

Disease Management by Biotechnological Methods

The use of genetically modified organisms and or modern techniques (genetic engineering, tissue culture etc.) with biological systems for disease control is known as biotechnology. Genetic engineering or Genetic manipulation is the deliberate alteration of the composition of a genome by man. A growth of cells in a laboratory nutrient medium is known as tissue culture i.e. the technique of growing of plants *in vitro*. Cells of plants can be cultured in special nutrient medium and whole plants can be regenerated from cultured cells. Plant biotechnology is used for rapid clonal propagation of plants. It can help to produce industrial plant products under tissue culture conditions. Biotechnological methods are employed to control important plant diseases which are not amenable to control by usual methods.

Genetic engineering

Genetic Engineering is the technology by which a particular gene is isolated from one organism and inserted into the genome of another organism and made to express at the right time.

Vectors for transfer of genes

Genetic engineering has been used to manage plant virus diseases. For transfer of genes to plants vectors are needed in which the gene to be transferred will multiply several folds. The most effective gene vector developed is the Tumour inducing plasmid of *Agrobacterium tumefaciens* from which the Tumor inducing genes have been removed. *A.tumefaciens* induces tumors (crown galls) through di-plasmid (tumor-inducing) which is a circular double stranded DNA molecule containing up to 2,00,000 base pairs organized into several genes.

The Ti-plasmid is transferred from the bacterium into the cell. A specific region of the plasmid, the T-DNA, is transferred from the plasmid to the nucleus of the plant cell. It becomes integrated into the plant nuclear genome, and is transcribed. Cauliflower mosaic virus (CaMV) is the only plant virus with double-stranded DNA genome. As it has DNA genome, it is used as a possible vector in introducing foreign genes into plant. It is possible to insert a non-viral gene into CaMV genome and obtain expression of the gene in the infected plant. The viral promotor regions from CaMV are effective for

obtaining expression of other genes in plant cells. The genes to be expressed is now fused to a promotor element from CaMV and a gene of *A.tumifaciens*. They are then introduced into the plants using *A. tumefaciens* Ti-DNA transformation.

DNA construction

Messenger RNA is extracted and exposed to an enzyme reverse transcriptase which synthesizes a complimentary single stranded DNA. The complimentary DNA (cDNA) is exposed to another enzyme, DNA polymerase, which produces the double stranded cDNA. The cDNAs are inserted into the plasmids of *A. tumefaciens*.

Coat-protein expression in transgenic plants

Example: Transgenic tobacco plants expressing coat protein gene protected the plants against TMV. Transgenic tobacco plants showing resistance to alfalfa mosaic virus and tobacco rattle virus have also been developed. Transformation using a gene encoding the viral nucleocapsid protein of tomato spotted wilt virus (TSWV) has yielded transgenic tobacco plants that are resistant to TSWV. The expression of the viral genome in transgenic plants gives resistance to virus infection. Transgenic tobacco plants transformed with a DNA copy of the satellite RNA of cucumber mosaic virus (CMV) are shown to produce large amounts of satellite RNA following inoculation with CMV and symptom development is greatly reduced.

Satellite RNA expression in transgenic plants

Satellite RNAs are associated with several viruses. They are packaged into virus particles along with the genomic RNAs of the helper virus. They are not part of the viral genome and have no obvious sequence relationships with the helper virus. The presence of the satellite RNA suppresses the disease severity in many hosts. Hence transgenic plants which express satellite RNA have been produced to manage virus diseases. e.g., Transgenic plants of tobacco expressed the synthesis of satellite tobacco ring spot virus and reduce the virus disease incidence. Satellite RNA expressing tobacco plants against Cucumber Mosaic Virus (CMV) and Tobacco aspermy virus have been synthesized.

MIC RNA expression in transgenic plants

A DNA copy is made of one or more sections of the viral genome that include the initiation codon for proteins vital to virus replication. The DNA copy is inserted in the host-cell genome, Cells then produce an 'antisera RNA' called mic RNA (mRNA-

interfering complementary to 5' end of the gene). The miRNA hybridizes in vivo with the viral mRNA blocking translation. The miRNA is inserted into the plants using the Ti plasmid of *A. tumefaciens*. Plants regenerated from the transformed cells will be resistant to the particular virus. This possibility is also being exploited for the control of virus diseases.

Use of RFLP markers for cloning resistance genes

Molecular markers *viz.*, isozymes and DNA markers (Restriction Fragment Length Polymorphisms - RFLPs; Random Amplified Polymorphic DNA - RAPD and others) are being used in several areas relevant to identification of disease resistance genes. Some of the disease resistance genes using random DNA markers have been identified.

Disease resistance genes mapped using RFLP markers

Plant	Pathogen
Tomato	<i>Fusarium oxysporum</i>
Citrus	<i>Phytophthora</i> spp.

Detoxification of pathotoxin

Pathogens that produce pathogenesis-related phytotoxins usually also have the capacity to metabolize *i.e.* detoxify, these compounds. The search for genes encoding the enzyme(s) performing the key catabolic step(s) should thus lead to a convenient source of resistance, which can be engineered into plants to protect them from the effects of the toxin. A gene encoding a tabtoxin acetyltransferase from the pathogen, *Pseudomonas syringae* pv. *tabaci* which causes wild fire disease of tobacco was isolated and transferred into tobacco under a strong constitutive promotor. The transgenic plants expressed this gene and, when treated with either the pathogen or its toxin, did not produce the chlorotic lesions typical of wild fire disease.

Activation of plant defense mechanism-Phytoalexins

Phytoalexins have long been known to accumulate in certain plants upon infection by pathogens. The production of phytoalexins is also triggered by mechanical stimulation, ultraviolet (UV) irradiation, stress and a variety of chemical elicitors. Phytoalexins are part of the localized hypersensitive response at the site of damage or

pathogen ingress, which involves cell trauma and death. The importance of phytoalexins in the defense response is underscored by experiments and pathogenicity in *Nectria haematococca* was correlated to its ability to detoxify the phytoalexin, pisatin, by way of demethylation. By transferring the demethylase gene from *Nectria*, *Aspergillus nidulans*, a non-pathogen on peas, was rendered insensitive to pisatin.

Defense related genes

a. Single gene defense mechanism

There are some defense proteins which do not require any intermediate step both for their synthesis and their expression require only few steps and those genes encoding such proteins are called single gene defense mechanism. Chitinases and glucanases are those proteins belonging to single gene defense mechanism.

Chitinases and glucanases

Chitinases are abundant proteins found in wide variety of plants. Although the physiological function of chitinases is not known, there is strong correlative evidence that they are defense proteins with antifungal activity. Chitin is a major structural component of cell walls of many fungi. The low constitutive activity of chitinase found in many plants can be dramatically induced by wounding or by infection of the tissue with fungal pathogens. Chitinase in concert with β -1,3-glucanase (capable of degrading glucans present in fungal cell wall), degrades fungal cell walls and inhibits fungal growth at hyphal tips and has been shown to associate with hyphal walls in plants.

The chitinase and glucanase enzymes are having direct action against several fungal pathogens compared to other defense related proteins. Since lytic enzymes are encoded by single genes, these defense should be high amenable to manipulation by gene transfer. The first reports of success with this approach was the expression of bean vacuolar chitinase gene under the control of the strong constitutive gene under the control of the strong constitutive promoter of the cauliflower mosaic virus (CaMV) 35 S transcript in tobacco and *Brassica napus*, which resulted in decreased symptom development by *Rhizoctonia solani*, the causative agent of post-emergence damping off.

An endochitinase gene (from genomic tomato DNA library) was introduced into *Brassica napus*. var. *oleifera*. The transgenic *Brassica* showed enhanced resistance against several fungal pathogens like *Cylindrosporium concentricum*, *Phoma lingam* and

Sclerotinia sclerotiorum under field conditions when compared to non-transgenic plants. More recently, chitinase gene from *Manduca sexta*, tobacco horn worm, has been cloned into *P. fluorescens* to increase their antagonistic potential against *R. solani*.

b. Multigenic defense mechanism

Defense responses such as phytoalexin biosynthesis or lignin deposition in the cell wall require the action of many genes.

Peroxidases

Anionic peroxidases in the cell wall catalyze the production of phenolic radicals for the oxidative polymerization of lignin from cinnamyl alcohols. In tomato, there is a marked induction of two linked genes encoding highly anionic peroxidases in an incompatible interaction with an avirulent form of *Verticillium albo-atrum*, with only weak induction in the compatible interaction with a virulent form of this vascular pathogen. Expression of one of these genes in transgenic tobacco under the control of either its own promoter or the CaMV 35s promoter resulted in massive increase in anionic peroxidase activity and these plants apparently showed a significant increase in resistance to *Peronospora parasitica* as judged by symptom development and fungal sporulation.

Activation of defense genes by chemicals

Several classes of compounds have the potential to act as inducers of natural resistance mechanisms in horticultural crops and chemicals with such indirect modes of action may offer attractive alternatives or supplement to existing contact/systemic fungicides in integrated disease management. Increase was found to occur in response to salicylic acid treatment as well as oligosaccharides and glycoproteins originating from either fungal cell wall or host cell walls, the so called elicitors. Recently, chitosan seed treatment has been found to induce defense related genes like chitinase and glucanase in tomato and consequently the Fusarium crown and root rot diseases were significantly reduced. Pre-treatment with 2, 6-dichloroisonicotinic acid was highly effective in significantly reducing both anthracnose (caused by *Colletotrichum lindemuthianum*) and rust (caused by *Uromyces appendiculatus*) diseases in bean plants.

Cell and tissue culture

Tissue culture approach is one of the oldest techniques in the field of molecular biology and it is applied in several ways for the development of disease resistance cultivars in agriculture and horticulture.

a. Somaclonal Variation

In the past two decades, several advances have been made in culturing of isolated plant cells and tissue under controlled conditions in vitro. When plants are regenerated from cultured cells, they exhibit new phenotypes, sometimes at high frequencies. If these are heritable and affecting desirable traits, such "somaclonal variation" can be incorporated into regular breeding programmes.

However, the finding of specific traits by these methods is largely left to chance and hence inefficient. Rather than relying on this undirected process, selection in vitro aims to specific traits by subjecting large populations of cultured cells to the action of a selective agent in the petridish. For purpose of disease resistance, this selection can be done by fungal pathogens, culture filtrates of pathogens or isolated phytotoxins that are known to have a role in pathogenesis. The selection will allow only those cells to survive and proliferate that are resistant to the challenge. Plants regenerated from resistant cells often display a resistant phenotype when evaluated with either the toxin or the pathogen itself.

Disease resistant plants from tissue culture

Plant	Culture System	Selection	Resistance to Pathogen
Potato	Protoplasts	SCV	<i>Phytophthora infestans</i> <i>Alternaria solani</i>
	Callus	CF	<i>Fusarium oxysporum</i>
Tomato	Callus	Fusaric	<i>Fusarium oxysporum</i>
	Protoplasts	acid	
Banana	Meristem	SCV	<i>Fusarium oxysporum</i>
Strawberry	Callus	SCV	<i>Fusarium oxysporum</i>

(SCV- plant regeneration without selection; CF crude culture filtrate)

Although this method has obviously yielded some impressive results, it also has its drawbacks; *viz.* i. Many pathogens do not produce pathogenesis specific toxins useful for selection ii. Culture filtrates are rather artificial and neither pathogens nor plant cells grown together *in vitro* behave quite as they would in a natural environment iii. The selection approach can only detect mutations in plant genes that are expressed at the time that selection is applied.

In order to be useful, new resistance traits, whether selected or not, must be heritable sexually or in the case of vegetatively propagated crops must be transmitted through vegetative propagules. The pathogens produced toxins can be used to screen calluses (cultured cells) which may regenerate resistant plants. The toxins will kill the calluses, but the mutant toxin resistant calluses will survive. The toxin-resistant calluses yield disease resistance plants. Vidhyasekaran obtained brown spot resistant rice plants using *Helminthosporium oryzae* toxin. Similarly, *H. maydis* resistant maize plants, *H. sacchari* resistant sugarcane plants and *Phytophthora infestans* resistant tobacco plants have been evolved.

b. Anther culture

In this method, the plants are produced directly from microspores (immature pollen grains). Through anther or microspore culture, one has immediate access to unique and rare combinations of genes representing the recombination of the genetic material contributed by the parents of the cross. Through anther culture, followed by chromosome doubling, such gene combinations can be fixed in their homozygous state as instant inbreds in a single step. Over the past two decades, anther culture has become widely accepted as a tool in cultivar development. This technique can be particularly useful for producing plants with novel combinations of resistance genes for managing fungal diseases.

c. Protoplasmic fusion

This generates hybrid cells by merging the total cellular components of somatic cells from which the cell walls have been removed to produce protoplasts. The incompatibility preventing sexual fertilization between species is thus avoided and viable hybrids have been created, even between unrelated distance species. Disease resistance genes have thus been transferred by protoplasts fusion from wild species into potato.