

CICR TECHNICAL BULLETIN NO: 17

BIOTECHNOLOGICAL APPROACHES FOR COTTON IMPROVEMENT

G. Balasubramani

J. Amudha

A. B. Dongre



**Central Institute for Cotton Research
Nagpur**

Downloaded from www.cicr.org.in

BIOTECHNOLOGICAL APPROACHES FOR COTTON IMPROVEMENT

FOREWARD

Cotton crop is an important segment of the Agricultural profile in India contributing nearly 80% raw material required for the textile industry which in turn generates approximately 30% of foreign exchange earnings of the country. Conventional breeding programmes have produced steady improvements in agronomic traits but the potential benefits of genetic engineering in cotton have been slow in coming.

The development of genetic engineering procedure for cotton, others and we have concentrated on characteristics that will modify or improve the agronomic practices for this high input crop. This includes the development of insect and herbicide resistant cotton plant. Improvements in quality characteristics or plant performance may come later with increasing knowledge of the molecular biology of plant development and physiology. This bulletin deals with the development of insect and herbicide resistant transgenic plants, the natural plant defense chemicals, improvements of fibre quality, plant tissue culture, gene construct, explants and methods for transformation and apprehension over terminator gene technology.

Hope this bulletin will give the real problems and prospects for the development of transgenic plants and possibilities and challenges ahead for terminator gene technology all over the world. I congratulate Dr. G. Balasubramani, Dr. (Mrs.) J. Amudha and Dr. A.B. Dongre for their sincere efforts in compiling information and bringing out this esteemed bulletin on Biotechnological approaches for cotton improvement.

Nagpur
07-07-2000

M.S.KAIRON
DIRECTOR

PREFACE

Agricultural planners and scientists are looking to the potentials of biotechnology to address the problems that currently exist and are anticipated in Cotton. Improvements in pest control strategies are the most obvious area for attention. Many companies have made progress on herbicide resistance and insect resistance imparted via the Bt toxin. The other areas of interest are also receiving attention includes biological control of pest, fibre quality modification and stress tolerance. Cotton seed protein and oil quality is a potential area for improvement.

We have made an attempt to present sufficient basic information to understand various biotechnological approaches used for cotton crop improvement. Hope this bulletin will help the biotechnologists, geneticists, breeders and researchers engaged with this crop. We are extremely grateful to Dr. R. S. Paroda, Director General ICAR and Secretary DARE, Dr. Mangala Rai DDG (CS), ICAR and Dr. M. S. Kairon, Director, ICAR, Nagpur for their constant guidance and encouragement to bring out this publication. Finely we thank, Dr. Ramasundaram ARIS-incharge, Mrs. Rama Iyer, Stenographer, Mr. Aunp and Miss. Aarthi for their untired help rendered during preparation of this bulletin.

Nagpur
07-07-2000

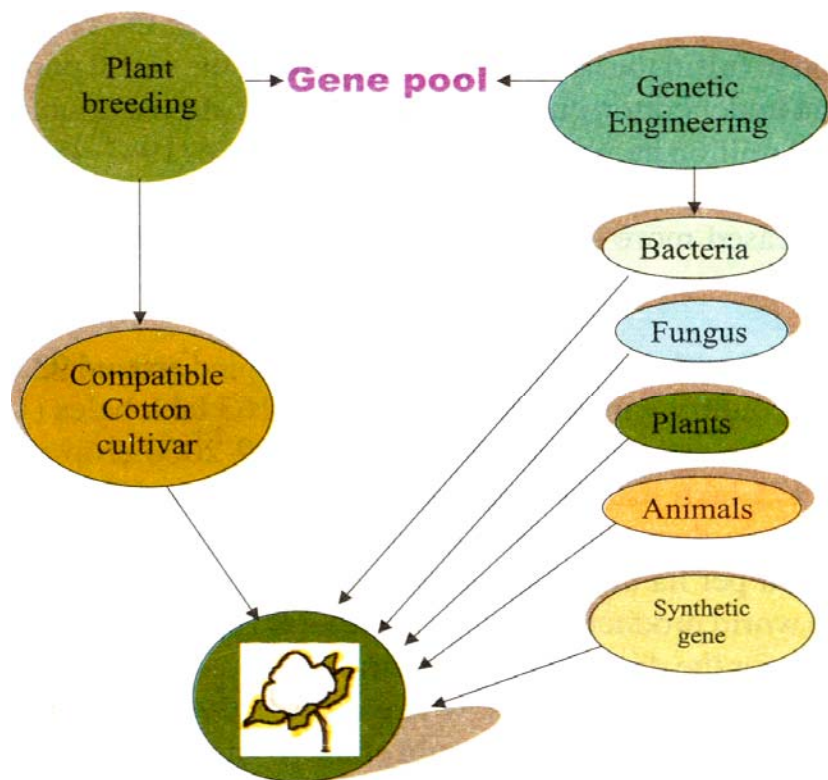
G.Balasubramani
J.Amudha
A.B.Dongre

Introduction

Cotton (*Gossypium* spp.) has a high economic value as a fibre crop, grown in over 90 countries, involving more than 180 million people. Cotton cultivation is acquiring more and more significance because of the continued increase in the requirement of cotton to fulfill the needs of ever-increasing the world population. The area under cotton cultivation in India has almost doubled from about 4.4 million ha. at the time of independence (1947-48) to about 9.0 million ha. during 1990s. Simultaneously, the production has also increased more than six fold from 25 lakh bales to 160 lakh bales.

During the year 1998-99 cotton occupied around 93.62 lakh ha in the country with the production over 163 lakh bales (1 bale = 170 kg). The estimated production for 1999-2000 crop season is around 175 lakh bales which will be more than sufficient to meet the domestic demand. However the productivity at around 300-330 kg lint per ha in India during last five years, falls short of the average world productivity of about 585-600 kg lint per ha. Further, the target for the domestic and export requirement set for 2000 AD is around 180-190 lakh bales, whereas in 2020 AD is 230-240 lakh bales. Much of this increase should come from an already shrinking natural resource base, whose sustainability is declining alarmingly. To meet the demands, the research efforts have to be geared up and given a new direction and thrust to achieve the targets within the time frame. Secondly, there is an urgent need to change the plant protection measures using biotechnological approaches. Since insect pest is the major factor that destabilized cotton yield annually. It is estimated that 30-40% of the total crop productivity is lost by insect pests.

Genetic engineering for cotton improvement



Genetic engineering and newer transformation methods allow any gene from any source to be incorporated into the cotton genome, whereas cross breeding is restricted to compatible cotton cultivars

Genetic engineering provides an alternate and powerful method for gene transfer from any organisms into cotton, whereas cross breeding is restricted to compatible cotton cultivars. The transferred foreign gene(s) integrates efficiently in to the plant chromosome. Then the integrated traits are inherited and expressed like any other plant genes. Thus, there is potential to improve cotton insect pest resistance traits (which is unique and extremely desirable at the present time), herbicide tolerance, fibre characteristics, oil content, tolerance to environmental extremes and even more fundamental physiological processes such as water and nutrient balance. The wealth of interesting genetic, physiological and agronomic problems available for study in cotton ensures that, there will be a rapid transition from the molecular analysis of gene structure to transform methods in more biological and agricultural applications.

Plant Tissue Culture

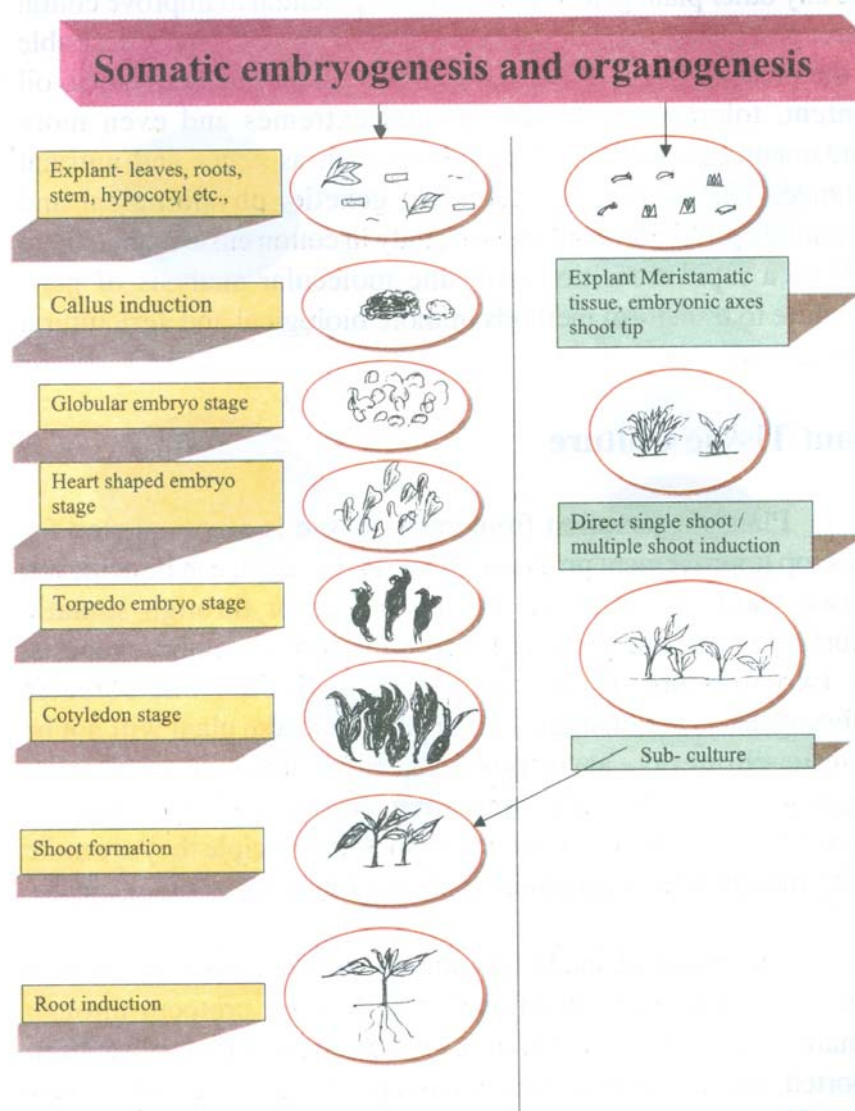
Plant regeneration from callus tissue is a critical step for any crop improvement program. Plant regeneration can be achieved in two ways viz., through organogenesis or through somatic embryogenesis. The preferred method is somatic embryogenesis, for two seasons. First, the plants derived from somatic embryogenesis are of single cell origin. Thus, the plant will not be genetic chimeras, as is possible with those derived from organogenesis. Secondly, since non-zygotic embryos have no vascular connection with maternal tissues, in principal they are more easily manipulated than plantlets derived through organogenesis.

The major obstacle to cotton crop improvement through gene transfer is, lack of efficient regeneration protocol through somatic embryogenesis. Though, regeneration in cotton has been reported, the response is restricted to specify genotype like Coker lines. The genetic transformation in cotton was succeeded in the Coker lines 312, 310 and 201 by *Agrobacterium* mediation and transformed plants were regenerated. Few investigators have reported successful transformation via organogenesis using biolistic method to circumvent cultivar dependent regeneration from somatic embryogenesis. However, development of tissue culture protocols to regenerate a complete plant from single cell via somatic embryogenesis is desirable for genetic transformation of cotton. Therefore efforts are required to extend plant regeneration from callus to various cultivars or species of cotton grown commercially in all parts of our country.

Secondly plant cell cultures are prone to chromosomal genetic and molecular changes. Both epigenetic as well as heritable changes have been found. Such variations pose a serious problem for the maintenance of genetic uniformity in plants regenerated *in vitro*. Through biotechnological approach plants are often transformed with a single gene and it is very important to retain the agronomic performance of the variety that is being transformed. True to type plants can be regenerated from embryogenic callus and cell cultures, only then the regeneration system is rapid and efficient. Long time maintenance of callus and cell cultures often results in plants that are morphologically abnormal and functionally sterile, thus making them unsuitable for cultivation. Therefore, work needs to be aimed at shortening the regeneration period so that the regenerated plants would have reduced potential somaclonal variation and

maximum similarity to the original cultivars with addition to the transferred traits(s)

A third barrier to cotton transformation has been low transformation frequencies and optimization of methods for using a selectable marker such as kanamycin resistance. In a system such as tobacco, transformed tissue at any stage of development can be selected on high level of kanamycin (upto 1000 mg/ml), while in cotton such levels are highly toxic. Efforts are required to increase the transformation frequencies and it would permit to look among a number of transformants for high expressing individuals, eg., for higher insecticidal activity or higher tolerance to herbicides. Superior individuals will be used directly as new cultivars or for breeding into other cultivars.



Genetic engineering of cotton is costly and time consuming. It probably takes 3-5 years to bring new technology from the lab into land. In this context, development of hybrid cotton lines is very important for purpose of transgenic variety protection. Research in this area has been successful and commercial production of F1 hybrid cotton lines will be possible in the near future.

Gene Constructs for Transformation

Recombinant DNA technology utilizes a few simple but powerful method, which allow researchers to identify, purify and determine the structure and regulation of genes and their products. It also provides the means for transferring genes from one organism to another and perhaps, for designing new genes. To achieve the effected transfer of a gene into a host, appropriate gene constructs need to be made to facilitate integration and expression of the foreign genes. It is preferable to include a selectable or screenable marker gene to identify the transformed cells. Selectable markers are preferred, as they allow under ideal condition, establishment and proliferation of transformed cells. These cells may regenerate into transgenic plants following transfer to appropriate media containing phytohormones. The transformed plant are subjected to root induction in hormone free media or low auxin containing media to make it complete plant and further subjected to hardening process to withstand the ambient temperature.

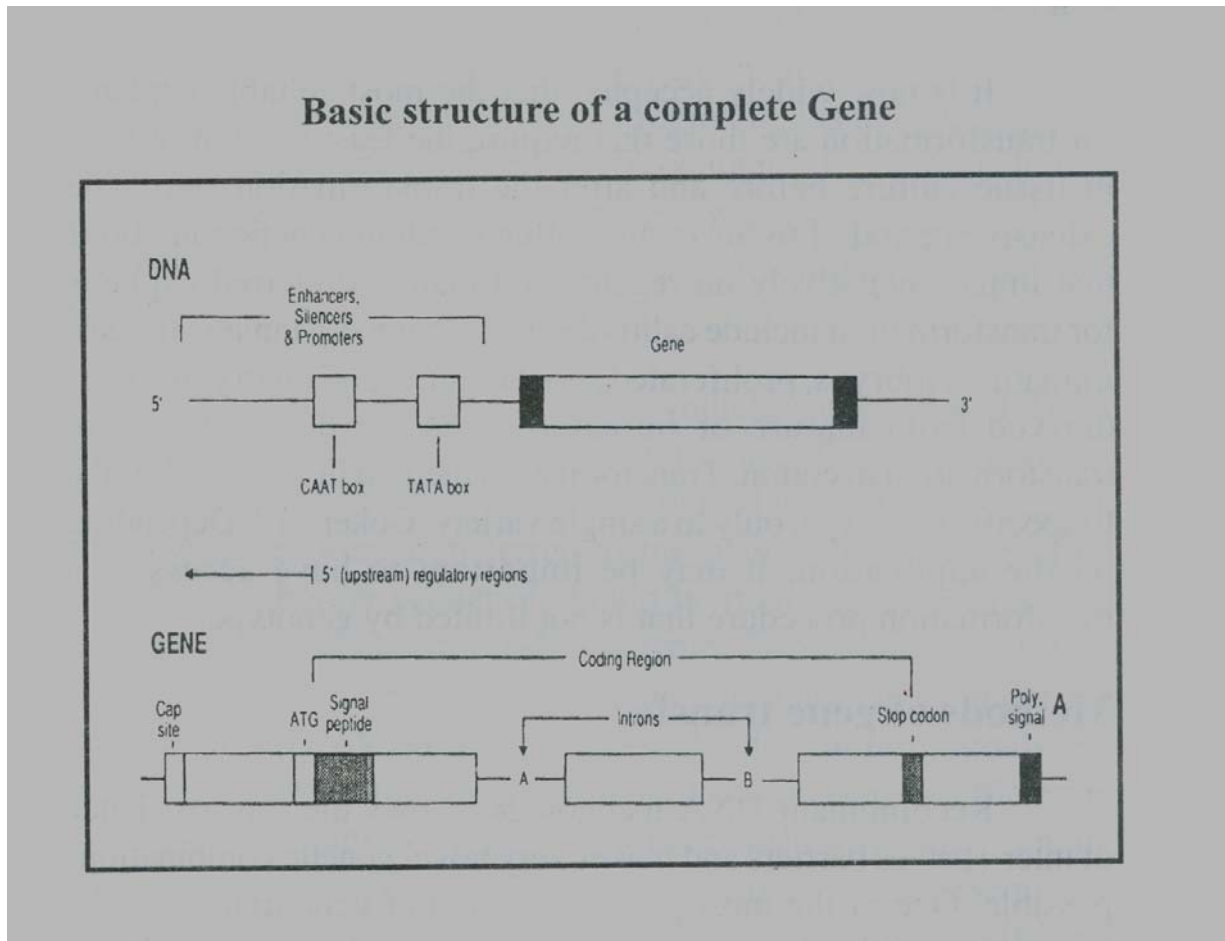
A typical gene construct will include a promoter, a coding sequence i.e. transgene and terminating signal. Transgenes may be introduced into plant tissues on separate plasmid or on cointegrated vectors. In the latter case, multiple genes, including selectable or screenable markers are present on the same plasmid. Commonly used promoters for plant transformation are the cauliflower mosaic virus (CaMV) 35S promoter, which is a constitutive promoter, suitable for driving the expression of foreign genes in dicotyledons and the maize ubiquitin promoter which drives strong expression of transgenes in monocotyledons. Organ/tissue - specific promoters are also available to drive expression of transgenes in particular part of the plant and specific examples include, the vicilin and phytohemagglutinin promoter, derived from pea and bean respectively, suitable for seed-specific expression. The high molecular weight glutenin promoter from wheat is also suitable for seed-specific expression and the alpha-amylase promoter, for driving expression in the aleuron of cereal grains. The most common selectable marker gene encodes proteins that detoxify metabolic inhibitors such as antibiotics or herbicides. Commonly used screenable markers include the beta-glucuronidase and luciferase genes and more recently the green fluorescent protein from jellyfish.

Explants for Transformation

It is now widely accepted that the most suitable explants for transformation are those that require the least amount of time in tissue culture before and after the transformation step. The extensive period of tissue culture often result in genetic mutations that impact negatively on regenerated plants. Preferred explants for transformation include callus derived form meristamatic tissues, immature embryos, proliferated shoot culture, and embryonic axes derived from mature or immature seed for direct meristem transformation in cotton. Transformation method in cotton is limited to specific genotype, only to a single variety, Coker 312. Depending on the application, it may be important to have access to a transformation procedure that is not limited by genotype.

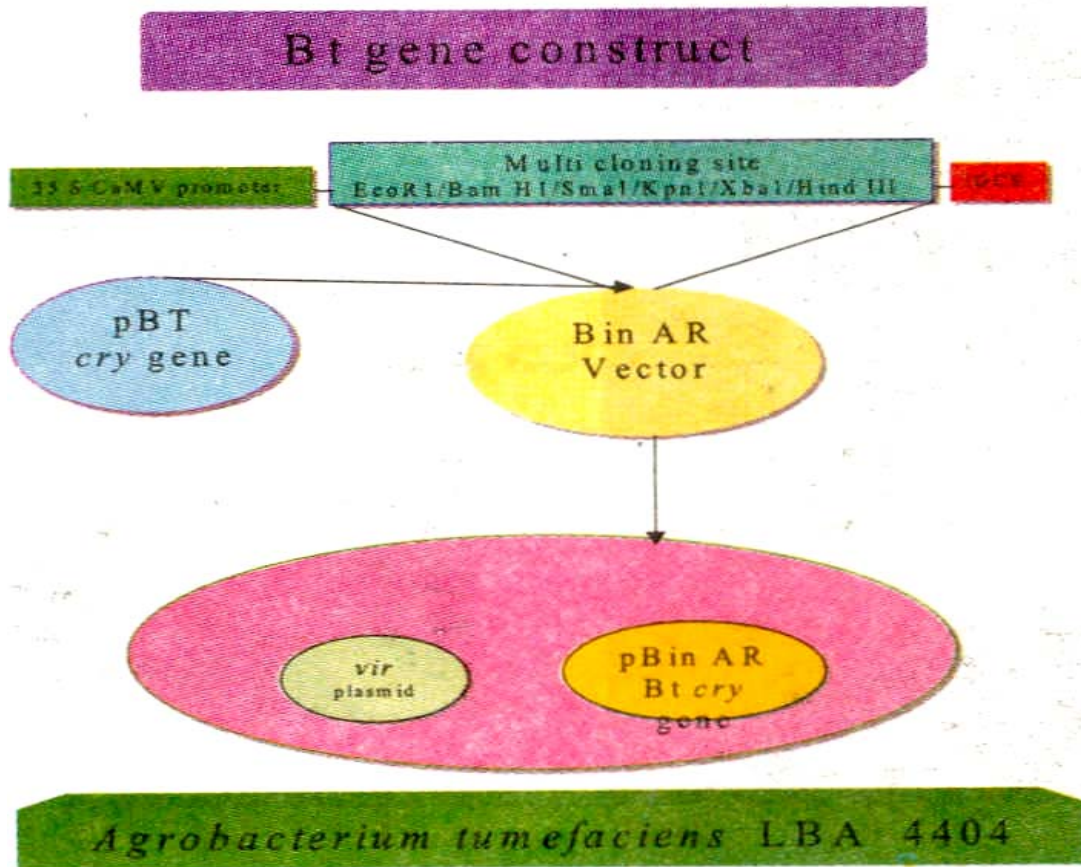
Methods of Gene Transfer

Recombinant DNA technology allows the transcendence of inter-species barriers and makes very novel genetic combinations possible. One of the most popular method of gene transfer is the use of the soil bacterium *Agrobacterium tumefaciens*, which can transfer genes to many plants at wound sites. The utility of this bacterium as a gene transfer system was first recognized when it was demonstrated that the crown gall disease were actually produced as result of the transfer and integration of genes from the bacterium into the genome of the plant cells.



Agrobacterium-mediation

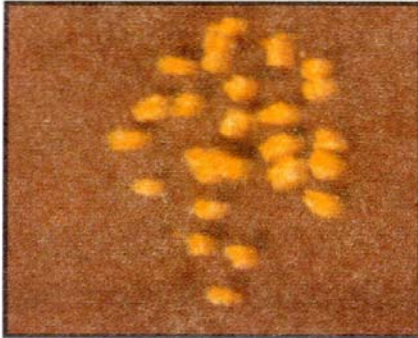
Virulent strains of *Agrobacterium* contain large Ti (Tumor inducing) plasmid, which is responsible for the DNA transfer and subsequent disease symptoms. Genetic and molecular analysis showed that Ti - plasmid contain two sets of sequence necessary for gene transfer to plants, one is T-DNA (transferred DNA) regions that are transferred to the plant and another is *vir* (virulence) gene which are not themselves transferred during infection. The T-DNA regions are flanked by border sequences that were shown to be responsible for the definition of the region that is to be transferred to the infected plant cell. The T-DNA contain 8-13 genes including a set for production of phytohormones, which are responsible for formation of the characteristic tumors when transferred to infected plants.



When the phytohormone biosynthetic genes from T-DNA were excised, the bacterium does not cause disease symptoms. Additionally, when other genes were placed in the T-DNA region, the new genes were transferred to the plant. The main criterion was that the border sequence should be maintained, because the enzyme that excises the T-DNA from Ti-plasmid apparently recognizes border sequence. Bacterial antibiotic genes under the control of T-DNA promoters and polyadenylation signals are inserted in between the 25 bp repeat border sequence and efficiently expressed in plant cells in a dominant fashion in any genetic background. This

phenomenon enables nearly any desired sequence of DNA fragment (gene) placed in T-DNA region for efficient transformation and direct expression of inserted coding sequences.

Transformation and Regeneration



Co-cultivation with *Agrobacterium*



Transformants in selection medium



Multiple shoot induction



Hardening stage -1



Hardening stage -II



Flowering stage of transgenic plant

Most importantly, *Agrobacterium* mediated gene transfer in plant is an excellent system since, the integration of T - DNA is a precise process, the plasmid routinely undergo rearrangement and concatenation reactions before insertion and can lead to chromosomal rearrangements during insertion in plant system. The stability of expression of most genes that are introduced by *Agrobacterium* have been found to be stable over at least five generations during cross breeding and seed increase on genetically engineered tomato, oilseed and cotton. This stability is critical

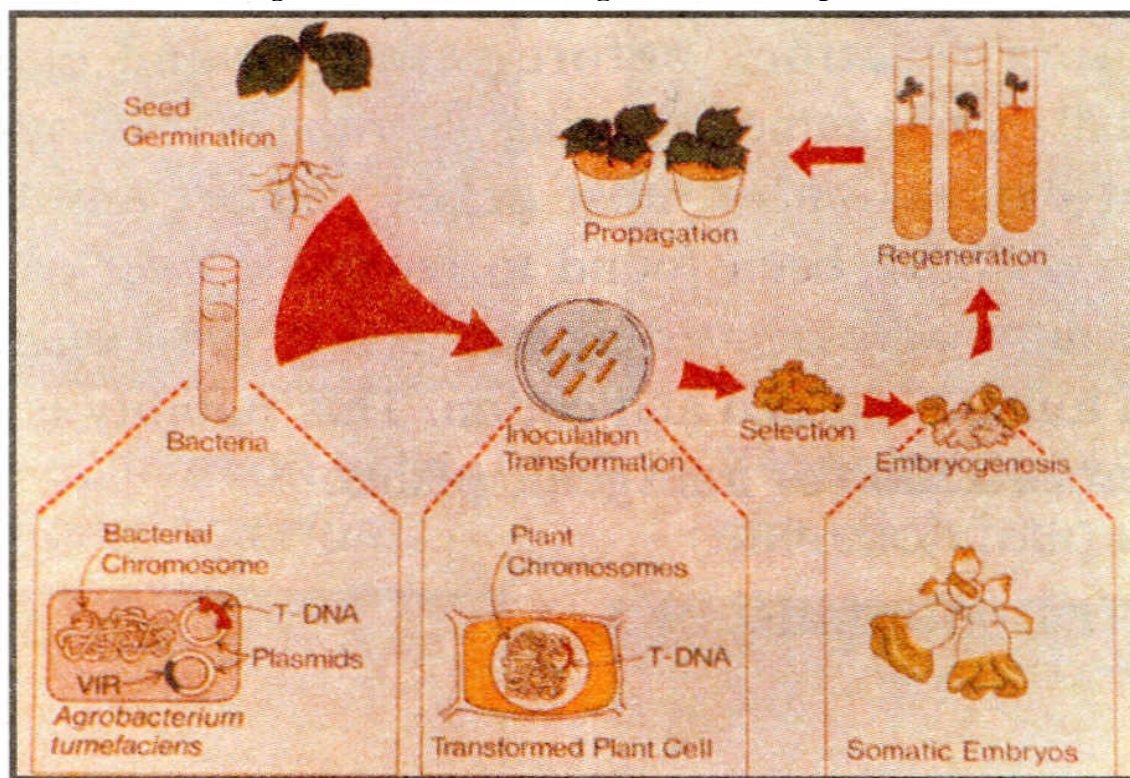
to the commercialization of transgenic plants.

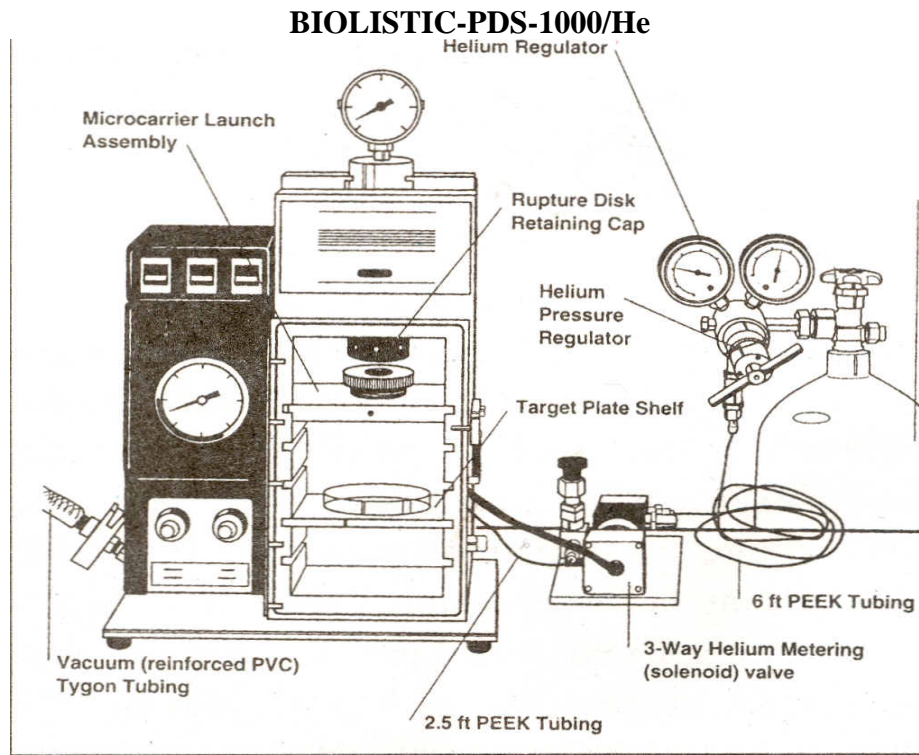
Biolistic Transformation

Biolistics (biological ballistics) is a process by which DNA or other biological materials are delivered into cells in association with high velocity microprojectiles. Gold particles (1.0 - 1.5 mm.) coated with the DNA of interest, usually a plasmid construction are accelerated with sufficient force for them to penetrate the outer cell walls of a target tissues/organs. The technique was successfully used for the development of transgenic plants in cotton, corn and soybean. Biolistics has also enabled transformation of the plant chloroplast genome.

Microprojectile bombardment can even be used to wound plant tissues, allowing more efficient transformation like *Agrobacterium*. Most methods of transformation are restricted to a limited range of explant type. In contrast, microprojectile mediated gene delivery permits the transformation of cells from wide range of sources including cell suspension, callus, meristem tissues, immature embryos, coleoptiles and pollen. Transient expression of foreign genes in plastids of higher plants using the chloramphenicol acetyltransferase (cat) gene under the control of a chloroplast specific promoter are delivered by particle bombardment.

Agrobacterium-mediated gene transfer in plants





Although biolistic is rapidly being adapted to new applications, improvements in the technology are still needed to make it more efficient. In plant system, transient gene expression is relatively easy to achieve, but usually only a few percent of the transiently expressing cells can be recovered of stable transformants. One factor that limits the recovery of stable transformants is injury to the cells. During bombardment, a portion of cells/tissues are commonly dislodged and or killed at the epicenter of the blast, creating a central zone without transformation. It is likely that a portion of the cells outside of this zone are also injured and impaired from subsequent division and growth. However, the modified or improved biolistic system in the name of PDS-1000- He - marketed by BIO-RAD Laboratory, which utilizes bursts of helium gas as the accelerating force and it was found gentle to the cells. Shock attenuating mechanisms placed within the sample chamber also moderated cell injury. The best recommendations for reducing cell injury during biolistic treatment are the helium accelerator should be configured with the flying disc, using 1000 psi or less, long target cell distances, medium rupture membrane to flying disc distances (9 mm), gold particles, baffles or meshes and of course, healthiest cells/organs.

Bt-Transgenic Cotton

Insect damage is a serious problem in cotton causing an estimated cost of Rs.600 crores/year by yield losses and insect control costs. In India, on an average 6-8 insecticidal sprays are applied in cotton crop and bollworms have developed resistant to many of the insecticides. Extensive and indiscriminate usage of organic pesticides have resulted in

deleterious consequences to human health, biodiversity and environmental quality. Since we are in need of safe and sustainable agriculture, it is imperative to deploy effective and eco-friendly strategies to manage insects. The most effective and cheapest method for protecting a crop from pests is through "Genetic resistance" of the crop to insect pests. In this case, the plants produce the resistance factors naturally and no additional producer inputs are required.

Genetic engineering provides us with valuable tools to develop transgenic crops carrying resistance to insect pests. Insecticidal protein genes present in a soil bacterium called *Bacillus thuringiensis* (BT) can be introduced into crop plants with great precision, without losing their insecticidal property and yet being safe to man and other organisms. Once they are integrated into plant chromosome, they are inherited and expressed like any other plant genes.

Through genetic engineering, genes encoding insecticidal crystal proteins (ICP) from a number of Bt strains have been cloned sequenced and expressed in a variety of hosts including bacteria such as *E. coli*, *B. subtilis* and *P. fluorescens* and plants such as tomato, tobacco and in cotton, where they give increased tolerance to insects pests. The ICP are classified as *cry* I to *cry* V based on amino acid sequence homology and insecticidal activity. Most *cry* I proteins are synthesized as protoxins (130-140 K Da) then proteolytically cleaved into an active toxin fragments (60-70K Da) in the insect midgut. Initial attempts to express Bt toxin from nuclear encoded chimeric genes in plants resulted in extremely low expression levels. Because Bt gene are high in Adenine/Thyamine (A/T) content and they exhibit abnormally low gene expression in plants due to number of factors such as premature transcription, termination, aberrant RNA splicing, mRNA instability and inefficiency in codon usage. In general, plants have a higher GC- (Guanine/Cytosine)content than that found in the delta - endotoxin gene, but cotton is having an even pronounced preference for high GC content in coding region. Modification of the coding sequence to increase the GC content of the Bt-gene results in a dramatic increase in expression of the insecticidal protein. Monsanto company, USA, reported first the development of transgenic cotton plants with Bt gene var.Kurstaki HD-1 *cry* I A (b) and *cry* I A (c) truncated genes in Coker 312 (American genotype). Transgenic cotton plants expressing upto 0.05 - 0.1 % of their total protein showed remarkable protection (70-87 %) of squares and bolls. In our research programme, synthetic Bt *cry* I A (b) gene have been transferred into Indian elite cotton cultivar LRK 516, however, the expression level of Bt toxin protein was very low (0.004% of their total protein). Therefore a strategy have been developed to make Bt- multigene construct for transformation and high level expression in the transgenic plants to protect the crop from insect and to break the development of resistance by the insects against the Bt-protein.

Field Performance of Bt-Cotton

Transgenic cotton plants carrying modified genes from *Bacillus thuringiensis* Berliner variety Kurstaki (BTK genes) that code for the delta endotoxins (insecticidal protein) Cry I A (b) from strain HD-1 and Cry I A (c) from strain HD- 73 have already been evaluated in the field for resistance against the devastating cotton bollworms. Initial BTK gene insertions into the cotton genome resulted in tissue expression of these insecticidal proteins that was too low to provide a useful level of insect control. Recently, genetic engineers have increased the expression of these insecticidal genes in cotton plant tissues by altering the coding sequence. Modification of key regions of the structural gene without changing the amino acid sequence resulted in the most

dramatic increases in levels of protein synthesis in the plant cells. Immunological analysis indicate that the altered BTK genes of *cry I A (b)* and *cry I A (c)* expressed the insecticidal protein at 0.05 - 0.1 % of total soluble leaf protein. Moreover, the level of plant protection against bollworms were much higher and had the potential for reducing larval injury to cotton in commercial production.

Genetic Variability of Bt-Cotton

There are two important sources of heritable genetic variability that could influence insecticidal protein expression and efficacy against target pest. Lepidopteran insects are the effects from the BTK gene insertion position in the plant genome (it is called as positional effects) and effects from the tissue culture plant regeneration process and which are called as somaclonal effects. The effect of foreign gene(s) insertion position on cotton physiology, morphology and growth is caused by the influence of the foreign DNA. For example, *cry I A (c)* base sequences plus selectable marker and promoter sequence, on the native genes at or near the position of insertion in the cotton genome and indirect effects caused by foreign gene expression and interaction with the cotton genome.

The successful expression of a foreign gene in plants could be predicted to be the result of many different factors. These include an efficient vector, the appropriate promoter, leader sequences, 3' non-coding sequences, the presence of potential volunteer plant regulator sequences, codon frequency, the secondary structure of the mRNA and the gene product itself. The tissue culture and plant regeneration effects are the result of the heritable genetic variability produced when whole plants are regenerated from somatic cells using plant tissue culture and regeneration techniques. These two sources of variability can independently effect any aspect of the plant that is under genetic control. Thus, in a crop improvement program to develop, insect-resistant cotton cultivars from transformed and regenerated cotton plants, it is critical to carefully screen a range of transformed and regenerated plants for the desired expression of agronomic and insect resistant characteristics.

Plant Defense Chemicals

Many plant synthesis specific chemicals associated with defense against pests and pathogens. Initially, these chemicals were considered as secondary plant compounds with unknown functions and are now considered to be defensive chemicals that are acting either alone or in concert to contribute to the resistance of plants against insects or pathogens. These defensive chemicals are found as either constitutive component in various plant tissues or synthesized in response to attacking insects pests. The inducible chemicals, which can also occur constitutively, include such complex substances as antibiotics, alkaloids and terpenes, as well as proteins such as enzymes, enzyme inhibitors and lectins. These plant defense chemicals generate inter and intracellular signals to activate gene that code for their production.

Induced defensive response to pests and pathogens are activated by signals released during the early stages of attacks. These are several potential signal found in the plant system including oligosaccharides, lipids (eicosapentaenoic and arachidonic acid), glycoproteins, enzyme proteins and hormones (IAA, ABA). The understanding of signaling of both

environmental and developmental responses is one of the major goals of present - day research in plant biology.

Many of these plants defense chemicals are the products of complex biochemical pathways, such as the synthesis of phytoalexins and lignin, which require the induced synthesis of several biosynthetic enzymes. Single gene that code for an enzyme has been extremely useful to understand signal-transduction pathways. Further these single gene defensive proteins such as hydroxyproline - rich glycoproteins, glycine - rich glycoproteins, chitinases, beta-glucanases, other PR-proteins, protease inhibitors and lectins have some practical advantage over genes that code for complex pathways. Such single gene character are amenable to transfer from one plant species or genus to another and expressing them, either with their own pathogen - inducible or insect inducible promoters or with constitutive promoters.

Protease inhibitor proteins are among the defensive chemicals in plant tissues that are both developmentally regulated and induced in response to insect and pathogen attacks. Proteins that form complexes with proteases and inhibit their proteolytic activity. In plants, there are 8-10 protease - inhibitor families and that have been found specific for each of the four mechanistic classes of proteolytic enzymes i.e. serine, cysteine, aspartic and metalloproteases. Members of the serine and cysteine proteinase inhibitor families have been more relevant to the area of plant defense than metallo- and aspartyl proteinase inhibitors. Serine proteinases have been identified to extracts from the digestive tracts of insects from many families, particularly those of the Lepidopteran and many of these enzymes are inhibitors. In the order Lepidoptera, which includes a number of crop pests, the pH optima of the guts are in the alkaline range of 9-11 where serine proteinases and metallo exopeptidases are most active. Additionally, serine proteinase inhibitors have antinutritional effects against several lepidopteran insect species.

An array of protease inhibitors including inhibitors of trypsin, chymotrypsin, elastase and carboxypeptidases, acting in concert, may add to the defensive potentials of the plant tissues. Genes coding for some of these inhibitors have already been isolated characterized and may be used to transfer from one plant to another as potential natural defenses against insect and pathogens, however the specification and affinities of the inhibitors with target enzymes must be considered.

Lectin is a class of proteins that also act on the digestive system of insects. These proteins have traditionally been associated with the seeds of legumes, but lectin type proteins occur in many plants. The mode of deleterious action of lectins on insect may be related to the chitin in the gut of some insects. Chitin is polymer containing N-acetylglucosamine, one of the polysaccharides to which many lectins bind. The lectins are thought to disrupt transport in those cells containing chitin. Lectin genes from monocotyledons to cotton have already been transferred however, the efficacy of the lectins on selected cotton against insects to be studied well.

Improved of Fibre Quality

Textile industry needs are changing considerably as a result of innovations of synthetic fibre industry. Because of their ability to provide a wide range of improved fibres in a timely fashion has enabled them to capture 62% of the textile market, while cotton's share has shrunk to

32%. To address this challenge, strategies have been devised to improve existing fibre properties such as length, strength and micronaire value and more importantly to add new properties to fibre (e.g., dye - binding properties).

Works are being carried out to determine the biochemistry and biology of fibre quality and correlate the relationship of fibre quality to yield and maturity. The fibre biochemistry and biology are used to identify "genes" that regulate fibre quality and thereby develop new approaches to select for cotton varieties with desirable yield and fibre. There are three possible approaches :

1. Select a protein that is likely to have an effect on fibre development determines if genetic variation for this protein exists and associated that variability with a specific fibre trait.
2. Use of multivariate analysis and discriminate functions to test plant biochemical and morphological traits for their association with fibre traits and build a model which correlates plant trait with traits for fibre quality.
3. Identify Mendelian markers (e.g. RFLP, RAPD, AFLP, Isozyme etc.), such that regions of chromosomes can be followed during inheritance and associate with quantitative trait loci.

The properties of cotton fibre arise from the manifestation of thousands of genes in cotton. The conventional cotton breeding, part of the gene pool from one cultivar is exchanged with that of another compatible cotton cultivar. However, their diversity of traits among different cotton cultivars is limited. Thus there is only a narrow range of properties that can be enhanced through plant breeding and new properties from other organisms can not be added. Recombinant DNA technology and new transformation methodologies can overcome this limitation. The critical task is to identify genes that can modify relevant fiber properties.

In order to modify fibre properties through genetic engineering, in addition to transformation capabilities, appropriate genes and promoters are required. Promoters are DNA elements that direct the expression of genes in tissues. Tissue-specific promoters activate genes in a tissue - specific manner. A number of fibre-specific promoters from cotton have demonstrated that they can be used to drive expression of genes in a fibre-specific manner in transgenic cotton. Of the many hundreds of potential genes available from various sources, only a few have actual value in fibre modification. Genes that would be useful include hormone genes from *Agrobacterium*, biopolymer genes from *Alcaligenes eutrophus* and genes encoding several cell wall protein.

Fibre initiation and development are known to be influenced by hormones. Thus, perturbing the hormone balance in the fibre may affect its development and properties. *Agrobacterium* has biosynthetic pathways that produce plant hormones, auxins and cytokinins. These genes can be isolated and modified for expression in fibre using fibre-specific promoters. Another potential gene system for fibre modification is the synthesis of bioplastic (polyhydroxyalkanoic acids - PHAs). A vast number of prokaryotes and eukaryotes synthesise various types of PHAs. The PHA molecules are energy reserves and are produced independent of nutrition. A large number of different bacterial PHAs have been detected some of which are co-polymers. These co-polymers are of economic importance because they behave like polypropylene and yet are biodegradable. Production of these polyesters in cotton fibre may result in a hybrid fibre that has properties of both cotton and polyesters. The PHA biosynthetic

genes from *Alcaligenes eutrophus* and a number of other bacterial strains have been identified. These genes can be transferred into cotton plants to produce bioplastic in the transformed plants and alter many of its properties.

Apart from fibre modification we need to improve a variety of cotton with superior dye-binding, thermal and surface properties to make cotton a more versatile fibre for textile industry. The genes necessary for these traits are not present in cotton. However, many bacteria, fungi and plants possess some of these traits, which can be transferred into cotton to enhance the existing properties or create entirely new one. In the near term we can expect novel fibre products and environmentally friendly acceptable fibre created by the introduction of genes from exotic sources into cotton.

Virus Resistance

The development of an understanding of the molecular structure of plant viruses has opened up new avenues to the engineering of virus resistance. For example, inoculation of plants with virulent strains of some viruses in the presence of an excess of virus coat protein has been shown to afford some resistance to the virus. Coat protein-mediated protection operates at the stage of uncoating of virus particles, a step common to all virus infections, and so may be applicable to DNA viruses as well as the RNA viruses. Extension of this finding to introducing coat protein genes from the virus to the host genome and thereby pre-arming the host with a supply of coat protein was a logical next step. There are now reports of virus resistance being incorporated into a range of species by introduction of coat protein genes into the plant genome, using *Agrobacterium* mediated transformation.

Antisense RNA in its simplest form can be produced by inverting a cDNA copy of a mRNA with respect to the promoter in an expression vector to give a full-length copy of the complement of a mRNA sequence. Fragments smaller than full length can also be effective. Antisense RNA molecules are thought to interact with mRNA molecules by base-pairing to form double standard RNA. Inhibition of expression of endogenous plant genes by expression of antisense RNA leading to observable phenotypic effects is also possible.

The presence of some kinds of satellite RNA in virus culture has the effect of decreasing the severity of disease symptoms. The aim of satellite RNA-mediated resistance is to introduce cDNA copies of a satellite RNA into plants so that the satellite RNA is replicated when the target virus infects the transgenic plants, thereby ameliorating the effects of the virus.

Inhibition of gene expression by ribozyme-mediated cleavage of a specific mRNA has also been demonstrated *in-vivo*. Recently, it has been reported that transgenic *Nicotiana tabacum* plants that express ribozymes against TMV show some resistance to TMV infection. The identification of a conserved sequence situated 5' to the initiation site of subgenomic mRNAs among 15 tymoviruses is of particular relevance for preventing virus infection of plants. This conserved sequence is 16 nucleotides long and has been named the tymobox. The tymobox sequence was used to design a ribozyme that was effective in clearing various different tymovirus-derived RNAs *in vitro*. Ribozymes could therefore be useful in engendering resistance to several viruses within a group where conserved sequences exist.

Other novel antiviral strategies probably still remain to be exploited. Expression of antibodies in transgenic plants has been described and they may be effective in interfering with virus infections. Endogenous plants, which confer resistance to virus infections probably still await identification and exploitation. Elucidation of the structures of virus-encoded proteins such as polymerases and proteases could eventually facilitate the design of novel polypeptides, which could bind to them and inhibit their functions. Further advances will probably lead to the development of other novel resistance strategies.

Herbicide Resistance

Herbicide treatments are an integral part of modern agriculture because they provide cost-effective increase in agricultural productivity by at least 12 %. Increased yield results from reduced weed competition for water, light and nutrient. Large quantities of herbicides are used even in developing countries where labour for weed control is very cheap. The treatment of herbicides destabilizes the function of soil eco-system and the major biological cycle of organic matter breakdown and nitrification is the most sensitive to these chemicals.

Genes that give plants tolerance to herbicides have been isolated and incorporated into cotton plants. For a number of technical and practical reasons, resistance to herbicides was among the first traits to which these new genetic approaches were applied. First, a specific target of herbicide action had been identified through physiological and biochemical studies. Secondly, genetic studies had shown that resistance to herbicides was a dominant trait exhibiting the simple Mendelian inheritance pattern of a mutation in a single nuclear gene. Dominance makes genetic selection of herbicide resistance mutants or transformants easier. The potential utility of herbicide resistance genes as dominant selectable genetic marker for research in plants (as antibiotic resistance genes in bacteria).

A part from insect resistance herbicide resistant cotton is also under cultivation. Monsanto Company, USA (Roundup ready cotton) has already developed the non-selective herbicide glyphosate tolerant cotton (GTCOT). The mode of action of glyphosate lies in the inhibition of the enzyme 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase, which is key catalyst in the production of aromatic amino acids. Since animals do not synthesize aromatic amino acids glyphosate has low toxicity to humans but broad toxicity to plants. Two different routes have accomplished resistance to glyphosate. In the first, a strong constitutive promoter was placed in front of a natural EPSP synthase gene so that, the enzyme was overproduced in the transformed plants. In the second, a mutated bacterial EPSP synthase gene that changed one amino acid in the enzyme protein resulted in the enzyme being insensitive to the herbicide. With an appropriate promoter, plants transformed with this gene were resistant to glyphosate. The principal motivation in developing glyphosate resistance in cotton is related to the broad spectrum of activity of the herbicide and its relatively low level of environmental contamination. Since the activity of the herbicide is directed against an enzyme in the chloroplast most plants are susceptible to it.

Another important broad-spectrum herbicide is bromoxynil, which is a benzonitrile compound that inhibits photosynthetic electron transport. An USA Company Calgene has developed transgenic cotton resistant to this herbicide. The gene (*bxn*) was isolated from the soil bacteria *Klebsiella ozeanae*, which codes for the enzyme nitrilase. This enzyme detoxifies the

compound by conversion to 3, 5-di-bromo-4-hydroxy benzoic acid in a single step (i.e. removal of nitrile atom from the compound and there by detoxifies it). A high level resistance in cotton to this herbicide allowed to control broad leaf weeds.

The sulfonylurea group herbicide are potent at low rates of application. The mode of action of this class of herbicide is inhibition of the enzyme acetolactate synthase (ALS). This enzyme is a key step in the synthesis of the branched chain amino acids, valine, leucine and isoleucine. Resistance to the sulphonylureas is usually associated with a mutated form of ALS and has been found in a number of cell cultures under selection pressure from the herbicide. Single amino substitution confers resistance. Phytogen Company has isolated the cotton ALS gene and used directed mutation at the critical codon to impart resistance. In the case the native promoter was maintained as well as the leader sequence for transport to the chloroplasts, the normal location of ALS.

Imidazolinones and sulfonylureas are among several newer classes of herbicides, which effectively control broad-leafed weed. These herbicides also act by inhibiting acetolactate synthase (ALS) also called acetohydroxy acid synthase (AHAS). The herbicide 2, 4-dichlorophenoxy acetic acid (2, 4-D) and several related phenoxy compounds have been used extensively for more than 40 years to control broad leaf weeds. Since monocotyledonous plants are naturally tolerant to 2, 4-D it is applied to control weeds, which grow among cereals, sugarcane, turf grasses and to manage forest undergrowth. Because 2, 4-D is highly volatile and tends to drift in the prevailing winds, nearby crops that are susceptible are often damaged. Cotton is notoriously sensitive to 2, 4-D and it will receive heavy damage from drift. One approach to reduce this damage is to incorporate resistance to 2, 4-D into cotton cultivars. Numerous strains of soil bacteria contain plasmid borne genes confer the ability to catabolize 2, 4-D. The gene *tfda* which degrade 2, 4-D has been isolated from pJP4 a plasmid found in *Alcaligenes eutrophus*. This gene *tfda* was shown to encode 2,4-D monooxygenase, which degrades 2,4-D into 2,4-dichlorophenol and glyoxylate. The gene was transferred into cotton cv Coker 312 by Agrobacterium mediation. This gene was transferred into cotton showed tolerance to 1.5 kg/ha 2,4-D, which is three times the highest recommended level used for wheat, corn, sorghum and pasture.

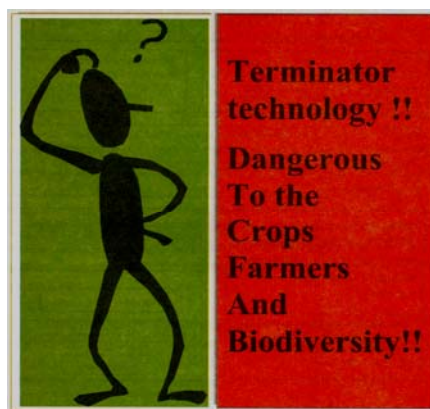
Terminator Gene Technology

The terminator gene technology is the biggest and the most controversial news in the transgenic plants and in the hybrid seed research. In March 1998 the US patent office granted the US Department of Agriculture and Delta and Pine Land Company (DPL) a patent for genetic engineering process that kill seeds. It is claimed that, the technology described in the patent has worked well with tobacco and cotton. Melvin Oliver, a scientist with USDA, ARS in Lubbock, Texas patented the work, through cooperative research with DPL. The patent claims a very broad protection and is valid for plant cells, tissues, seeds and whole plants of both transgenic and conventional crop varieties of any species. USDA and DPL also announced that they would make this technology ideally available to many seed companies through licensing agreements. According to them this will provide competition between the different seed suppliers and will be targeted for use in countries like China, India and Pakistan. The Rural Advancement Foundation International describes the technology as a "technology protection system". The patented processes will terminate the farmers independence and threaten the food security of over a billion

resource poor farmers in developing countries like India, where farmers are saving seeds to plant the following year.

In this background the Indian government has banned the entry into the country of any seed material that may carry the terminator gene and has also decided not to grant a patent to DPL for the terminator gene technology. The implementation of these decisions are being made to develop molecular probes to detect terminator genes in the seed, entering the country from outside. The technology in the patent could be applied in a number of ways. But in general, it involves three steps.

1. Transfer of terminator gene into a crop / transgenic plant.
2. The seed companies initiate the terminator process before selling the seeds to the farmer.
3. Farmers plant seeds grow plants and harvest mature but sterile seeds which is unfit for cultivation.

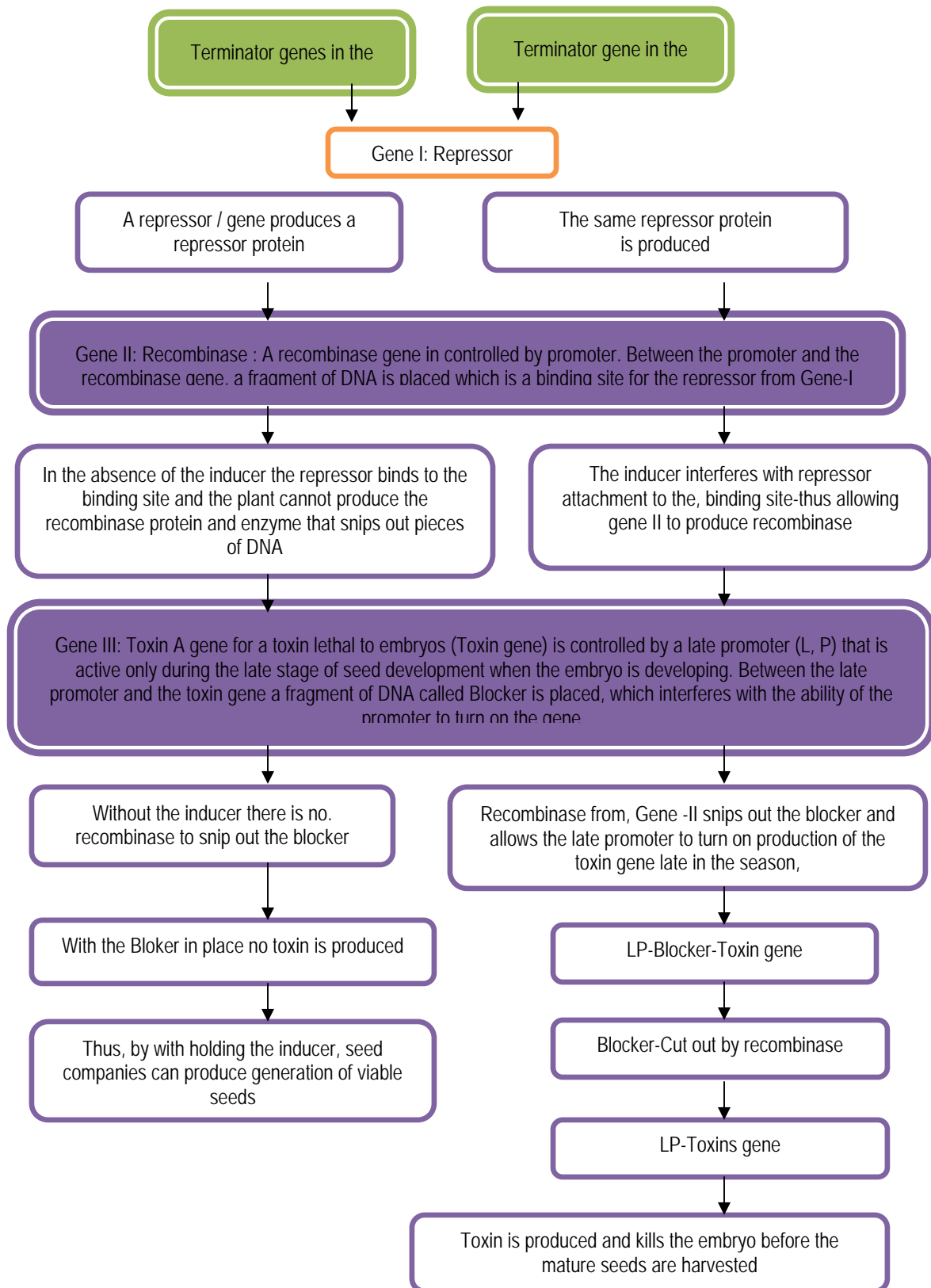


The strategy being the technology is to kill only the embryos and leaving other important seed components such as oils and proteins intact. The technology success depends on a cleverly controlled sequence of interactions among the spliced in genes. The last engineered gene comes into play very late in seed development when a special switch under the control of the inducer turns on the gene causing it to produce toxin. The toxin kills the embryo that is part of each mature seed.

An Outline of the Terminator gene Technology

The terminator gene technology consists of three genes with their on /off switches. Before selling to the farmers a seed company treats the seeds with a chemical inducer probably tetracycline to initiate the terminator gene interactions.

Terminator gene technology has more troubling implications for the developing countries where seed saving is more widely practiced especially in India. Poor farmers are frightened anticipation of a danger at the prospect of seed markets, dominated by multinational corporations selling sterile seeds. They fear increased seed costs, must be repurchased every year and loss of control over own food supplies. What poor farmers need is inexpensive, locally adapted seed that can be easily saved for cultivation.



In India, for example, concern about the terminator gene technology and other application of genetic engineering led farmers to uproot and burn genetically engineered Bt-Cotton. The intensity of the reaction is based partially on the misconception that the terminator gene is already in commercial crops. Although its implementations is still some more years away, the reaction of farmers are an indication of the likely response to the terminator gene crop when it comes for cultivation.

The USA also feels that the terminator gene containing crops are a serious threat to the right of the farmers as breeder, users and managers of bio-diversity to save, exchange and improve seeds in the time-honored way. It also points out that pollen from the crops carrying the terminator gene could infect the fields of farmers who either reject or cannot afford the technology. All these concerns about the impact of terminator gene technology on poor farmers have also led the United nations funded consultative group on International Agricultural Research to recommend that its sixteen member institutes ban the technology in their crop Improvement research programs.

More recently, Zencea of UK indicated that it would seek patents in more than 50 countries for its improved plant germplasm invention. This technology will prevent plant growth, rather than killing the embryo of seed. The technology makes use of a gene from the fat tissues of a rat, which will block the normal plant growth unless the blocking process is deactivated by a chemical treatment. The technique has been dubbed as "verminator" by RAFI and appears to be wider and more flexible than the terminator, though intended to serve the same process.

The Indian government has already decided that it will not grant a patent to the US firm DPL (Delta and Pine Land Ltd.) for its controversial terminator gene technology. The US firm DPL applied to the Indian patent office for this particular biotechnological product, but the government has banned the gene in imported seed material. Recently the Agriculture Ministry's plant quarantine office instructed that the import permit authority should ask importers to get an additional declaration of Phyto-Sanitary certificate that the seed is free from the terminator gene.

GLOSSARY

Biotechnology: The application of recently developed skills in microbial and biochemical technology to applied biology, i.e. to the exploitation of biological systems and processes for our own use

Biolistic: Process by which DNA molecules are propelled into a recipient cell using coated microprojectiles shot from a gene gun. The method of propulsion may vary and ranges from electric discharge to helium blast.

CaMV: Cauliflower mosaic virus. One of the few DNA plant virus. Its 35 S promoter is often used for transformation experiments. It is a constitutive promoter.

Callus: An unorganized, proliferate mass of differentiated plant cells; a wound response.

cDNA: Complementary Of copy DNA; DNA made by reverse transcriptase enzyme from RNA.

Genetic engineering: Manipulating the genetic material (genes) using *in vitro* process. It involves gene isolation, DNA cloning and usage of tissue culture technology. It may result from transfer of gene from its normal location into a cell, which does not possess it. **In vitro:** Latin: meaning in glass (i.e. inside a test tube)

In vivo: Latin: in real life

In situ: Latin: in the natural location

Meristem: Organized zone of mitotic division giving rise to cell clusters capable of further differentiation into new organ types.

Organogenesis: In plant tissue culture, a process of differentiation by which plant organs are formed *de novo* or from preexisting structures.

PCR: (Polymerase Chain Reaction) A method for amplifying DNA of any organism using two specific oligonucleotide primers: (about 10-15 base pairs in length) which flank the region of interest. The method has extreme value in diagnostics forensics and general molecular biology like sequencing, probe preparation, genome mapping etc. The inventor K. Mullis received the Nobel prize in 1993.

Plasmid: Circular, covalently closed DNA molecule commonly found in bacteria. Plasmids are used as cloning vector in genetic engineering.

Primer: Short sequence of DNA used to initiate DNA replication.

Promoter: Regulatory region of a gene involved in the control of RNA polymerase binding to the target gene.

rDNA : Recombinant DNA; made by joining of DNA fragments from different species using restriction endonuclease and cloning approaches.

RAPD: (Random amplified polymorphic DNA) The method is used to distinguish organisms, gene mapping and diagnostics. It uses single primers (Nine nucleotides and larger) and amplification products are generally visualized using agarose electrophoresis and ethidium bromide fluorescence. Usually 4-10 products are generated.

Regeneration: In Plant culture, a morphogenetic response to a stimulus that results in the production of organs, embryos or entire plants.

Selectable marker: Gene encoding a protein that detoxifies a chemical - the chemical is usually included in culture media to allow preferential growth of cells that have integrated foreign DNA.

Southern hybridization: Also called Southern blotting, a method employing gel separation of

restricted DNA fragments, their blotting onto a membrane support dissociation into single stranded DNA and hybridization (reassociation) with labeled probe. Regions of homology are detected usually by autoradiography. Invented by E. SOUTHERN in 1975.

Screenable marker: Gene encoding a protein that results in a visible product, allowing identification of cells that express the gene.

Somatic embryogenesis: In plant culture, the process of embryo initiation and development from vegetative or non-gamete cells.

Transformation: This term implies the transfer of a gene to a new cellular environment coupled with the expression of that gene to alter the phenotype of the recipient cell may be transient or stable as judged by inheritance.

Transgenic plant: A plant transformed with foreign gene(s) (economically important gene/marker gene) and expressing in the new genetic background.

---- End of the reports ----