrophoresis (migration in an electric current) and separated by size. If DNA from a particular strain of yeast, for example, was cut by a particular restriction enzyme and separated by electrophoresis repeatedly, the fragments always migrated in the same order; 10 samples on the same gel would produce identical patterns. However, if the same enzyme was used to cut the DNA of another strain, a few differences would appear when compared with the banding pattern of the first. This was found to be due to small but appreciable differences in the size of certain fragments. These were named restriction fragment length polymorphisms (RFLPs). In Edinburgh, Southern (1975) devised a technique that transferred DNA from the electrophoresis gel to a nitrocellulose membrane where the RFLPs could be repeatedly detected by various radioactive nucleic acid probes.

RFLPs were quickly seized as potential markers and a milestone paper (Botstein *et al.*, 1980) appeared with the radical proposal that RFLPs could be used to map human genes. The challenge was accepted and molecular biologists everywhere began to search for markers and genes. Table 1 shows some of the more notorious diseases that were linked to markers and placed with varying degrees of accuracy on the map of the human genome. Whereas much of the early work was done with funding only from medical philanthro-

pies in the US, France, Britain and other countries, investment by various governments has begun. The US has promised $US 200 million a year for 15 years. Unofficial estimates predict progress in the human genome to be such that mapping would be complete in six to 10 years, and sequencing each gene would be 50% complete by then. Donnis-Keller *et al.* (1987) reported an RFLP map with markers placed at an average of one for every 10% crossover (10 centimorgans). About four thousand human genes have been cloned, mapped or sequenced. This represents 4% of the estimated 100 000 genes mapped after 10 years of effort. However, with new infusions of funds, international co-operation, automated RFLP technology, application of new techniques (PCR, AP-PCR, RAPDs) and future discoveries, the 15 year goal may even be conservative.

Table 1

Human diseases and genetic map locations

Disorder	Chromosome	Date
Huntington's chorea	4 telomere	1987
Familial adenomatous polyposis	5 deletions	1987
Schizophrenia	5 (polygenic)	1987
Juvenile diabetes	6	1988
Cystic fibrosis	7	1985
Friedreichs' ataxia	9	1988
Type II alcoholism	11	1987
Manic depression	11 short arm (poly)	1987
Sickle-cell anemia	11	1983
Mental retardation	13 deletion (poly)	1986
Retinoblastoma	13 deletions, linked to esterase D	1980
Cancer accelerator	17 deletions (poly)	1987
Cancer accelerator	18 deletions (poly)	1988
Alzheimer's disease	21 long arm (poly)	1987
Duchenne's muscular dystrophy	X top, deletions	1985
Manic depression	X linked to G6PD & color blindness	1986
Mental retardation	X fragile X tip	1969

Data from:

Bishop, JE, and Waldholz, M. *Genome.* Simon & Shuster. New York 1990. 52 pp.
Wingerson, L. *Mapping our genes: the genome project and the future of medicine.* Dutton, New York. 1990. 338 pp.

These advances are having collateral effects on plant breeders. Many organisations have already developed RFLP maps; tomato, potato, maize, sorghum, rice, wheat, oats and barley are crops that have received emphasis. The tomato has been sufficiently mapped so that it is now possible to relate RFLP markers to quantitative traits like sugar content (Paterson *et al.*, 1989). Tomato breeders now have maps and statistical programmes that allow the selection of parents by statistical analysis of their gene maps, which provide a probability figure predicting the sugar content of fruit from progeny of a specific cross. Maize breeders, who have been integrating phenotypic, isozyme and RFLP markers to produce the most detailed crop map, are using it in conventional breeding programmes and in planning improvements in seed protein quality.

Although it was fairly easy to develop a potato map once the closely related tomato was mapped, sugarcane breeders are not as fortunate. Whereas tomatoes, potatoes and maize have relatively few chromosomes, the genus *Saccharum* may have up to 192 (D. Burner, personal communication) and no commercial variety today has less than 100. The cytological phenomena of high polyploidy and aneuploidy, a moderately large DNA content (five times that of a dandelion but half that of wheat) and irregular meiosis present a combination of potential problems not encountered in other crops.

Interest turned to action in the physiology section at the Hawaiian Sugar Planters' Experiment Station, when scientists began to map the cytoplasmic genome of sugarcane. This was the result of the observation by veteran breeder Albert Mangelsdorf, who noted that almost all modern commercial varieties, despite being complex interspecific hybrids, were traceable to the two *S. officinarum* females, one Javanese and one Indian, that were founders of all modern varieties. Concern was manifest because of the possibility of a restricted cytoplasmic gene pool and vulnerability to a defect (like the maize T-cytoplasm susceptibility to southern corn blight). Progress was slow because of difficulties in purifying cytoplasmic DNA, and work in the lab shifted to isozyme markers and the exploration of possibilities with RFLPs. Exploratory contacts were made with Native Plant Industries and with Stephen Tanksley at Cornell University on the prospects of establishing an RFLP map of the nuclear genome.

At the insistence of Werther Annicchino, president of Copersucar, serious discussions about the need to develop molecular genetics began in Brazil in 1988. Following a suggestion by N.W. Simmonds, plans were developed to map the sugarcane genome (all of the genes in the sugarcane breeding pool). These culminated in an agreement in which Copersucar and the Hawaiian Sugar Planters' Association contracted Drs Tanksley and Sorrells of the Plant Breeding department, Cornell University, NY, to map the sugarcane genome.

The work, now in its third year, has been carried out largely by visiting scientists from Brazil and Hawaii. Wu *et al.* (1991) have demonstrated the technical feasibility of cane mapping and Burnquist (1991) produced a chart of the first eight linkage groups. Because of the exploratory nature of the work, progress has been slower than hoped for, with fewer than 40 markers proving useful. Da Silva, a Copersucar breeder, continues the work at Cornell.

To make progress more rapid, an international group met in Beltsville, Maryland, USA, on 15 March 1991 to consider a research proposal from the California Institute of Biological Sciences involving newly developed technology that might be applied to cane mapping. The South African Sugar Association, along with organisations from Brazil, Hawaii and Louisiana in the United States, agreed to fund a two year project. Additionally, the group decided on a formal agreement under which information, probes and data banks would be freely interchanged among all parties involved. This agreement was formally signed in Brazil on 9 May 1991 and provides a welcome change of attitude, promising co-operation in an area of science stained with secrecy and legal disputes.

Another area of plant molecular genetics involves the movement of genes from one organism to another and their incorporation into the genome of the receptor. Because the introduction and incorporation of an exotic gene results in permanent change, the organism is said to be transformed. Although the flashes made by the sunbean and the glowing petunia are fading, other plant transformations have been achieved that may have practical application. Genes from bacteria that convey herbicide resistance have been used in the transformation of a number of plants, and some of these have progressed to field testing (Table 2).

Table 2
Permits issued for environmental testing of transformed plants in the United States.*

Character	Organisation	Year
	Alfalfa	
AMV resistance	Pioneer	1989
Glufosinate tolerance	Northrup King	1989
	Cantaloupe	
CMV, PRV	Upjohn	1990
CMV, PRV, WMV-2, ZYMV	Upjohn	1990 (pending)
	Corn	
Clavibacter xyli cynodontis	Crops Genetics Int.	1987, '88, '89
expressing BT endotoxin	"	1990 (pending)
Marker genes	BioTechnica	1990 (pending)
	Cucumber	
CMV	NY State Expt. Stat.	1989, '90
	Cotton	
Glyphosate tolerance	Monsanto	1989, '90 (pending)
Bromoxynil tolerance	Calgene	1989, '90 (pending)
BT resistance	Agracetus	1989
BT resistance	Monsanto	1990 (pending)
BT resistance	Calgene	1990 (pending)
BT resistance	Northrup King	1990
Sulfonylurea tolerance	Du Pont	1990
	Poplar	
CAT gene	Iowa State Univ.	1989
	Potato	
X, Y, leaf roll virus	Monsanto	1989, '90 (pending)
BT resistance	Monsanto	1989 Marker gene
	USDA/ARS	1990
	Soybean	
Glyphosate tolerance	Monsanto	1989, '90
	Squash	
CMV, PRV	Upjohn	1990
CMV, PRV, WMV-2, ZYMV	Upjohn	1990 (pending)
	Tobacco	
Bromoxynil tolerance	Calgene	1987
BT/CpTi	Calgene	1989
BT resistance	Ciba-Geigy	1990
BT resistance	Sandoz Crop Prot.	1988
BT resistance	Rhom & Haas	1989
CAT wi proteinase inhibitor II promoter	Iowa State Univ.	1988
CAT	Iowa State Univ.	1989
Chitinase gene	Du Pont	1988
DHDPAS gene	BioTechnica	1989
Etch, mottle viruses	Univ. Kentucky	1990 (pending)
Glyphosate tolerance	Calgene	1987, '89
Mouse metallothionein	Univ. Kentucky	1989, '90 (pending)
Sulfonylurea tolerance	Sandoz	1989, '90 (pending)
	Tomato	
AMV resistance	Agrigenetics	1988
Antisense for pectalytic gene	Calgene	1990 (pending)
Antisense gene for polygalacturonase	Calgene	1988, '89, '90
Bromoxynil tolerance	Calgene	1987
BT resistance	Agrigenetics	1988
BT resistance	Monsanto	1988, '89, '90
Glufosinate tolerance	Canners Seed	1990 (pending)
Glyphosate tolerance	Calgene	1989
Glyphosate tolerance	Monsanto	1988
Sulfonulurea tolerance	Du Pont	1987, '88
TMV resistance	Monsanto	1987
TMV resistance	Uphohn	1990
TMV/ToMV resistance	Monsanto	1989
	Walnut	
Marker gene	Univ. Calif., Davis	1990

AMV Alfalfa mosaic virus
BT *Bacillus thuringiensis* delta endotoxin
CAT Chloramphenicol acetyl transferase gene
CMV Cucumber mosaic virus
PRV Papaya mosaic virus
TMV Tobacco mosaic virus
ToMV Tomato mosaic virus
WMV-2 Watermelon mosaic virus
X Potato X virus
Y Potato Y virus
ZYMV Zucchini yellow mosaic virus

* Data from *The 1990-1991 Biotechnology Regulations Handbook* by EL Korwek, CEEM. 1990.

The δ-endotoxin gene from *Bacillus thuringiensis* (Bt gene) has been cloned and various plants have been transformed. Protection against lepidopteran larvae has been obtained in cotton, potato, tobacco and tomato, and these plants are in licensed field tests. Protection in maize has been achieved by transforming the bacteria, *Clavibacter xyli* subsp. *cynodontis*, with the Bt gene and inoculating maize seedlings. The bacteria grow in the vascular system of the corn plant and supply a natural insecticide. This system has been field tested for four years, and recent results showed that although protection against insects was effective, the bacterial carrier produced adverse effects in some varieties of maize.

Protection against virus diseases has been achieved through transformation in many crops (Table 2). If the gene that regulates the production of the coat protein of a virus is cloned and introduced into a plant that is normally susceptible to the virus, the plant then produces the protein and becomes resistant to infection by the same virus. Coat protein genes from 11 viruses have been used to transform eight crops (Table 2).

Protection against bacterial and fungal diseases is less advanced, but it has been found that lytic peptides from the coelom of the Cecropia moth kill bacteria and fungi (JM Jaynes, personal communication). Insect compounds proven toxic include cecropins, attacins, lysozyme, sarcotoxins, melittin, and defensins. Tobacco has been transformed with a designer gene called Shiva I which imparted disease resistance (Nagpala *et al.*, 1990). Of interest to cane growers would be the lytic peptide gene that kills *Clavibacter xyli*, a bacterium related to the causal agent of ratoon stunting disease. Of interest to processors would be the possibility of these exotic peptides carrying over into the process stream.

Transformation of monocots is not as advanced as in dicots. Table 2 shows permits issued for field testing of transformed plants. Of the 12 transformed crop plants to be field tested there was only one monocot (maize), and that transformation was with marker genes. Maize has recently been transformed (Gould *et al.*, 1991) by introducing the GUS (β-glucuronidase) gene through the bacterium *Agrobacterium tumefaciens*, a vector commonly used with dicots but seldom effective with monocots. Rice has also been transformed (Battraw and Hall, 1990) with GUS (but not field tested); transformation was by the physical method of electroporation. Electroporation involves removing the cellulose wall from plant cells and exposing the protoplasts, bathed in a nutrient solution containing foreign DNA, to electric current. Some DNA penetrates holes in the cell membrane, becomes incorporated in the plant genome and the protoplasts are grown to cells, callus tissue and whole plants.

Transformation in sugarcane was first reported by Chen *et al.* (1987) who were successful in putting the CAT gene into sugarcane protoplasts by electroporation. Although the protoplasts eventually produced callus, regenerated plants were not obtained. Recently, the GUS gene was introduced (Irvine and de Almeida, 1991) to sugarcane callus in Texas by coating tungsten particles with DNA and injecting them into the tissue by using an airless sprayer. A few regenerated plants appear to be transformed when tested for the presence of the GUS enzyme by fluorometric analyses. Preliminary reports from Hawaii and Australia mention transformation by particle guns. Particle injection appears to be a promising technique for testing the transformation of plants by genes with potential utility.

Although there are some interesting exotic genes from microbes, animals and unrelated plants that might be introduced into the breeding material used for sugarcane varieties, perhaps the real value of this technology to the breeders will

be the movement of genes among *Saccharum* and related genera without the necessity of crossing. There are wild and commercial canes with useful genes but the plants do not flower, and are thus unavailable for breeding. There are also wild canes that have specific traits of great value (disease resistance and cold tolerance for example) but also have a vast number of undesirable genes which would accompany the desirable genes during sexual hybridisation. Once the desiarable gene was identified and located by mapping, it would be cut out, cloned and inserted into the gene pool. Alternatively, there does not have to be a separate transformation for each newly cloned gene. Parent clones of sugarcane can be engineered as repositories to carry a large number of desirable genes and could be used as foundation breeding stock. The individual genes of polygenic traits might be accumulated in a few sugarcane clones, therefore simplifying the breeder's choice and making breeding and selection much more efficient. Map-based transformation would make it possible to search, locate, excise and insert a gene into an otherwise acceptable sugarcane clone. This could provide the breeder with rapid resistance to a new disease or insect invader, or correct a deficiency in an otherwise acceptable parent.

For thousands of years, crops have been improved genetically by crossing the best with the best. This prescription was not improved upon until the rediscovery of Mendel's concept of inheritance. Over the years, plant breeders built upon principles of crop improvement, developing knowledge of Mendelian and quantitative genetics for the efficient breeding and selection of varieties from large populations. These fundamental biological and mathematical principles are the foundation of traditional plant breeding and provide the systematic methods of observation, data collection and statistical analysis of plant performance across wide ranges of environments.

In the last two decades, molecular genetics has developed additional tools for the breeder to use in the identification, localisation and genetic transfer of specific genes or gene groups. These approaches, including transformation and genome mapping, will complement and supplement conventional breeding and selection for crop improvement.

A close interaction and collaboration between plant breeders and molecular biologists will be essential; the formation of networks of scientists working on the same crop problems will increase efficiency and speed. Sugarcane breeders have always been excellent at international co-operation. In the future, they will intensify their co-operation in order to realise the full potential of traditional breeding supplemented by molecular genetics.

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