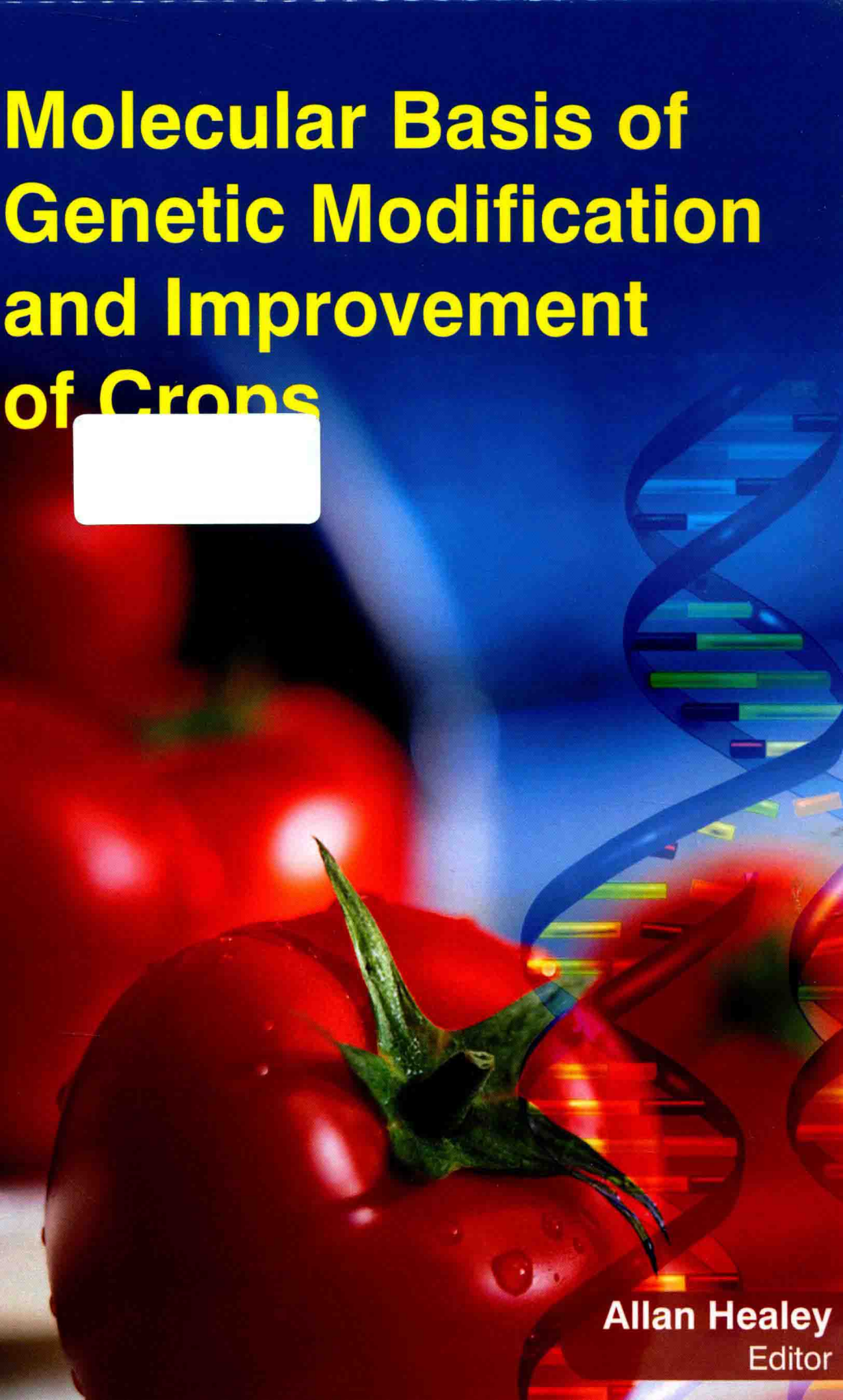
Molecular Basis of Genetic Modification and Improvement of Crops



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Preface

Crop production is threatened by global climate change, and recent demands for crops to produce bio-fuels have started to affect the worldwide supply of some of the most important foods. How can we support a growing human population in such circumstances? One potential solution is the improvement of crops to increase yield from both irrigated and non-irrigated lands, and to create novel varieties that are more tolerant to environmental stresses. Recent progress has been made in the isolation and functional analyses of genes controlling yield and tolerance to abiotic stresses. In addition, promising new methods are being developed for identifying additional genes and variants of interest and putting these to practical use in crop improvement. The identification of genes that are responsible for important agricultural traits has been mostly conducted by traditional molecular genetics for discrete traits and by quantitative trait locus (QTL) mapping for complex traits. In general, the traditional methods are powerful enough to reveal the genes that are involved By contrast, the transgenic approach does not require hybridization, but does require identification of the gene responsible for an advantageous trait. Even genes from non-plant species can potentially be used. In principle, combinations of several beneficial genes can be transferred into the same plant. However, not all crop species or cultivars are easily transformed. In cereal crops other than rice, transformation methods have either not been established (for example, in sorghum) or existing methods have only low efficiency (for example, in wheat and barley). Even in rice, some cultivars are transformed only at low levels. Thus, technical advances in gene manipulation will be required for improvement of those plants in the future. Another potential limitation is that the function of homologous genes is often not exactly the same among plant species (or, indeed, among plant and non-plant species), which might cause unexpected and unwanted side-effects. For practical application, marker-assisted backcrossing of a transgene into a commercially viable variety is often needed. Among the traits affecting crop yields, we focus on those that are linked to genetic programmes controlling the size and number of reproductive organs

rather than traits that are indirectly involved in yield stability, such as the semi-dwarf trait. However, it should be noted that moderate reduction of plant height in cereal crops is important to avoid lodging and thereby to increase yields, as represented by the 'Green revolution' trait.

Genes involved in this form of yield improvement in wheat or rice are known to encode a factor that interferes with the signal transduction pathway of the growth hormone gibberellin (Ga) or with production of Ga, as reviewed previously. In terms of developmental aspects, terminal-branching pattern and fruit-size control seem to be the predominant determinants for the yield improvement of fruits and grains. In rice, for example, two basic traits largely affect grain yield: the number of grains per panicle and the size of the grains. The number of grains mostly depends on the branching pattern of panicles, which is determined by the activity and size of the shoot meristem as well as the timing of transition from shoot to flower, whereas the size of the grains reflects cell-division activity. Studies on the genetic control of inflorescence structure in maize and other grasses have recently been reviewed.

The book will be an indispensable source for all professionals, researchers and students in this subject and for anyone working in the related areas for acquiring an up-to-date overviews.

—Editor

Contents

Chapter 1

Introduction

Reverse Breeding and Doubled Haploids (DH)

A method for efficiently producing homozygous plants from a heterozygous starting plant, which has all desirable traits. This starting plant is induced to produce doubled haploid from haploid cells, and later on creating homozygous/doubled haploid plants from those cells. While in natural offspring recombination occurs and traits can be unlinked from each other, in doubled haploid cells and in the resulting DH plants recombination is no longer an issue. There, a recombination between two corresponding chromosomes does not lead to un-linkage of alleles or traits, since it just leads to recombination with its identical copy. Thus, traits on one chromosome stay linked. Selecting those offspring having the desired set of chromosomes and crossing them will result in a final F1 hybrid plant, having exactly the same set of chromosomes, genes and traits as the starting hybrid plant. The homozygous parental lines can reconstitute the original heterozygous plant by crossing, if desired even in a large quantity. An individual heterozygous plant can be converted into a heterozygous variety without the necessity of vegetative propagation but as the result of the cross of two homozygous/doubled haploid lines derived from the originally selected plant. patent

Genetic Modification

Genetic modification of plants is achieved by adding a specific gene or genes to a plant, or by knocking down a gene with RNAi, to produce a desirable phenotype. The plants resulting from adding a gene are often referred to as transgenic plants. If for genetic modification genes of the species or of a crossable plant are used under control of their native promoter, then they are called cisgenic plants. Genetic modification can produce a plant with the desired trait or traits faster than classical breeding because the majority of the plant's genome is not altered. To genetically modify a plant, a genetic construct

must be designed so that the gene to be added or removed will be expressed by the plant. To do this, a promoter to drive transcription and a termination sequence to stop transcription of the new gene, and the gene or genes of interest must be introduced to the plant. A marker for the selection of transformed plants is also included. In the laboratory, antibiotic resistance is a commonly used marker: plants that have been successfully transformed will grow on media containing antibiotics; plants that have not been transformed will die. In some instances markers for selection are removed by backcrossing with the parent plant prior to commercial release.

The construct can be inserted in the plant genome by genetic recombination using the bacteria *Agrobacterium tumefaciens* or *A. rhizogenes*, or by direct methods like the gene gun or microinjection. Using plant viruses to insert genetic constructs into plants is also a possibility, but the technique is limited by the host range of the virus. For example, Cauliflower mosaic virus (CaMV) only infects cauliflower and related species. Another limitation of viral vectors is that the virus is not usually passed on the progeny, so every plant has to be inoculated. The majority of commercially released transgenic plants, are currently limited to plants that have introduced resistance to insect pests and herbicides. Insect resistance is achieved through incorporation of a gene from *Bacillus thuringiensis* (Bt) that encodes a protein that is toxic to some insects. For example, the cotton bollworm, a common cotton pest, feeds on Bt cotton it will ingest the toxin and die. Herbicides usually work by binding to certain plant enzymes and inhibiting their action. The enzymes that the herbicide inhibits are known as the herbicides *target site*. Herbicide resistance can be engineered into crops by expressing a version of *target site* protein that is not inhibited by the herbicide. This is the method used to produce glyphosate resistant crop plants Genetic modification of plants that can produce pharmaceuticals (and industrial chemicals), sometimes called *pharmacrops*, is a rather radical new area of plant breeding.

Issues and Concerns

Modern plant breeding, whether classical or through genetic engineering, comes with issues of concern, particularly with regard to food crops. The question of whether breeding can have a negative effect on nutritional value is central in this respect. Although relatively little direct research in this area has been done, there are scientific indications that, by favouring certain aspects of a plant's development, other aspects may be retarded. A study published in the *Journal of the American College of Nutrition* in 2004, entitled *Changes in USDA*

Food Composition Data for 43 Garden Crops, 1950 to 1999, compared nutritional analysis of vegetables done in 1950 and in 1999, and found substantial decreases in six of 13 nutrients measured, including 6% of protein and 38% of riboflavin. Reductions in calcium, phosphorus, iron and ascorbic acid were also found. The study, conducted at the Biochemical Institute, University of Texas at Austin, concluded in summary: *"We suggest that any real declines are generally most easily explained by changes in cultivated varieties between 1950 and 1999, in which there may be trade-offs between yield and nutrient content."*

The debate surrounding genetically modified food during the 1990s peaked in 1999 in terms of media coverage and risk perception, and continues today-for example, *"Germany has thrown its weight behind a growing European mutiny over genetically modified crops by banning the planting of a widely grown pest-resistant corn variety."*. The debate encompasses the ecological impact of genetically modified plants, the safety of genetically modified food and concepts used for safety evaluation like substantial equivalence. Such concerns are not new to plant breeding. Most countries have regulatory processes in place to help ensure that new crop varieties entering the marketplace are both safe and meet farmers' needs. Examples include variety registration, seed schemes, regulatory authorizations for GM plants, etc.

Plant breeders' rights is also a major and controversial issue. Today, production of new varieties is dominated by commercial plant breeders, who seek to protect their work and collect royalties through national and international agreements based in intellectual property rights. The range of related issues is complex. In the simplest terms, critics of the increasingly restrictive regulations argue that, through a combination of technical and economic pressures, commercial breeders are reducing biodiversity and significantly constraining individuals (such as farmers) from developing and trading seed on a regional level. Efforts to strengthen breeders' rights, for example, by lengthening periods of variety protection, are ongoing. When new plant breeds or cultivars are bred, they must be maintained and propagated. Some plants are propagated by asexual means while others are propagated by seeds. Seed propagated cultivars require specific control over seed source and production procedures to maintain the integrity of the plant breeds results. Isolation is necessary to prevent cross contamination with related plants or the mixing of seeds after harvesting. Isolation is normally accomplished by planting distance but in certain crops, plants are enclosed in greenhouses or cages (most commonly used when producing F1 hybrids.)

Participatory Plant Breeding

The development of agricultural science, with phenomenon like the Green Revolution arising, have left millions of farmers in developing countries, most of whom operate small farms under unstable and difficult growing conditions, in a precarious situation. The adoption of new plant varieties by this group has been hampered by the constraints of poverty and the international policies promoting an industrialised model of agriculture. Their response has been the creation of a novel and promising set of research methods collectively known as participatory plant breeding. Participatory means that farmers are more involved in the breeding process and breeding goals are defined by farmers instead of international seed companies with their large-scale breeding programs. Farmer's groups and NGOs, for example, may wish to affirm local people's rights over genetic resources, produce seeds themselves, build farmers' technical expertise, or develop new products for niche markets, like organically grown food.

Intellectual Property Rights (IPRs) for Plant Breeding

Plant breeding in recent years has been on the threshold of a major change, firstly, due to prospects provided by biotechnological approaches and *secondly,* due to recent emphasis on 'participatory plant breeding'. This trend is going to continue and will have a major impact on plant breeding. Plant breeding will also be influenced by the provisions of a number of international instruments (treaties) including the 'Convention on Biological Diversity (CBD)'. This change in the emphasis in plant breeding will demand and allow investments by private industry in a big way. The role of farmers in the form of participatory plant breeding and the equitable sharing of benefits by them in the form of farmers' rights will also be witnessed. Consequently, the plant genetic resources (PGRs) and the products of plant' breeding will have to be adequately covered by intellectual property rights and protection. In several developing countries including India, plant breeding work has so far been primarily designed to promote agricultural production by the farmers, so that it is mainly concentrated at Agricultural Universities and Research Institutes.

Consequently, in these countries, no intellectual property law existed in the past to protect the products of plant breeding. However, this state could not continue indefinitely, and the intellectual property law has already started responding to the changes being brought about by biotechnology industry. It will also be necessary to reward equitably the plant breeders, the farmers and the biotechnologists, for

their efforts, so that they are encouraged to invest and work further, utilizing the new techniques. Under TRIPS (Trade Related Intellectual Properties) agreement also, as members of WTO, many developing countries will have to provide protection for plant varieties either 'by patents or by an effective sui generis system or by any combination thereof'. In view of this, the plant variety rights and protection were widely discussed in the last decade of the 20th century and in the early years of the 21st century.

At the international level. a major revision of PVP (plant variety protection) in the UPOV. Convention of 1991 seeks to provide for a better control of the plant breeder (UPOV= International Union for the Protection of New Plant Varieties). The patents and plant breeders' rights (PBRs) can no longer be operated in isolation from each other, so that it is necessary to understand the difference and relationship between the two systems. It has been recognized that most varieties presented for plant variety protection, may not qualify for patent protection, since it may be difficult to prove that the production of a variety really involved an innovative step. It will also be difficult to describe the method of breeding in a way that may be repeatable. Despite these difficulties, in some developed countries (e.g., USA, Australia), both patents and plant breeders rights are used for plant variety protection. In European Union, although patents have been allowed particularly for transgenic crops, one is still uncertain whether patents for protection of plant varieties are freely available.

In several developing countries like India also, such a protection is in the process of taking legal status. The adoption of the agreement on Trade Related Aspects of Intellectual Property Rights (TRIPs) under the aegis of GATT (WTO), and the pressure of private industry, have led to enactment of 'Protection of Plant Varieties and Farmers' Rights Act' (PPV&FRA), which was approved by the Parliament in 2001. Indian Patent Act 1970 is also being amended.

It has been recognized that the enactment of patent legislation and strong Plant Variety Rights (PVR) similar to those in developed countries are probably not appropriate for developing countries like India, at least in the short term. In view of this, amend d draft of 'Protection of Plant Variety and Farmers' Rights Act (PPV & FRA)' was prepared and has now been approved by the Parliament. Necessary steps were being taken in the year 2002 to implement PPV &FRA. The amended Indian Patent Act was still to be ratified in the year 2002.

In agriculture sector, the Plant Breeders Rights (PBRs) are' used in most OECD (Organization of Economic Cooperation and Development) countries and some other developing countries. There are also arguments for and against the use of plant breeders' rights (PBRs) in countries like India. With the expansion of private seed industry in India, however, there is pressure from this industry to provide due reward for their investments, so that innovations in plant breeding may be encouraged. There are, however, fears that PBRs may hamper the free supply of seed to the farmers thus also leading to' loss of genetic diversity. However, hardly any data on the impact of PBRs on seed industry is available, to assess the effect of PBRs on loss of biodiversity. These and related aspects of IPR relevant to crop varieties and plant genetic resources (PGRs) will be discussed briefly in this chapter. The Indian PVP&FRA (Plant Variety Protection and. Farmers Right Act) 2001, which was approved by the parliament in August 2001 and given assent by the President of India in November 2001 will also be discussed.

History of Plant Breeding

What is Plant Breeding? For several thousand years, farmers have been altering the genetic makeup of the crops they grow. Human selection for features such as faster growth, larger seeds or sweeter fruits has dramatically changed domesticated plant species compared to their wild relatives. Remarkably, many of our modern crops were developed by people who lacked an understanding of the scientific basis of plant breeding.

Despite the poor understanding of the process, plant breeding was a popular activity. Gregor Mendel himself, the father of genetics, was a plant breeder, as were some of the leading botanists of his time. Mendel's 1865 paper explaining how dominant and recessive alleles could produce the traits we see and could be passed to offspring was the first major insight into the science behind the art. The paper was

largely ignored until 1900, when three scientists working on breeding problems rediscovered it and publicized Mendel's findings. Major advances in plant breeding followed the revelation of Mendel's discovery. Breeders brought their new understanding of genetics to the traditional techniques of self-pollinating and cross-pollinating plants. Corn breeders, particularly, tried numerous strategies to capitalize on the insights into heredity. Corn plants that had traditionally been allowed to cross-pollinate freely were artificially self-pollinated for generations and crossed to other self-pollinated lines in an effort to achieve a favourable combination of alleles. The corn we eat today is the result of decades of this strategy of self-pollination followed by cross-pollination to produce vigorous hybrid plants.

The art of recognizing valuable traits and incorporating them into future generations is very important in plant breeding. Breeders have traditionally scrutinized their fields and travelled to foreign countries searching for individual plants that exhibit desirable traits. Such traits occasionally arise spontaneously through a process called mutation, but the natural rate of mutation is too slow and unreliable to produce all the plants that breeders would like to see.

In the late 1920s, researchers discovered that they could greatly increase the number of these variations or mutations by exposing plants to X-rays. "Mutation breeding" accelerated after World War II, when the techniques of the nuclear age became widely available. Plants were exposed to gamma rays, protons, neutrons, alpha particles, and beta particles to see if these would induce useful mutations. Chemicals, too, such as sodium azide and ethyl methanesulphonate, were used to cause mutations.Examples of plants that were produced via mutation breeding are given in the table below.

Crop Mutation	*Cultivar Name*	*Method Used to Induce*
rice	Calrose 76	gamma rays
wheat	Above	sodium azide
	Lewis	thermal neutrons
oats	Alamo-X	X-rays
grapefruit	Rio Red	thermal neutrons
	Star Ruby	thermal neutrons
burmuda grass	Tifeagle	gamma rays
	Tifgreen II	gamma rays
	Tift 94	gamma rays
	Tifway II	gamma rays

Contd...

Crop Mutation	*Cultivar Name*	*Method Used to Induce*
lettuce	Ice Cube	ethyl methanesulphonate
	Mini-Green	ethyl methanesulphonate
common bean	Seafarer	X-rays
	Seaway	X-rays
lilac	Prairie Petite	thermal neutrons
St. Augustine grass	TXSA 8202	gamma rays
	TXSA 8212	gamma rays

Quite a few flower cultivars have been developed via mutation breeding, among them some of the cultivars of *Alstroemeria*, begonia, carnation, chrysanthemum, dahlia, and snapdragon. Mutation breeding was particularly popular in the United States during the 1970s. Although interest has waned somewhat in recent years, occasional varieties continue to be produced using these methods. For example, the new herbicide-resistant wheat variety Above was developed using exposure to sodium azide. Mutation breeding efforts continue around the world today. Of the 2,252 officially released mutation breeding varieties, 1,019, or almost half, have been released during the last 15 years.

Another method for increasing the number of mutations in plants is tissue culture. Tissue culture is a technique for growing cells, tissues, and whole plants on artificial nutrients under sterile conditions, often in small glass or plastic containers. Tissue culture was not developed with the intention of causing mutations, but the discovery that plant cells and tissues grown in tissue culture would mutate rapidly increased the range of methods available for mutation breeding. It was during the 1970s also that haploid breeding was heavily utilized. Spontaneously occurring haploid plants, those having half the normal number of chromosomes, were discovered in the 1920s, but haploid breeding was not a practical technique until methods for the controlled production of haploid plants were developed.

Once a haploid plant has been obtained, its chromosomes are artificially doubled to return the plant to the normal number of chromosomes. Such a plant is valuable because the chromosomes that were created by artificial doubling are exact copies of the chromosomes that were present in the haploid plant. Haploids have been used in creating cultivars of barley, maize, tobacco, asparagus, strawberries, and tall fescue grass. Often these plants are more useful in basic research than in commercial applications, but the haploid-derived

barley cultivar Tangangara was released for commercial production in Australia in 1996. While most breeders cross-pollinate plants of a single species, some breeding methods rely on crosses that can be made between two species within the same genus.

A cross between *Musa acuminata* and *Musa balbisiana*, both members of the genus *Musa*, produced the bananas with which we are familiar. Less commonly, the cross is between members of two different genera. A cross between wheat, *Triticum aestivum*, and rye, *Secale cereale*, produced the grain called triticale, which contains a copy of all the chromosomes from both species.. A variation on the wide crossing procedure is to select plants that have single chromosomes or chromosome arms substituted from one species into another.

Many modern wheat cultivars, for example, contain a chromosome arm from rye, which adds resistance to several diseases. Transgenic technology provides the means to make even more distant "crosses" than were previously possible. Organisms that have until now been completely outside the realm of possibility as gene donors can be used to donate desirable traits to crop plants. These organisms do not provide their complete set of genes, but rather donate only one or a few genes to the recipient plant. For example, a single insect-resistance gene from the bacterium *Bacillus thuringiensis* can be transferred to a corn plant to make Bt corn. A description of Bt corn is available on our Current Transgenic Products page. Transgenic plants were first created in the early 1980s by four groups working independently at Washington University in St. Louis, Missouri, the Rijksuniversiteit in Ghent, Belgium, Monsanto Company in St. Louis, Missouri, and the University of Wisconsin. On the same day in January 1983, the first three groups announced at a conference in Miami, Florida, that they had inserted bacterial genes into plants. The fourth group announced at a conference in Los Angeles, California, in April 1983 that they had inserted a plant gene from one species into another species.

The Washington University group, headed by Mary-Dell Chilton, had produced cells of *Nicotiana plumbaginifolia*, a close relative of ordinary tobacco, that were resistant to the antibiotic kanamycin. Jeff Schell and Marc Van Montagu, working in Belgium, had produced tobacco plants that were resistant to kanamycin and to methotrexate, a drug used to treat cancer and rheumatoid arthritis. Robert Fraley, Stephen Rogers, and Robert Horsch at Monsanto had produced petunia plants that were resistant to kanamycin. The Wisconsin group, headed by John Kemp and Timothy Hall, had inserted a bean gene into a sunflower plant. These discoveries were soon published in scientific