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REVIEW

BIOTECHNOLOGICAL APPROACHES FOR THE MANAGEMENT OF FUNGAL PATHOGENS IN HORTICULTURAL CROPS

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India has a variety of climate and soil on which a wide range of horticultural crops is grown. Total production of horticultural crops is much below the annual requirement of our population. Horticultural crops cover about 6.8 per cent of the area and contribute to 18 per cent of India's gross agricultural output. India produces 70 million tonnes of horticultural crops annually. Using the recommended *per capita* consumption of 60 g of fruits and 280 g of vegetables daily as a basis, India needs 17.5 million tonnes of fruits and 81.8 million tonnes of vegetables (Venkataraman, 1992). At this level, the extra requirement will be at least 30 million tonnes of fruits and vegetables. Notwithstanding this, Indian exports to the Middle and Far East and Western Europe of fruits and vegetables, both fresh and processed has increased in the past decade from Rs. 17.2 crore in 1977-78 to Rs. 163.8 crore in 1988-89. Therefore, in the next few years, if the production and quality of the horticultural produce are not enhanced by keeping pace with increasing population and increasing export demands, the cost of produce is bound to go up several folds.

There are several constraints which significantly reduce both the quality and yield of the horticultural crops. The main problems are insects and diseases. Bacteria, fungi and viruses cause most diseases in horticultural crops and fungi cause significant reduction both in yield and quality of the produce. Currently, chemical fungicides are used in many cases to control the fungal pathogens. Most of the times, excessive chemicals have to be applied in order to achieve market competitiveness. Very often it leads to environmental pollution and human health concerns.

The modern high yielding crop varieties appear to lack much of the natural disease resistance of old land races or related wild species. One probable reason is that in the course of breeding for high yield and other desirable traits, resistance genes have been lost (Davis *et al.*, 1990). Secondary plant products (Williams *et al.*, 1989), such as tannins, phenolics, alkaloids, saponins etc., which are now known to be components of plant defense systems, are often undesirable in crops because of their bitter taste, or interference with digestion or food processing. Consequently, efforts have been made by crop breeders to reduce the content of such compounds with the side effect that the improved crop cultivars have become more susceptible to diseases, necessitating disease control by chemical means in order to get increased yield. In several cases, resistance genes have been lost inadvertently because breeders did not make conscious effort to select for their continued presence. Sometimes, the loss of resistance genes from a particular germplasm has made itself painfully noticed only when a sudden outbreak of disease has devastated the crop. There are several ways to improve the genetic resistance of horticultural crop plants against fungal pathogens. This review focuses on some of the molecular approaches that cell and molecular biologists have been developing over the past decade and discusses their potential for enhancing the disease resistance against important fungal pathogens affecting the horticultural crops.

I. Tissue and cell culture approaches

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 resistant cultivars in agriculture.

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 this method is largely left to chance and hence inefficient. Rather than relying on this undirected process, selection *in vitro* aims at specific traits (Wenzel, 1985) by subjecting large populations of cultured cells to the action of a selective agent in the Petri dish. 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 (Daub, 1986). 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 (Table 1).

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 not 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.

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 combination 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 (Hu and Zeng, 1984). This technique can be particularly useful for producing plants with novel combinations of resistance genes for managing fungal diseases.

c) Protoplast 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 distant species (Harms, 1985). Disease resistance genes have thus been transferred by protoplast fusion from wild species into potato (Austin *et al.,* 1985, 1988).

II. Molecular biology of disease resistance

In fact no other technology has changed biological research in the past 15 years as much as has what is collectively called molecular biology. Over the past 10 years, a variety of methods have been developed for the introduction and expression

Table 1. Disease resistant plants from tissue culture

Plant	Culture System	Selection *	Resistance to Pathogen	Reference
Potato	Protoplasts	SCV	*Phytophthora infestans*	Shepared *et al.* (1980)
			Alternaria solani	Shepard *et al.* (1980)
				Matern *et al.* (1978)
	Callus	CF	*Fusarium oxysporum*	Behnke (1980)
Tomato	Callus Protoplasts	Fusaric acid	*Fusarium oxysporum*	Shahin and Spivey (1986)
Banana	Meristem	SCV	*Fusarium oxysporum*	Hwang (1991)
Strawberry	Callus	SCV	*Fusarium oxysporum*	Toyoda *et al.* (1991)

*SCV = Plant Regeneration Without Selection; CF = Crude Culture Filtrate

of foreign genes in plants that include some of the major horticultural crops like potato (De Block, 1988 ; Newell *et al.*, 1991) and sugar beet (Krens *et al.*, 1988). These approaches in molecular biology are now becoming available on an ever-widening scale as powerful new tools for management of fungal plant pathogens.

Identification of genes which confer resistanc to fungal pathogens

a) 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. Due to inherited mutational changes, the DNA base sequence differs between the individual members in a population. These differences are detectable as bands of DNA when the DNA was digested with restriction endonucleases and on a gel according to their size they are electrophotetically separated. By hybridizing with a radiolabelled or fluorescent DNA probe, the sizes of the cut DNA can be highlighted. The resulting variability has been termed RFLP. The degree of RFLP diversity is inversely proportional to the genetic relatedness of the individuals analysed. Using such markers it is possible to follow the segregation of allelic forms of genes through a series of crosses. Based on the segregation behaviour (recombination frequencies), linkage maps of these random DNA markers can be contructed. Specific phenotypes, such as disease resistance traits, can be correlated with flanking RFLP markers with which they cosegregate. The resolution of this correlative mapping depends solely on how densely the markers are distributed over the genome and the amount of diversity in a population. Several RFLP markers have been identified for several plants such as carrot, lettuce, tomato and potato (Landry *et al.*, 1987 ; Gebhardt *et al.*, 1989 ; Miller and Tanksley, 1990). The RFLP markers are used in two ways. For the RFLP markers to serve as road signs on the genetic map it is not necessary to know their gene function, if any. Once the linkage of a desired trait with a set of RFLP markers has been established, individuals harbouring that trait can be identified and selected within a segregating breeding population simply by following these correlated markers. This tagging of genes works not only with single gene traits, but also combinations of many genes can be correlated with specific RFLP patterns. This is important, as it allows racking of multigenic traits that are inherited in a quantitative manner (Tanksley *et al.*, 1989). Some of the disease resistance genes using random DNA markers have been identified (Table 2).

Depending on the saturation of RFLP map and the genome size of the plant, the distance between two markers may average from 100 kilo bases (kb) to several million basepairs of DNA. By cloning a set of overlapping DNA fragments it is possible ultimately to bridge the gap between two known adjacent RFLP markers and to identify the genes that occupy that region. Identification can be done by a series of transformations of the cloned fragments into a sensitive host in which the encoded phenotype e.g. a disease resistance trait is expressed. This technique of "chromosome walking" is by no means a trivial task but intensive research is in progress in numerous laboratories using the strategy to clone the genes determining resistance to major crop diseases (Grill and Somerville, 1991). Other cloning strategies, including transposon tagging (Soller and Beckmann, 1987; Balcells *et al.*, 1991) are also being used for the same purpose (Ellis *et al.*, 1988). Transposable elements from maize and *Antirrhinum* have been shown to excise and reinsert in the genome of transgenic plants such as tobacco, petunia and *Arabidopsis* (Masterson *et al.*, 1989). This means that transposon tagging and cloning of genes can take advantage of the small genome size and well developed RFLP map of *Arabidopsis* (Chang *et al.*, 1988; Grill and Somerville, 1991). Once cloned, the putative resistance genes are available for detailed molecular and functional characterisation. When these genes have been engineered back into a

Table 2. Disease resistance genes mapped using RFLP markers

Plant	Pathogen	Reference
Tomato	*Fusarium oxysporum*	Sarfatti *et al.* (1989)
		Sarfatti *et al.* (1991)
Citrus	*Phytophthora* spp.	Durham *et al.* (1992)
		Jarrel *et al.* (1992)

susceptible host plant, they then be tested for their ability to provide protection from pathogen attack.

b) Cloning of genes by knowing their function through gene products

Plant disease, especially in wild species, is the exception rather than the rule (Lamb *et al.*, 1989), suggests that plants have evolved very effective mechanisms to defend against most pathogenic agents. In general, plants utilize a variety of morphological, structural and chemical defenses to prevent or limit the disease incidence. Inducible host responses include (i) accumulation of low molecular weight compounds such as phytoalexins (ii) deposition of cell well materials which serve as structural barriers for limiting pathogen penetration and (iii) production of chitinase and β-1.3-glucanase enzymes effective against cell walls of invading fungi. In the former method of isolating genes, the functions of genes need not be known. Further, using resistant donor plants, the genes can be identified. In the second method, the gene product and its function have to be elucidated before identification of the genes which confer resistance. For this purpose, mRNA codes for the particular protein, (the protein has to be purified and tested for its antifungal nature against the particular fungal pathogen) can be purified by isolating the polyadenylated fraction of total RNA. DNA complementary (cDNA) to the RNA sequence is synthesized *in vitro* by the use of reverse transcriptase (RT). RT or more specifically, RNA dependent DNA polymerase is an enzyme which uses an RNA template to generate a hybrid double stranded molecule consisting of the original RNA and its complement.

The RNA component of the hybrid is removed by treatment with RNase H (an enzyme that specifically degrades RNA from an RNA/DNA hybrid) and second strand of DNA is synthesized using DNA dependent DNA polymerase. Once the double stranded cDNA is synthesized, then the DNA is ready for cloning. For cloning, certain vectors have been developed. The λgt 11 carry the lacZ gene as an indicator. Cloning the cDNA into gt 11 inactivates lacZ. However, the *lac* promoter remains intact and the cDNA becomes a transcriptional fusion within the *lacZ* gene. Upon expression from the *lac* promoter, clones carrying the cDNA in both the proper orientation and reading frame will produce a fusion prot[illegible] consisting of a portion of the β-galactosidase ge[illegible] and the cDNA directed protein. Antibody to t[illegible] cDNA protein can then be used to select positi[illegible] clones which express fusion protein and therefo[illegible] carry the gene of interest.

The Polymerase Chain Reaction (PCR) dire[illegible] *in situ* amplification of DNA fragments by a fact[illegible] of up to 10^6 fold. For PCR amplification, tw[illegible] primers (prepared based on the known ge[illegible] sequence infromation) are required. The prime[illegible] hybridize to opposite strands of the DNA and a[illegible] oriented so that primer extension proceeds acro[illegible] the target fragment, towards the opposing prime[illegible] By repeating the thermal cycles of denaturati[illegible] primer annealing and polymerase extension, the ta[illegible] fragment is exponentially amplifed with a doubl[illegible] at each cycle. At the end of the amplification proc[illegible] the relative concentration of the fragment [illegible] interest will be so high compared to the total D[illegible] population that, using the PCR mix for libr[illegible] construction, a small number of clones will have [illegible] high probability of containing the target fragment.

DISEASE RESISTANCE (R) GENES:

By transposon tagging (using a dissociatio[illegible] element from maize) Cf- 9, a disease resistanc[illegible] gene from tomato was isolated. This gene has bee[illegible] found to be effective against *Cladosporium fulvu[illegible]* (harbouring Avr9 gene). The Cf-9 encodes [illegible] putative membrane anchored extra-cytoplasmi[illegible] glycoprotein that contains 28 imperfect leucine ric[illegible] repeats (LRR) and has homology to the recepto[illegible] domain of several *Arabidopsis* receptor like protei[illegible] kinases and to plant antifunga[illegible] polygalacturonase-inhibiting proteins. The recepto[illegible] like structure of the cf-9 protein indicates that i[illegible] might bind either directly to the fungal *Avr9* gene product or with another plant protein that itself binds *Avr9* (Jones *et al.*, 1994). A tomato line lacking cf-9 gene was engineered that expressed the *C.fulvum* Avr 9 gene under the control of a plant gene promoter (Staskawicz *et al.*, 1995). When this line was crossed with a line containing both Cf-9 and Ds element, most of the progeny died because the interaction of the *Avr9* gene product with Cf-9 gene product resulted in the elicitation of systemic hypersensitive reaction. However, mutants carrying a Ds-inactivated tagged Cf-9 gene survived.

Defense related genes

Plant synthesize an array of compounds when the pathogen infects them. Few genes are activated to produce mRNAs which result in synthesis of the specific proteins and these genes are called defense related genes. There are two types of defense mechanisms *viz.*, (a) Single gene defense mechanism. (b) Multigene defense mechanism.

(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 (Muthukrishnan *et al.* 1993; Mahendran *et al.*, 1997; Samiyappan 1996; Vidhyasekaran *et al.*, 1996). Chitin is a major structural component of cell walls of many fungi (Mauch *et al.*, 1988). 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 (Roby *et al.*, 1987; Samiyappan 1996). 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 (Boller *et al.*, 1983) and has been shown to associate with hyphal walls in planta (Wubben *et al.*, 1992). In addition to a possible antifungal role, many chitinases appear to be developmentally regulated, with specific isoforms appearing in certain organs only during certain stages of the plants' life; this is particularly evident in the flower. An endogenous role for chitinase has not yet been demonstrated, although specific isoforms may play a role in embryo development (De Jong *et al.*, 1992).

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 systems should be highly amenable to manipulation by gene transfer. The first report of success with this approach was the expression of bean vacuolar chitinase gene under the control of the strong constitutive promoter of the cauliflower mosaic virus (CaMV) 35 S transcript in tabacco and *Brassica napus*, which resulted in decreased symptom development by *Rhizoctonia solani*, the causative agent of post-emergent damping off (Broglie *et al.*, 1991).

Recently, Grison *et al.*,(1996) reported that 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.

(b) Multigene defence mechanism

Defense responses such as phytoalexin biosynthesis or lignin deposition in the cell wall require the action of many genes. Phytoalexins are low molecular weight antimicrobial compounds that are both synthesized and accumlated in cells after exposure to microorganisms (Lamb *et al.*, 1989). Prospects for increasing the expression of the multigenic defences depend on the identification of the determining step, manipulatuion of which impacts flux through the multistep pathway leading to the formation of the developing agent or the identification of regulatory genes that condition the coordiante expression of functionally interdependent defense genes.

Phenylalanine ammonia-lyase

Phenylalanine ammonia-lyase (PAL) catalyzes the first reaction in the synthesis of wide range of natural products based on the phenylpropane skeleton including lignin monomers and certain phytoalexins (e.g. furanocoumarins in Solanaceae and umbelliferae and pterocarpans in Leguminosae). Several cDNA clones of PAL gene have been obtained from bean (Cramer *et al.*, 1989) sweet potato (Tanaka *et al.*, 1989) and potato (Fritzemeier *et al.*, 1987). Analysis of a series of transgenic tobacco plants in which PAL activity was reduced to varying degrees following

introduction of heterologous (bean) PAL sequences revealed a direct relationship between the level of PAL activity and accumulation of phenylpropanoid products, indicating that PAL is a major rate determining step in this complex biosynthetic pathway (Elkind *et al.*, 1990). Further, transgenic plants with suppressed phenylpropanoid biosynthesis show increased susceptibility to the fungal pathogen *Cercospora nicotianae* (Lamb *et al.*, 1992). These studies show that it is possible to alter flux through a multistep metabolic pathway, and hence impact the biological activity of the pathway products, by manipulation of the expression of a single gene encoding a key regulatory enzyme, and it will be of considerable interest to determine whether PAL activity show responding increase in phenylpropanoid accumulation and pathogen resistance.

Peroxidases

Anionic peroxidases in the cell wall catalyze the production of phenolic radicals for the oxidative polymerization of lignin from cinnamyl alcohols (Lagrimini *et al.*, 1987). 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 intereaction with a virulent form of this vascular pathogen (Kolattukudy, 1992). 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 (Kolattukudy, 1992).

Activation of defense genes by chemicas:

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 (Mahendran *et al.*, 1997) as well as to oligosaccharides and glycoproteins originating from either fungal cell walls (Anderson *et al.*, 1991) or host cell walls (Ryan, 1988), 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 (Benhamou *et al.*, 1994). 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 (Dann and Deverall, 1995).

CONCLUSION

More than 10 genes are involved in phytoalexin synthesis. Therefore many genes may have to be transferred to develop transgenic plant producting phytoalexins, Hence, in several leading laboratories in USA, Japan, Netherlands and Germany efforts are being taken in developing transgenic plants containing chitinase or glucanase genes which do not require intermediate step for their expression or overproduction. Further, it is ideal to incorporate some of the antimicrobial genes like chitinase and glucanase in combination with important horticultural crops so as to get enhanced level of pretection against fungal pathogens. Such combinatorial deployment of antimicrobial genes, each giving partial protection, may in fact be desirable since this should reduce the selection pressure on the pathogen and hence prevent the development of resistance or avoidance mechanisms, such as minor modifications in cell wall structure or production of inhibitors to reduce the effectiveness of lytic enzymes.

In India biotechnological approaches for disease management in horticultural crops have been very slow and started only recently. In many of the laboratories, only tissue culture based research is going on. However in some of the Universities and Government Research Institutes, research on anti-microbial genes, their isolation, manipulation and expression are in progress, but mostly on cereal crop like rice only. Hence, the possibility of extending such type of biotechnological research to horticultural crops can be explored by generous funding and support from

...th Government and Private Agencies in the ...ming years.

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