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BIOTECHNOLOGY IN PLANT RESISTANCE TO INSECTS

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Abstract: *For centuries, humans have searched for crop plants that can survive and produce in spite of insect pests. Knowingly or unknowingly, ancient farmers selected genes for pest resistance in their crops, sometimes by actions as simple as collecting seed from only the highest yielding plants in their fields. With the advent of genetic engineering and several tools of biotechnology viz, anther culture, embryo culture, protoplast fusion, Somaclonal variation, marker assisted selection and genetic transformation for insect resistance now can be moved into plants more quickly and deliberately. Genetic engineering viz, durable insect resistance, tissue specific expression, vertical insect resistance, marker assisted selection for plant resistance to insect and inhibitors of several digestive enzymes may be used to develop insect resistant crops now and in the future. Advances in biotechnology will accelerate the development of insect-resistant plants. The acceptability to biotechnology products may be greater along with the increase in more understanding of biotechnological processes.*

Keywords: *Pest resistance, Somaclonal variation, genetic engineering, enzyme inhibitors and protoplasm fusion.*

Introduction: The development of insect resistant plants has been started in 1782 since Havens published a paper about a wheat cultivar resistance to Hessian fly. Since then, numerous insect-resistant plants have been developed by the international and national research center, private sector through conventional method or biotechnology. For example, International Rice Research Institute (IRRI) has developed and released numerous rice variety resistant to brown planthopper, green leafhopper, rice stem borer, and rice gall midge. Several transgenic insecticidal cultivars obtained through biotechnology approach have also been developed, such as Bt-corn, Bt-cotton, and Bt-rice.

In breeding program for insect resistance plants, the first step is to identify the parents or donors of resistance. These may be cultivated germplasm, landraces, weeds races, wild species, or different species. The breeding method used to develop insect resistance plants based on the following factors: the source of donor of resistance, the efficiency and certainty of selection of progenies. If the donor of resistance is commercial varieties, they may be improved by pure line or mass selection or hybridized with elite germplasm. If they are land races, weed races, or wild species, they have to be hybridized with the elite germplasm. If the donor of resistance is different species, biotechnology method may used, such as genetic transformation or protoplast fusion. If the donors have not available, biotechnology method may be used, such as somaclonal variation for insect-resistance. If the efficiency and certainty in the selection of desired traits were needed, the use of biotechnology method such as marker assisted selection and anther culture may be appropriate. Biotechnology approach includes protoplast fusion, somaclonal variation, molecular assisted selection, and genetic. For anther culture and embryo culture, at the beginning they used organism of F1 plants to produce pollen grain and wild and cultivated species to produce embryo. However, since in the following steps they may be using cells culture, so we consider

the anther culture and embryo culture as a biotechnology approach.

Anther Culture: Anther or pollen grain could be cultured in vitro using artificial medium. On the artificial medium anther may form callus, shoot, root, and finally the entire plants. All the plant are haploid. This approach is possible to speed up the formation of homozygous population of insect-resistant plants.

Embryo Culture: Wild species are often more resistant to insects. Wide hybridization to transfer genes conferring insect-resistant from wild species to cultivated plants will produce abnormal inter-specific hybrid embryos. The embryo can be rescued by culturing it on the nutrient medium to generate the entire plants. Several genes for insect resistance have been transferred from wild to cultivated germplasm (Table 1).

Table 1. Genes conferring insect resistance transferred from wild to cultivated species

Recipient	Alien donor	Trait
Bread wheat	*Secale cereale*	Resistance to greenbug
	S. cereale	Resistance to Hessian fly
	Aegilops squarrosa	Resistance to Hessian fly
Rice	*Oryza officinalis*	Resistance to BPH
	O. officinalis	Resistance to WBPH
	O. australiensis	Resistance to BPH
	O. minuta	Resistance to BPH
Peanut	*Arachis monticola*	Resistance to chewing insect
Lettuce	*Lactusa virusa*	Resistance to Aphids
Cotton	*Gossypium armourianum*	Boll weevil, leafworm, bollworm

Protoplast Fusion: Protoplasts are plant cells that could be isolated by digested the wall enzymatically. The traits of resistant to the pest may be present in the one of two species that cannot be hybridized sexually. The two species may be formed hybrids through protoplasts fusion. The protoplasts may be cultured on artificial medium and some protoplasts will grow into entire plants. The plants may be carried the resistant traits.

Somaclonal Variation: Somaclonal variation may be used to select insect-resistant variants. Insect-resistant somaclones can be selected through the following steps (1) calli or cell suspension derived from high yielding variety were grown for several or long term cycles, (2) the long-term cell lines were regenerated into plants, and (3) the regenerated plants were evaluated against target insects. About 2000 plantlets of sugar cane were evaluated for resistance to the sugar cane borer under artificial infestation as well as natural infestation in field plots. Some somaclones showed resistance to sugar cane borer. The same method has been used to obtain somaclones of sorghum showed resistance to the fall armyworm.

Marker Assisted Selection: Nucleic acid probe, antibodies, or enzymes may be used as a marker assisted selection in breeding program. Those probes may be used to determine the genetic constitutions of plants or plant extracts, includes the present of resistant traits. The precise information provided by this method will increase the speed and certainty of selection progeny carry resistance traits in conventional plant breeding. The probes that tight linkage to the resistance traits have been identified (Table 2).

Table 2. The probes for marker assisted selection

Crop	Trait of resistance	Genes	Probes
Rice	WBH	Wbph-1	RG146
	BPH	Bph-10(t)	RG457
	Gallmidge	Gm-2	RG476, RG776, RG 224
Mungbean	Bruchid beetle	-	PA 882
Wheat	Hessian fly	H-23	XksuH4, XksuG48(A)
		H-24	XcnlBCD 457, Xcnl CDO 482, Xksu G48 (B)

Genetic Transformation: Genetic transformation refers to the introduction of cloned DNA segments or genes from plants, bacteria, or animals into a new genetic background. The DNA segments or genes introduced into the protoplast or cell may confer

resistance to insect. The genes may be delivered to the protoplast or cell by using one of the following methods: Agrobacterium, biolistic, electroporation, PEG, vortex, and microinjection. Several resistant plants have been developed through genetic Transformation.

Table 3. Resistant plants develop through genetic transformation

Plants	Traits
Alfalfa	Insect resistance
Cabbage	Insect resistance
Cotton	Resistance to bollworms and budworm (Bt toxin)
Eggplant	Insect resistance
Maize/Corn	Resistance to corn borer (Bt toxin)
Potato	Resistance to Colorado potato beetle (Bt toxin) Resistance to Potato tuber moths
Soybean	Insect resistance
Sugar cane	Insect resistance
Sweet potato	Insect resistance
Tobacco	Insect resistance
Tomato	Insect resistance
Rice	Insect resistance (Bt toxin)

Strategies to Develop Insect-Resistant Plants: Sooner or later an insect resistant plants will become susceptible due to the development of new bio-types. To anticipate the breakdown of an insect-resistant plants, various strategies should be adopted in developing those plants. One strategy is to prolong the useful life of insect-resistant plants. This can be realized by developing durable insect-resistant plant. The durable resistant plant is governed by polygenes. Durable resistance is also referred to poligenic resistance or horizontal resistance. The other strategy is to develop varieties with different genes conferring insect resistant. This may be achieved by developing vertical resistant plants. The vertical resistant plant is governed by major genes. The more vertical resistant varieties with different genes is available, the more easy farmer access to new varieties to replace the previous varieties.

Durable Insect-resistant Plants: The level of resistance of durable insects-resistant plants is generally not very high. Because of this low selection pressure, the development of new biotypes is very slow. Polygenes resistance could be obtained from the landraces. Once crossing has been made between cultivated and landraces, we face to the difficulty in the selecting the desired segregants. This may be due to not all the polygenes or QTL from landraces are transferred to cultivate and the level of resistance is diluted. The drawback is that the screening techniques currently available are only applicable to identify segregants with high level of resistance not segregants with low level of resistance. Molecular-assisted selection offers great role to facilitate the development of durable insect-resistant plants. However, the first step should be done is to tagged the QTL for insect resistance with molecular markers. Molecular marker based selection will assist in the accumulation of polygenes. QTLs for resistance to rice brown planthopper have been tagged with RFLP markers.

Vertical Insect-resistant Plants: Vertical insect-resistant plants are easier to develop since major genes are easier to transfer from one variety to others. To anticipate the emergence of new insect biotypes that overcoming resistant plant, numerous vertical insect-resistant plants with different genes should be develop through conventional or biotechnology. Numerous rice varieties with different genes resistance to BPH have been produced by IRRI. Insect-resistant transgenic plants developed through genetic transformation currently available are generally vertical resistance with the major genes, such Bt-gene.

To anticipate the development of new biotype overcoming Bt-plants, others resistant plants with different genes should be develop. Pyramiding of major genes may be one of strategy to develop the longer useful life of resistance plants. This approach needs reliable method to determine the present of the two genes. If bioassay method will be adopted, several insect biotypes should available for this purpose. The use of molecular markers is very reliable method to diagnose the integration of the two genes in the plants. Pyramiding of major genes may be achieved through genetic transformation, for example pyramiding the two Bt genes or combining genes encoding a toxin and a gene encoding a repellent.

Tissue-specific Expression: The constitutive expression of resistance genes at all time and in the whole tissue may be caused a great selection pressure that leads to the development of new

insect biotypes. This problem may be solved by expressing the resistance traits in a specific tissue, or at certain growth stages, or only in response to insect feeding. Promoters to regulate these expressions are available.

Marker Assisted Selection for Plant Resistance to Insect: Co-evolution of host plants and their pests is a continuous process. The host evolution in the nature is relatively slower than that of its pests. In this race, therefore, the host often loses out and thus remains in a disadvantageous position. In a few cases, the host somehow succeeds in managing to protect itself by evolving newer genetic mechanisms, but this happens at the cost of productivity. Weedy traits co-evolve with the host resistance /tolerance for imparting greater adaptability and thus increased fitness for survival, although with a penalty on yield performance. Plant breeders have been striving to improve crop productivity simultaneously by increasing yield potential and by reducing the losses due to pests through conventional means. With the development and use of novel bio technological tools and techniques now available to the breeders, this process of developing high yielding and stable crop varieties with pest resistance is being greatly facilitated. One such area is the use of molecular markers. The objective of this brief review is to describe the important molecular markers and highlight their applications in breeding crop plants for pest resistance with specific examples,

Molecular Markers: Molecular markers can be defined as the differences in the nucleotide sequence of DNA at corresponding sites on the homologous chromosomes that follow a simple Mendelian pattern of inheritance [1]. These differences are detected by employing various techniques such as Southern blot hybridization, Polymerase Chain Reaction (PCR) and DNA sequencing. Majority of the markers in use today are based on PCR. Depending on the method of detection of the sequence variation, the molecular markers have been given different names.

Molecular Mapping of Genes for Pest Resistance: Mapping and tagging of the genes conferring resistance on the host plant against pathogens, nematodes and insect pests is a prerequisite for their marker-assisted transfer to desired genetic backgrounds. Host plant resistance to the pests can be monogenic or polygenic. Molecular markers have been used for mapping genes for both monogenic and polygenic resistance. For mapping monogenic resistance, three approaches are being employed: (i) use of markers selected from a complete genome map and study of their co-segregation with the trait in a segregating population, (ii) use of random unmapped markers to differentiate near isogenic lines followed by validation of marker trait linkage in a segregating population, and (iii) use of random markers to differentiate two DNA bulks made from phenotypically different segregants identified in a mapping population followed by confirmation of linkage using the individual plants of the population. The first method is the most direct but needs a complete linkage map. The second method needs a pair of near isogenic lines, which have to be generated. This requires several years/seasons. The last method is considered fastest. Although RAPD markers were first used in conjunction with bulked segregant analysis (BSA) of a F2 mapping population, AFLP is more commonly being used in the recent years. Most of the genes for monogenic traits have been mapped using the third approach in crop plants

Experimental Approaches for Marker Assisted Breeding for Pest Resistance: Molecular markers can be used in a variety of ways in different plant breeding schemes. In most of the cases reported so far, markers have been used in backcrossing programmes. There are not many attempts made so far to analyze the theoretical considerations and provide experimental steps in marker-assisted breeding. There can be three distinct situations in breeding for pest resistance using markers: (i) Transfer of the desirable gene/QTL from a donor to a suitable agronomic back ground through strict backcrossing, (ii) Recombining pest resistance gene from one of the parents with agronomic traits from both parents in pedigree breeding or a combination of limited back-crossing with pedigree method and (iii) Advanced backcross method particularly for simultaneous detection and transfer of QTL for pest resistance available in a related species. The first method has been highlighted in Fig. 1. Here, one has to use the markers for selection of the desirable segregants (heterozygous resistant in case of dominance of resistance over susceptibility or heterozygous carrier when resistance is recessive to susceptibility) in every backcross generation. The number of plants to be genotyped can be reduced by exercising selection for other phenotypic traits of the recipient parent such as plant height, flowering and maturity. After the

third backcross, more than 99% of the recurrent parent genome can be recovered in case both foreground and background selections are practiced.

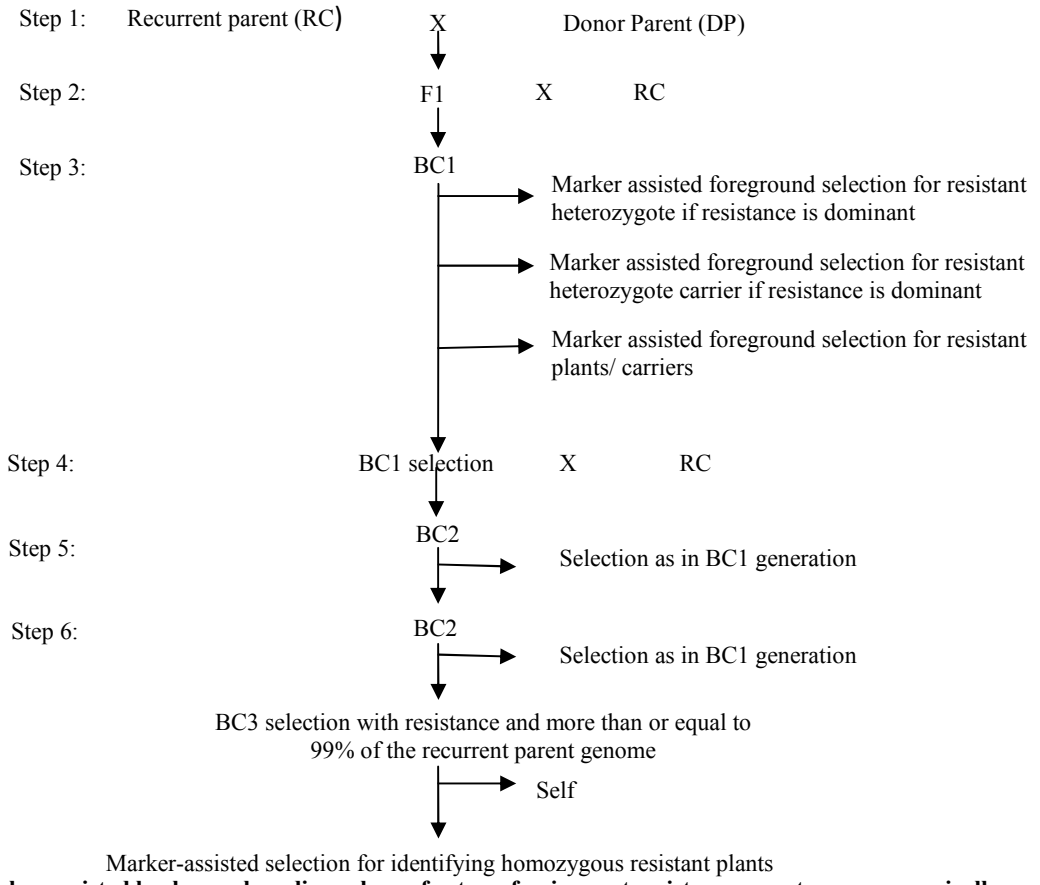

Fig. 1: Marker-assisted backcross breeding scheme for transferring pest resistance gene to an agronomically superior variety.

Use of Markers in Breeding for Pest Resistance: Plant breeding involves creation of genetic variation and selection of the desirable variants/recombinants, which are subsequently evaluated and Released for commercial cultivation. Selection of the desirable variants/recombinants has been based on the phenotype. Success of such selection largely depends upon the skill of the concerned plant breeder. Selection of a genotype carrying desirable gene or gene combination via linked marker(s) is/are called marker-assisted selection. Breeders sometimes practice marker-assisted selection when an important trait, that is difficult to assess phenotypically, is tightly linked to another Mendelian trait* which can be easily scored. For example, a gene for purple coleoptile color in some traditional rice varieties is closely linked to a gene that confers resistance to brown plant hopper (BPH). In a segregating population like F2, about 95% of the planti showing purple coleoptile are found resistant to BPH. In this case, coleoptile colour is a morphological marker, which is used to assist selection for BPH resistance. Morphological markers are however, limited in number, are specific to particular genotypes and are dominant. Morphological markers may also show tissue and developmental stage specific expression, pleiotropy and even at times, adverse effect on plant growth, vigour and viability. Molecular markers do not suffer from these limitations and thus offer advantages over the morphological markers. Molecular marker assisted selection involves scoring for the presence or absence of a desired plant phenotype indirectly based on DNA banding pattern of linked markers on it gel or on autoradiogram depending on the marker system.

In soybean Marker Assisted Selection offers the potential to reduce linkage drag and to pyramid genes with similar phenotypic effects into elite genotypes. One such example was seen in soybean breeding programme where a QTL conditioning corn earworm resistance in the accession PI229358 and a synthetic *Bacillus thuringiensis crylAc* transgene from the recurrent parent 'Jack-131' were pyramided into BC2F3 plants by marker-assisted selection, Segregating

individuals were genotyped at SSR markers linked to an antibiosis/an tixenosi s QTL on linkage group M, and were tested for the presence CryAc IAS was used during and after the two backcrosses to develop a series of BC2F3 plants with or without *cryAc* Transgene and the QTL conditioning

Genetic Engineering: With the advent of genetic engineering, practically any gene from any organism can be isolated, sequenced, artificially synthesized and cloned into a simple prokaryotic system such as *Escherichia coli* and *Agrobacterium tumefaciens*. A scheme of gene (DNA) isolation, construction of recombinant DNA (r DNA) and its cloning is presented in Fig. 2. Transgenic plants (genetically engineered, modified or transformed) are those which carry functional foreign genes. These foreign genes can either be genomic or *chimeric* which carry the information for the synthesis of a particular protein from one species and *regulatory gene* (promoter, etc.) which is important for the expression of structural genes from another species. These promoter sequences determine how much protein is to be synthesized by a structural gene. In general, the structural genes whose products are required in greater amount have strong promoters and those which synthesize lesser protein have weak promoters.

In genetic engineering, the rate of expression of a structural gene can be increased by putting a strong promoter upstream in a particular gene. When the recombinant DNA (chimeric gene) containing the desired gene under the control of a strong promoter is inserted into the host organisms, the organism will then produce the desired protein in large amount. Promoter sequences of nopaiine synthase gene (NOS) from A. *tumefaciens* and cauliflower mosaic virus 35S subunit gene (CaMV 35S) that allow constitutive expression of structural gene have been widely used. In a truncated gene, some DNA sequences from structural genes are deleted, the truncated gene being able to produce a product which performs the same primary function as the complete product of the gene. It has been observed that in a transgenic plant, intact gene sometimes docs not express whereas the truncated gene is able to express itself.

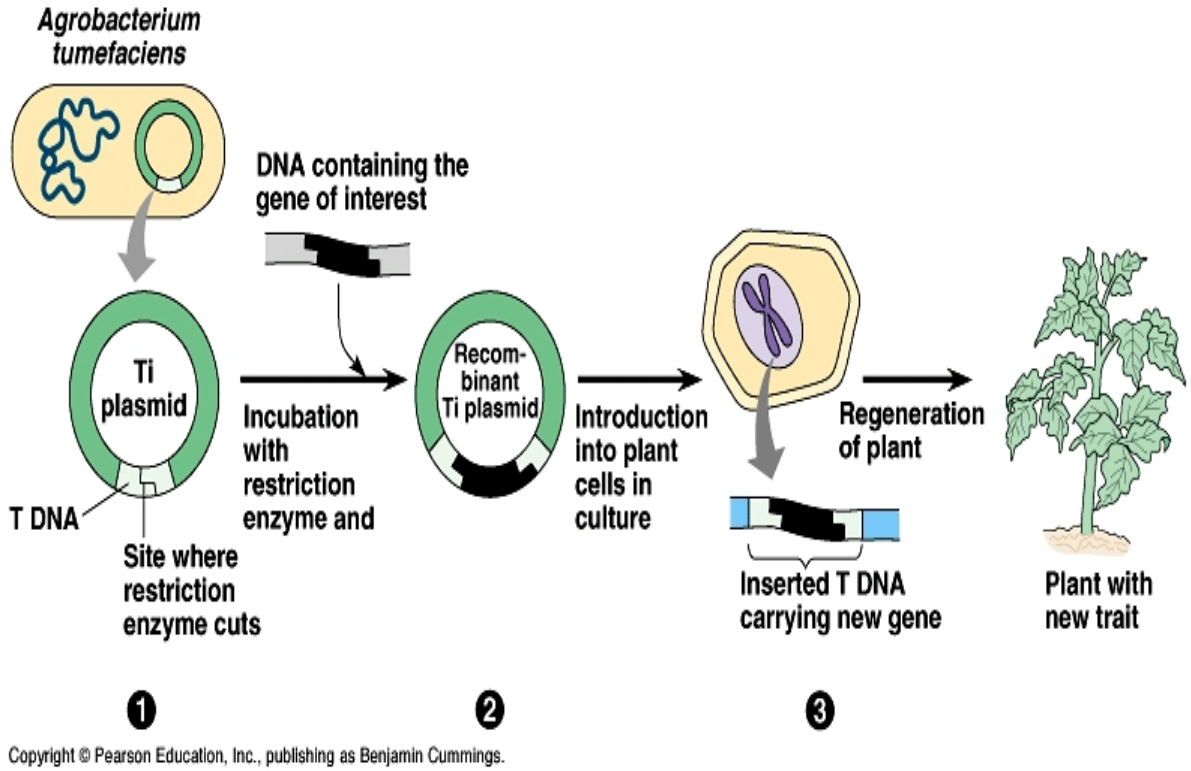

Fig. 2: Gene transformation

Vector-mediated Gene Transfer: A vector is a vehicle which transports the foreign genes into the recipient cells, protoplasts or intact plant. It is a DNA molecule, capable of replication in a test organism, into which a gene is inserted to construct a recombinant DNA molecule. This method is also called indirect method of gene transfer, A vector could be either DNA virus such as caulimovirus or geminivirus or plasmids such as Ti (tumor inducing) and Ri (root

inducing) plasmids of *A. tumefaciens* and A *rhizogenes,* respectively.

(i) ***Agrobacterium-mediated Gene Transfer****: Agrobacterium* is the natural genetic engineer of plants. For a long time, perhaps millions of years, the common soil bacterium, *A. tumefaciens* has been doing what molecular biologists are now striving to do. It has been inserting foreign genes into plants and getting the plants to express these genes in the form of proteins. In the process, the bacterium causes the plant cells to proliferate and form a gall or tumor, most commonly at or near the junction of the roots and stem, the crown.

A tumefaciens is a gram negative soil bacterium which infects a wide range of dicot plant species. It has been demonstrated that a virulent bacterium in addition to its chromosomal DNA carries Ti plasmid. During infection, Ti plasmid transfers a portion of its transfer DNA (TDNA) into the plant cell and becomes integrated into the chromosomal DNA of plant [2]. TDNA segment of Ti plasmid carries a number of genes encoding enzymes for the synthesis of phytohormones such as cytokinins and auxins which stimulate the growth and division of the plant tissue resulting in the formation of characteristic tumors and the production of specific metabolites called opines such as octopine and nopaline. Foreign genes inserted within the T-region of the Ti plasmid are transferred to and stably integrated into the plant genome.

(ii) ***DNA Viruses as Vectors:*** The genomes of caulimoviruses such as cauliflower mosaic virus (CaMV) and geminivrus such as tomato golden mosaic virus (TGMV) are double stranded DNA which make these viruses as potential transformation vectors. There is only one example where methotrexate resistant dihydrofolate reductase (DHFR) gene of E. coli has been successfully cloned into an intergenic region of CaMV. In this method, viral DNA uses the ability of the *Agrobacterium* to transfer DNA from bacterial cells to plants. The combined use of the tumor inducing plasmids and viral DNA opens the way to molecular biological approaches that are not possible with either system alone.

Vectorless Gene Transfer: *A. tumefaciens* is established as a vector for gene transfer in many dicotyledonous plants but is not accepted as a vector in monocotyledonous plants. However, recently this bacterium has been successfully used in the transformation of *Zea mays* L. Some non-biological methods for introduction of foreign genesinto the plants have been developed. These methods use chemical or physical means to facilitate the entry of DNA into plant cells.

(a) ***Direct uptake of DN:*** In the presence of some chemicals like polyethylene glycol (PUG), plant's protoplast is capable of taking up DNA. Using this chemical, kanamycin resistance gene in the form of plasmid DNA has been inserted into the protoplast and regenerated fertile tobacco plants have been recovered [3]. This gene was inherited in a Mendelian fashion. The synchronized protoplasts have significantly higher frequencies of DNA uptake than unsynchronised protoplasts.

(b) ***Elcctroporation:*** In this technique a high voltage current is applied in a pulsed manner which creates tiny holes in the plant cell membrane. These holes are large enough for DNA molecule to diffuse into the cell. The cells recovered from the electric shock can be regenerated into whole plants.

(c) ***Microinjection:*** Injection of DNA directly into the plant cell nucleus or intact plant organ has been used to develop transgenic plants.

(d) ***Microprojectile bombardment:*** This is also called *biolistic approach.* In this technique, metallic particles carrying DNA are accelerated to high velocity by a particle gun apparatus. Using microprojectiles carrying chloramphenicol acetyltransferase (CAT) gene, it has been demonstrated that extracts from treated tissue showed varied levels of chloramphenic acetyltransferase activity indicating that micro projectiles can effectively deliver DNA into intact tissue. This methodology has been used to develop transgenic plants of soybean having *GUS* and *NPTH* genes, however, microprojectiles were accelerated using high voltage electric discharge.

Transgenic Plants: A transgenic plant is simply a normal plant with one or more additional genes from diverse sources Scientists have bred crops for resistance against insect pests for a long time. The application of transgenic plants through genetic engineering is the latest concept in insect pest management [4]. These transgenic plants produce insecticidal or Antifeedants proteins continuously in the plants under field conditions. The particular advantages genetic engineering offers are that derived genes can be transferred without co-transfer of undesirable characteristics and it enables the transfer of genes across species barriers. In this approach, identification of useful genes to be transferred is a limiting factor.

Because of this reason, the genes conferring insect resistance transferred by this approach are mostly limited to Bt endotoxine gene and cowpea protease inhibitor (CpTi) gene.

Insect Resistant Transgenic Plants Expressing *Bacillus thuringiensis* Toxin: Bt preparations have been used for many years as an "organic" insecticide that is sprayed onto plant tissues. However, the utility of Bt as a conventional insecticide is limited by instability of the protein when exposed to UV light and poor retention on plant surfaces in wet weather. The high level of toxicity of the Bt toxin protein, and the ease of isolating its encoding gene from bacterial plasmids, made it an obvious choice for initial experiments attempting to produce insect-resistant transgenic plants [5].

Genetic Engineering of Plants to Express *Bt* Toxins: Whereas the isolation of genes encoding *Bt* toxins was an easy task, subsequent engineering of transgenic plants that expressed these toxins proved much less straightforward. in fact, considerable to the Bt toxin genes has proved necessary in order to obtain adequate expression to confer insect resistance on transgenic plants. The necessary modifications have fallen into two classes; alteration to the protein sequence of the Bt toxins and alteration to the gene sequences.

Bt toxins from different strains vary widely in their spectrum of activity. The most important are those produced from a strain *kurstaki* called *Btk* effective against lepidopteran pests of forest trees, vegetables, cotton and ornamentals. These products have been sold since the early 1960s and *account* for bulk of *Bt* applications. In the late 1970s, another strain called Bti (i for *isradensis)* was identified. The products of this strain are effective against dipteran insects. It is estimated that more than 2000 tones of these preparations have been applied worldwide with no undesirable effects. However, these formulations are expensive to produce, are of very poor persistence under field conditions and there is a need of carefully timed repeated treatments. In order to overcome these limitations, it was recognised that expression of *B. thuringiensis* toxin genes in transgenic plants might increase their utility in insect control.

Bt endotoxins are attractive candidates for insect-resistant crop development using transgenic technology because (i) they have an established safety record, (//) they act rapidly and are completely biodegradable and proven safe to humans and non-target organisms and the environment, and *(Hi)* the endotoxins are the products of single genes and are highly effective against the larvae of Leptdoptera, a major group of destructive insect pests. Major research, has been conducted using the lepidopteran specific 6-endotoxin of *Bt* strain *kurstaki*

Plant Genetic Systems, a Belgian Biotechnology Company, in July 1987 were the first to report development of transgenic plants of tobacco containing δ endotoxin in enough amount to kill the first instar larvae of *Manduca sexta* (Johannsen) and *Heliothis virescens* (Fabricius) within 3 days. Similar results were reported by a group of researchers from Monsanto Company using tomato, and Agrautus Company using tobacco. The transgenic tomato plants expressing *Bt* gene have been extensively evaluated under field conditions. Plants infested with tobacco hornworm showed very limited feeding damage compared to non-transgenic control plants which suffered heavy feeding damage and were almost completely defoliated within two weeks. Significant control of tomato fruit worm and tomato pinworm was also reported.

The first step in developing transgenic plants carrying 6-endotoxin is to look for local strains of *Bt* toxic to the insect to be controlled. Once the strain has been selected, then genome is identified and genes of toxin are isolated and introduced into the crop. With few exceptions, most of the graminaceous crops are not readily susceptible to infection by *A. tumefaciens* which is a vector for gene transfer commonly used with many dicot species. Genes can be transferred directly into protoplasts, without any *Agrobacterium* vector, by methods that permit DNA to cross the plasmalemma. Stable transformation of maize cells has been achieved through direct uptake of DNA into protoplasts that had been permeabilized by elcctroporation and plants had been recovered from maize protoplasts. The transgenic maize plants produced high levels of insecticidal protein and exhibited excellent protection against extremely high and repeated infestations of *Ostrinia nubilalis* (Hubner), a major pest of maize in North America and Europe.

Transgenic plants carrying *Bt* genes have now been produced in a wide range of crop species including tobacco, tomato, potato, cotton, maize, rice, broccoli, oilseed rape, soybean, walnut, larch, poplar, sugarcane, apple, peanut, chickpea and alfalfa with different crystal protein genes.

Table 4: Transgenic plants carrying Bt genes for insect resistance.

Plant	Gene (s)	Target insect pest(s)
Cotton	Cry 1A(b), Cry 1A(c)	*Helicoverpa zea, Pectinophora gossypiella, Spodoptera exigua, Trichoplusia ni*
Egg plant	Cry (III) b	*Leptinotarsa decemlineata*
Maize	Cry 1A(b)	*Ostrinia nubinalis*
Poplar	Cry 1A	*Lymantria dispar*
Potato	Cry 3A	*L. decemlineata*
Rice	Cry 1A(b), Cry 1A(c)	*Chilo suppressalis, Cnaphalocrocis medinalis, Scirpophaga incertulas*
Sugarcane	Cry 1A(b)	*Diatraea saccharalis*
Tobacco	Cry 1A(b)	*Heliothis virescens, H. zea, M. sexta*
Tomato	Cry 1A(c), Bt (k)	*H. zea, M. sexta, Keifera lycopersicella*

Insect-resistant Transgenic Plants Expressing Inhibitors of Insect Digestive Enzymes: Plants make extensive use of biochemical defences, based primarily on a rich and varied secondary metabolism, but also on the use of defensive proteins. Genes encoding endogenous plant defensive proteins were thus obvious candidates for enhancing the resistance of crops to insect pests. Interfering with digestion and thus affecting the nutritional status of the insect is a strategy widely employed by plants to defend themselves against pests. a major factor in inhibition of digestion is the presence of protein inhibitors of digestive enzymes (both proteinases and amylases) in plant tissues. These protein interact with digestive enzymes, binding tightly to the active site and preventing access of the normal substrates. In the case of proteinase inhibitors, binding is accompanied by hydrolysis of a target peptide bond in the inhibitor, which determines its specificity toward a particular type of protease. The enzyme inhibitor complex is both thermodynamically and kinetically very stable and thus stoichiometric inhibition of the enzyme is achieved. The inhibition of digestive enzymes not only has direct effect on the insect's nutritional status, but is also thought to lead to secondary effects where oversynthesis of digestive enzymes occurs as a feedback mechanism in an attempt to utilise ingested food. If the insect cannot overcome the inhibition of digestion, death by starvation occurs.

Protease Inhibitors: Protease inhibitors (Pis) are common to all classes of organisms. In plants, Pis are mainly restricted to storage organs (tubers, seeds, etc.). Their function would be two-fold, *(i)* to prevent uncontrolled proteolysis, and (ii) to protect plant tissues against foreign proteases. It has become clear that the defense response of plants to pathogens is an extremely complex process and PI represents only one aspect amongst many others. The role of protease inhibitors in conferring resistance to insects has been an area of great interest for transgenic technologies. Synthesis of protease inhibitors in the leaves of a number of plant species, including tomato, in response to insect attack clearly suggests that these compounds are of functional significance. Many insects, particularly members of Lepidoptera, depend on serine proteases (trypsin, chymotrypsin and elastase like endoproteases) as their primary protein digestive enzymes and genes encoding members of various different serine protease inhibitor families have been cloned and introduced into transgenic plants. Insects themselves also produce serine protease inhibitors which are active against, and presumably are involve^ in regulating, their own digestive proteases. It has been suggested that these could be turned against insects by expressing them in transgenic plants since these inhibitors have probably been evolved specifically to be very effective against the insect proteases. Other pests rely on thiol proteases (cysteine proteases) rather than serine proteases as their primary digestive protease. These have been targeted with thiol protease inhibitors (cysteine protease inhibitors).

1. Serine Protease Inhibitors: The first gene of plant origin to be transferred successfully to another plant species resulting in enhanced insect resistance was that isolated from cowpea encoding a double-headed trypsin inhibitor. Search for resistant genotypes of cowpea to the bruchid beetle, Callosobmchus maculatus (Fabricius) at International Institute of Tropical Agriculture (IITA), Nigeria led to the identification of only one accession designated as TVu 2027 possessing significant level of resistance. Gatehouse ct al. (1979) established that resistance to C. maculatus in TVu 2027 was associated with higher level of Bowman-Birk type of protease inhibitor called cowpea trypsin inhibitors (CpTi), which was 2-4 fold higher than in the susceptible lines. Polyacrylamide gel electrophoresis and isoelectric focusing of the trypsin inhibitor fraction from these varieties suggested that the differences were purely quantitative rather than qualitative.

The Bowman-Birk type protease inhibitors are small polypeptides of around 80 amino acid. These are generally found in legume seeds, but related proteins have been identified in cereals also. They are double headed inhibitors which means that each inhibitor molecule can inhibit two enzyme molecules. The type of serine protease inhibited may be different at the two active sites. The cowpea inhibitors comprise a small family of four major isoinhibitors which are encoded by a larger gene family. There may be only four active genes. Three of the isoinhibitors are trypsin/trypsin inhibitors, the fourth is a trypsin/chymotrypsin inhibitor. However, trypsin/trypsin inhibitors predominate.

Trypsin inhibitor was isolated from cowpea, purified and tested in artificial diets, against a wide range of both field and storage insect pests including members of Lepidoptera (Helicoverpa sp., Spodoptera sp.), Coleoptera (Diabrotica sp., Anthonomus sp.) and Orthoptera (Locusta sp.). Purified CpTi, when added to artificial diets, had detrimental effects on larval development, only when applied at concentrations found in resistant seeds. In all the cases, it was found to be an effective insecticide and also possessing no mammalian toxicity. CpTi becomes an ideal candidate or genetic transformation. It has been transferred into Nicotiana tabacum and has been found to impart resistance against *Heliothis virescens* (Fabricius), *H. zea, Spodoptera littoraiis* (Boisduval) and *Manduca sexta.*

2. Cysteine Protease Inhibitors. Two types of proteins in cereal grains that inhibit insect digestive enzymes and may play roles in preventing insect and microbial attack on cereal grains have been characterized at Kansas State University, USA. More than ten α-amylase inhibitors were identified from both wheat and rice extracts that exhibit unique selectivities toward insect and mammalian and microbial enzymes. Several were specific inhibitors of rice weevil, red flour beetle and yellow mealworm enzymes, and were substantially less inhibitory towards human, porcine and bacterial (α-amylase). Recently, a cysteine protease inhibitor, oryzacystatin, from rice has been isolated that inhibits nearly all the proteolytic activity in the rice weevil and flour beetle midgut. Gut proteases of the rice weevil and the red flour beetle are strongly inhibited by oryzacystatin. Oryzacystatin has been engineered into poplar frees for resistance towards *Cheysomela tremulae.*

Recently, a cc gene (corn cystatin) was introduced into protoplasts of rice and the cystatin activity of the transgenic rice plants was assayed against a crude midgut proteinase fraction from *Sitophilus zeamais* Motschulsky. The results showed that 50 per cent of the midgut protease activity in 5. *zeamais* was inhibited by 2 μg and completely inhibited by 5 μg of transgenic seed protein fraction, whereas untransformed rice seeds had no significant effect. Additional inhibitors from other plants, such as mungbean trypsin inhibitor, potato protease inhibitors I and II, arrowhead protease inhibitors and towel gourd trypsin inhibitors are being tested against digestive enzymes of rice insect pests, and are likely to be candidate genes for transforming rice and other crops.

α-Amylase inhibitors: Seeds of bean (*Phaseolus vulgaris* L.) contain proteins that inhibit the digestive enzymes of mammals and insects, and not plant a-amylase. There is a-amylase inhibitor (a-AI) in the seeds of several varieties of common bean, P. vulgaris, the a-Al forms a If 1 complex with insect and mammalian a-amylase but is not active against plant and bacterial a-amylase. Strong evidence was proposed by Moreno and Chrispccls (1989) that a-AI is encoded by an already identified lectin gene. Two polypeptides of phytohemagglutinin, a major seed lectin PHA-E and PHA-L, are encoded by two linked genes. A chimeric gene was made consisting of the coding sequence of the a-Al gene with 5* and 3' flanking sequences of the gene that encode PHA-L. This gene was transferred to the Hind III site of the vector Bin 19 and used to transform tobacco plants via A. tumefaciens. The PHA-L promoter directs the seed specific expression of chimeric genes in transgenic tobacco plants.

α-Amylase inhibitory activity could be detected in the transgenic seeds between 12 and 15 days after pollination and reached a maximum level by 20th day. The inhibitory content decreased slightly during the drying of the seeds. Immunoblotting of the seed protein extract from transgenic tobacco plants showed that several polypeptides were produced and processed in these seeds. Most of these polypeptides bind to a pig pancreas (a-amylase affinity column). Protein extract from transgenic plants inhibited procine pancreas amylase as well as a-amylase from *Tenebrio molitor* Linn while no a-amylase^ inhibitory activity was detected in extracts of untransformed (control) plants suggesting that inhibitory activity results from the presence of

transgenic products. It was also observed that a-AI produced in transgenic plants is active and stable in tobacco seeds. Since plant protein inhibitors arc known to inhibit the growth and development of larvae of *Helicoverpa zea* (Boddie), *Spodoptera exigua* (Hubner) and Colorado potato beetle when added to artificial diet, and bean lectins PHA and arcelin, two other plant defence proteins that are homologous to the gene (hat encodes the a-Ai, suggest that genes for digestive enzyme inhibitors can be used to control insect larvae, Recently, a gene encoding an a-amylase inhibitor from wheat has been expressed in tobacco, resulting increased protection against Spodaptera spp. and Agrotis, spp.

Lectins: Lectins are a group of plant proteins that bind to carbohydrates, including chitin. Some of these have shown to provide protection against insect attack. Seeds of common bean *(P. vulgaris)* contain a carbohydrate-binding lectin protein called l) hytohemagglutinin (PHA). While major part of adaptive significance of PHA is to protect bean seeds from insect attack but it is ineffective against bean weevil, *Acanthoscelides obtcctus* (Say) and Mexican bean weevil, *Zabrotes subfasciatus* (Boheman). In the accession PI 325690, several seeds were found to contain a protein. This protein was named as Arcelin, after Arcelia, the town in the stale of Guerrero near which PI 325690 had been collected. Several accessions of wild bean had clectrophorctic pattern, showing unique protein bands. Four protein variants which had electrophoretic mobilities similar to each other but different from the other major seed proteins, phascolin and lectin, were observed. All four variants which have not been described in cultivated beans were tentatively named arpelin proteins and designated as arcelin 1, 2, 3 and 4.

Arcelin 1 protein has toxic effects against an important bruchid pest, Z. *subfasciatus* and the resistance was due to larval antibiosis of arcelin 1 causing upto 97 per cent mortality of the first instar larvae. The presence or absence of PHA does not affect bruchid development. Since PHA was shown to have insecticidal activity against the cowpea weevil, there is a high degree of similarity in nucleotide and amino acid sequence between arcelin and PHA which indicates an evolutionary relation between these genes.

The deleterious effect of chitin binding lectins on insect development is mediated by binding to chitin in the peritrophic membrane that lines the midgut of insects, thus interfering with the uptake of nutrients. Genes for lectin production would be good candidates for transforming crop plants against insect attack.

Enzymes: Transgenic expression of various enzymes has been proposed as a crop protection strategy. The most obvious candidate is chitinase, since chitin is such an important structural component of insects. Expression of an insect chitinase in transgenic tobacco enhances resistance to some lepidopterans. Similar marginal protective effects have been observed from expression of bean chitinase (BCH) in transgenic tobacco. Transgenic potato plants expressing a gene encoding BCH were found to reduce fecundity of the glasshouse potato aphid, *A. solani,* although this reduction was not statistically significant. However, nymphs produced on these BCH exp[r]cshing plants were significantly smaller compared to those on control, non-transformed plants.

A bacterial endochitinase from *Serratia marcesens* has been shown to act synergist ic^lly with *Bt* toxin against 5. *littoralis* larvae, but not yet in transgenic plants. Cholesterol oxidase secreted from *Streptomyccs* has been shown to be acutely toxic to larvae of *Anlhonomus grandis* Boheman. A *Streptomyces* cholesterol oxidase gene has been expressed in protoplasts, but insecticidal activity in iransgenics has yet to be reported. Lipoxygenase has been shown to have some insecticidal effects. Although introduced lipoxygenases have been expressed in transgenic plants, enhanced resistance of such plants to insects has not been reported.

Conclusion Numerous insect-resistant plants have been developed through biotechnology. The use of biotechnology tools in breeding program will continue. Advances in biotechnology will accelerate the development of insect-resistant plants. The acceptability to biotechnology products may be greater along with the increase in the understanding to biotechnology processes.

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