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Inducing Fungus-Resistance into Plants through Biotechnology

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Abstract

Plant diseases are caused by a variety of plant pathogens including fungi, and their management requires the use of techniques like transgenic technology, molecular biology, and genetics. There have been attempts to use gene technology as an alternative method to protect plants from microbial diseases, in addition to the development of novel agrochemicals and the conventional breeding of resistant cultivars. Various genes have been introduced into plants, and the enhanced resistance against fungi has been demonstrated. These include: genes that express proteins, peptides, or antimicrobial compounds that are directly toxic to pathogens or that reduce their growth in situ; gene products that directly inhibit pathogen virulence products or enhance plant structural defense genes, that directly or indirectly activate general plant defense responses; and resistance genes involved in the hypersensitive response and in the interactions with virulence factors. The introduction of the tabtoxin acetyltransferase gene, the stilbene synthase gene, the ribosome-inactivation protein gene and the glucose oxidase gene brought enhanced resistance in different plants. Genes encoding hydrolytic enzymes such as chitinase and glucanase, which can deteriorate fungal cell-wall components, are attractive candidates for this approach and are preferentially used for the production of fungal disease-resistant plants. In addition to this, RNA-mediated gene silencing is being tried as a reverse tool for gene targeting in plant diseases caused by fungal pathogens. In this review, different mechanisms of fungal disease resistance through biotechnological approaches are discussed and the recent advances in fungal disease management through transgenic approach are reviewed.

Keywords: transgene, coat protein, RNA interference, chitinase, phytoalexins

Introduction

Plant pathogens are a real threat to worldwide agriculture. Significant yield losses due to fungal attacks occur in most of the agricultural and horticultural species. More than 70% of all major crop diseases are caused by fungi (Agrios, 2005). Crops of all kinds often suffer heavy losses. Fungal diseases are rated either the most important or second most important factor contributing to yield losses in major crops like rice (Lee *et al.,* 2007), wheat (Huang and Gill, 2001), barley (Smith, 2002), cotton (Cui *et al.,* 2000), groundnut (Mace *et al.,* 2006), and grapevine (Dhekney *et al.,* 2007). Fungal plant diseases are usually managed with the applications of chemical fungicides. For some diseases, chemical control is very effective, but it is often non-specific in its effects, killing beneficial organisms as well as pathogens, and it may have undesirable health, safety, and environmental risks (Manczinger *et al.,* 2002). A promising method for protecting plants against diseases is constructing and employing pathogen-resistant cultivars. Although a number of resistant cultivars have been developed through breeding programs, these cultivars become obsolete in a short time due to the rapid evolution of the phytopathogens and the emergence of virulent forms capable to overcome the plant resistance. Breeders are often confronted with the issue of using a limited number of plants in their breeding programs, undesirable traits transferred together with the valuable resistance genes, and, in recent years, also with the depletion of potential gene sources. Control of diseases is a subject of great interest for biotechnologists. The most significant development in the area of varietal development for disease resistance is the use of the techniques of gene isolation and genetic transformation to develop transgenic resistance to fungal diseases. Improvements in genetic transformation technology have allowed the genetic modification of almost all important food crops like rice, wheat, maize, mustard, pulses and fruits. Genetic engineering technology has proved to be beneficial in managing viral (Wani and Sanghera, 2010) and bacterial (Jube and Borthakur, 2007; Sanghera *et al.,* 2009) diseases in plants. The advances in gene engineering technologies and the understanding of the molecular nature of plant protection mechanisms have provided means for developing principally new strategies of plant disease control, in addition to the traditional approaches based on employing chemicals or classical breeding schemes. Biotechnology will enhance our understanding of the mechanisms that control plant's ability to recognize and defend itself against disease caused by fungi (Punja, 2007). The integration of biotechnology with traditional agricultural practices will be the backbone for sustainable agriculture

Plant biotechnology and fungal disease management

Plant biotechnology is a precise process in which special techniques are used to develop molecular- and cellular-

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based technologies to improve plant productivity, quality and health; to improve the quality of plant products; or to prevent, reduce or eliminate constraints to plant productivity caused by biotic and abiotic stresses (Azhaguvel *et al.,* 2006). Plant biotechnology involves the modification of plant performance for a particular purpose. Genome segments from plant pathogenic fungi are widely used as vectors with genes inserted to make transgenic plants. This is of paramount importance to ensure efficacy and genetic integrity of the product and to protect intellectual property. This can be achieved in a number of ways including:

a. Increasing or decreasing the expression of several genes that are naturally present in an organism.

b. Transferring genes between individuals of the same or different species.

Genetic engineering refers to artificial techniques capable of transferring genes from other organisms directly to recipient organisms (Gold, 2003). The techniques of genetic engineering can be used to manipulate the genetic material of a cell in order to produce a new characteristic in an organism. Genes from plants, and microbes can be recombined and introduced into the living cells of any of these organisms (Azhaguvel *et al.,* 2006). Transgenic recombinant plants are generated by adding one or more genes to a plant's genome and the techniques are frequently called transformation (Newell, 2000). Transgenic recombinant plants are identified as a class of genetically modified organism (GMO); usually, only transgenic plants created by direct DNA manipulation are given much attention for public discussions (Osusky, 2004). Genetic engineering has the potential to provide a cornucopia of beneficial plant traits, particularly an enhanced ability to withstand or resist attack by plant's pathogens (Chenault *et al.,* 2005; Punja, 2007). New approaches to plant disease control are particularly important for pathogens that are difficult to control by the existing methods. Genetic engineering can help farmers to increase crop yields and feed even more people (Amalu, 2004). The percentage of GMO plant resistant to diseases is approximately about 2% of total cultivated GMO plants (Gold, 2003).

Mechanism disease resistant genes on plant

Genetic engineering for fungal resistance has been limited. But several new advances in this area now present an optimistic outlook. Many reports (Makandar *et al.,* 2006; Yang *et al.,* 2009) show positive results relative to transgenic plants, expressing genes for fungal disease resistance (Tab. 1). Depending upon the mechanism of plant disease resistance, the transgenic plants have been grouped into the following categories:

(i) Pathogenesis-related (PR) proteins

PR protein genes appear to be a very potential source for candidate genes for fungal resistance. Van Loon and van Strein (1999), showed that a set of proteins is induced in tobacco plants after tobacco mosaic virus infection. Host plants contribute an enormous number of diseases resistance genes such as those encoding pathogenesis-related (PR) proteins, which have been used against fungal diseases (Van Loon and Van Strien, 1999). PR proteins were shown to be induced not only by pathogens but also by wounding, fungal cell wall elicitors, ethylene, UV light, heavy metals, etc. PR proteins are induced during hypersensitive response (HR) and also during systemic acquired resistance (SAR) and therefore are thought to have a role in natural defense or resistance of plants against pathogens. These proteins may play a direct role in defense by attacking and degrading pathogen cell wall components (Fig. 1). PR proteins that exhibit antifungal activity, including osmotin-and thaumatin-like proteins (TLP), and some uncharacterized PR proteins have been engineered into crop plants. The PR-5 proteins induce fungal cell leakiness, presumably through a specific interaction with the plasma membrane that results in the formation of transmembrane pores (Kitajima and Sato, 1999). Thaumatin-like proteins are also expressed in plants as response to a range of stress conditions and were demonstrated to have antifungal activity *in vitro* against several pathogens, including *Botrytis*, *Fusarium*, *Rhizoctonia*, and *Sclerotinia* (Koiwa *et al.,* 1997). When expressed in transgenic potato, osmotin was shown to delay the expression of disease symptoms caused by *Phytophthora infestans*. Other pathogenesis-related proteins/peptides include osmotin, thionins and lectins (Florack and Stiekema, 1994).

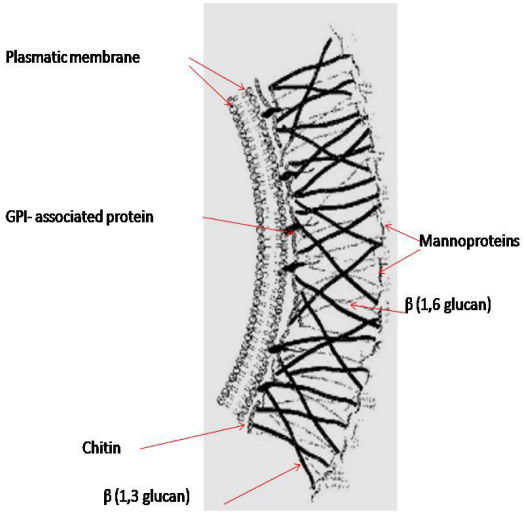

Fig. 1. Fungal cell wall (Selitrennikoff, 2001)

(ii) Antifungal proteins

Introduction of the chitinase gene in tobacco and rice has been shown to enhance fungal resistance in plants (Lee and Raikel, 1995; Nishizawa *et al.,* 1999). Chitinase

Tab. 1. Transgenic plants for fungal disease resistance

Transgene	Source	Target species	Pathogen	Reference
Vst1 (Stilbene (resveratrol) synthase)	*Vitis vinifera*	*Nicotiana tabacum*	*Botrytis cinerea*	Hain *et al.* (1993)
Aglul, RCH10	Alfalfa, Rice	*Nicotiana tabacum*	*Cercospora nicotianae*	Zhu *et al.* (1994)
PR-3(I)	Rice	Rice	*Rhizoctonia solani*	Lin *et al.* (1995)
Aglul, RCH10	Alfalfa, Rice	Alfalfa	*Phytophthora megasperma f. sp.medicaginis* (Pmm)	Masoud *et al.*, 1996
PR-3(I)	Rice	Cucumber	*Botrytis cinerea*	Tabei *et al.* (1998)
Vst1 (Stilbene (resveratrol) synthase) pss (pinosylvin synthase)	*Vitis vinifera, Pinus sylvestris*	*Hordeum vulgare, Triticum aestivum*	*Botrytis cinerea; Puccinia recondita f.sp. tritici & Stagonospora (Septoria) nodorum*	Leckband and Lorz (1998); Serebriakova *et al.* (2005)
RCC2	Rice	Grape vine	*Uncinula necator, Elisinoe ampelina*	Yamamoto *et al.* (2000)
Synthetic *D4E1*	*Cecropia* (insect)	*Nicotiana tabacum*	*Colletotrichum destructivum*	Cary *et al.* (2000)
AiiA	*Bacillus*	*Solanum tuberosum*	*Pectobacterium (Erwinia) carotovora*	Dong *et al.* (2001)
RC7 chitinase PR-3	*Oryza sativa*	*Oryza sativa*	*Rhizoctonia solani.*	Datta *et al.* (2001)
gf-2.8 (oxalateoxidase)	*Triticum aestivum*	*Glycine max*	*Sclerotinia sclerotiorum*	Cober *et al.* (2003)
Cry1Ab (*Bt* toxin)	*Bacillus thuringiensis*	*Zea mays*	*Fusarium spp*	Clements *et al.* (2003); Hammond *et al.* (2004)
Rpi-blb2 (NB-LRR)	*Solanum bulbocastanum*	*Solanum tuberosum*	*Phytophthora infestans*	Van Der Vossen *et al.* (2003, 2005)
Vf (*Cf*)	*Malus floribunda*	*Malus domestica*	*Venturia inaequalis*	Belfanti *et al.* (2004)
gf-2.8 (oxalate oxidase)	*Triticum aestivum*	*Populus euramericana*	*Septoria musiva*	Liang *et al.* (2004)
Rchit	Rice	*Pigeon pea*		Kumar *et al.* (2004)
9f-2.8 (oxalate oxidase) & TaPERO (peroxidase)	*Triticum aestivum*	*Triticum aestivum*	*Blumeria graminis f.sp. tritici*	Altpeter *et al.* (2005)
Chit	French Bean	Cotton	*Verticillium dahliae*	Masoud *et al.* (2005)
Chi11 (chitinase) Tlp (PR-4)	*Oryza sativa*	*Oryza sativa*	*Rhizoctonia solani*	Kalpana *et al.* (2006)
NPR1	*Arabidopsis thaliana*	*Triticum aestivum*	*Fusarium graminearum*	Makandar *et al.* (2006)
KP4	*Virus infecting Ustilago maydis*	*Triticum aestivum*	*Tilletia caries*	Schlaich *et al.* (2006)
Gfzhd101	*Clonostachys rosea*	*Zea mays*	*Fusarium graminearum*	Igawa *et al.* (2007)
Synthetic *D4E1*	*Cecropia* (insect)	*Gossypium hirsutum*	*Thielaviopsis basicola*	Rajasekaran *et al.* (2007)
Chi 18 (chitinase)	*Solanum tuberosum*	*Raphanus sativus* Linn	*Rhizoctonia solani*	Yang *et al.* (2009)

enzyme degrades the major constituents of the fungal cell wall (chitin and α-1, 3 glucan). Coexpression of chitinase and glucanase genes in tobacco and tomato plants confers a higher level of resistance than that imparted by either gene alone. Use of genes for ribosome-inactivating proteins (RIP), along with chitinase, has also shown synergistic effects. A radish gene encoding antifungal protein 2 (*Rs-AFP2*) was expressed in transgenic tobacco and resistance to *Alternaria longipes* was observed (Broekaert *et al.*, 1995). Transgenic tobacco plants harbouring human lysozyme gene showed enhanced resistance against the fungus *Erysiphe cichoracearum*-both conidia formation and mycelia growth were reduced, and the size of the colony was diminished (Fig. 2) (Nakajima *et al.*, 1997).

(iii) Phytoalexins

The low molecular weight compounds, such as phytoalexins, possess antimicrobial properties and have been implicated in imparting plant resistance to fungal and bacterial pathogens (Leckband and Lorz, 1998). Active oxygen species (AOS), including hydrogen peroxide, also play an important role in plant defense responses to pathogen infection.Transgenic potato plants expressing an H_2O_2-generating fungal gene for glucose oxidase were found to have elevated levels of H_2O_2 and enhanced levels of resistance both to fungal and bacterial pathogens - particularly to the verticillium wilt pathogen (Wu *et al.*, 1995). Further, overexpression of defense-response genes in transgenic plants has provided enhanced resistance to a variety of fungal pathogens (Muehlbauer and Bushnell, 2003). For example, transgenic wheat lines carrying a bar-

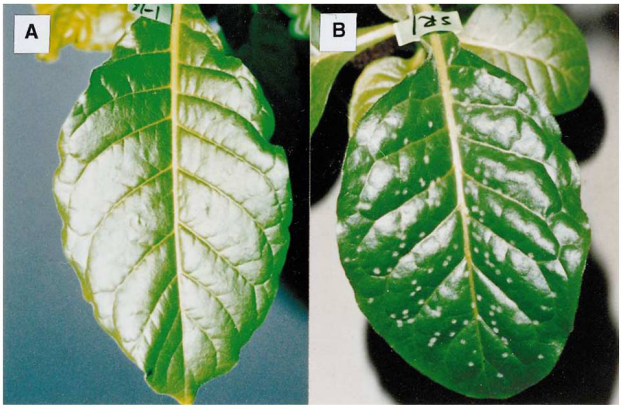

Fig. 2. Enhanced resistance of transgenic tobacco plants against *E. cichoracearum;* A. The transgenic tobacco plant, NT-7, which produced the human lysozyme; B. Wild-type tobacco 'SR1' (Nakajima *et al.,* 1997)

ley-seed class II chitinase exhibited enhanced resistance to powdery mildew (Bliffeld *et al*., 1999; Oldach *et al*., 2001). Varying levels of resistance towards powdery mildew were observed in transgenic wheat lines carrying a barley chitinase or a barley β-1, 3-glucanase (Bieri *et al*., 2003). With respect to fusarium head blight (FHB) a transgenic wheat line carrying a rice *tlp* and a line carrying a combination of a wheat β-1, 3-glucanase and chitinase exhibited delayed symptoms of FHB in greenhouse trials (Chen *et al*., 1999; Anand *et al*., 2003). In addition, transgenic *Arabidopsis* plants carrying an overexpressed *Arabidopsis* thionin have exhibited increased resistance to *F. oxysporum* (Epple *et al*., 1997). Transgenic wheat expressing the *Arabidopsis NPR1* gene, a gene that regulates defense responses, was shown to exhibit a high level of resistance to FHB in greenhouse evaluations (Makandar *et al*., 2006).

(iv) Antimicrobial Proteins

An antimicrobial protein with homology to lipid transfer protein was shown to reduce the development of *Botrytis cinerea* when expressed in transgenic geranium (Bi *et al.,* 1999). Antimicrobial peptides have been synthesized in the laboratory to produce smaller (10-20 amino acids in length) molecules that have enhanced potency against fungi (Cary *et al.,* 2000). The overexpression of defensins and thionins in transgenic plants was demonstrated to reduce the development of several different pathogens, including *Alternaria, Fusarium*, and *Plasmodiophora*, and provided resistance to *Verticillium* on potato under field conditions (Gao *et al.,* 2000).

(v) Plant ribosome-inactivating proteins and other peptides

Ribosome-inactivating proteins are plant enzymes that have 28S rRNA *N*-glycosidase activity, which depending on their specificity can inactivate conspecific or foreign ribosomes, thereby shutting down protein synthesis. Plant RIPs inactivate foreign ribosomes of distantly related species and of other eukaryotes including fungi. A purified RIP from barley inhibits growth of several fungi *in vitro*. Tobacco plants constitutively expressing a RIP encoding DNA sequence of barley showed better resistance to *R. solani* (Logemann *et al.,* 1993). Resistance levels improved when RIP was used in combination with either PR2 or PR3.

In addition, a synthetic cationic peptide chimera (cecropin-melittin) with a broad-spectrum antifungal activity has been produced (Osusky *et al.,* 2000). When expressed in transgenic potato and tobacco, these synthetic peptides have provided enhanced resistance against a number of fungal pathogens, including *Colletotrichum, Fusarium*, and *Phytophthora*. These peptides may demonstrate lytic activity against fungal hyphae, inhibit cell wall formation, and (or) enhance membrane leakage. The ability to create synthetic recombinant and combinatorial variants of peptides that can be rapidly screened in the laboratory could provide additional opportunities to engineer resistance to a range of pathogens simultaneously (Dhekney *et al.,* 2007). *MsrA2* peptide was shown to control *Fusarium* head blight (FHB) of wheat and barley grains caused by *Fusarium graminearum*. Trichothecenes genes-the virulence factors produced by the fungus-were introducing into wheat in order to increase the FHB defense mechanism in wheat spikes and reduced or prevented the initial infection (Osusky, 2004).

(vi) Resistance genes (R-gene)

The *R-gene* products that have been cloned from tomato, tobacco, rice, flax, *Arabidopsis*, and several other plant species shared one or more similar motifs: a serine or threonine kinase domain, a nucleotide binding site, a leucine zipper, or a leucine-rich repeat region, all of which may contribute to recognition specificity (Takken and Joosten, 2000). The plant's resistance *R-gene* product acts as a signaling receptor for the pathogen's avirulence (*Avr*) gene product in the presence of resistance-regulating factors such as RAR1 and SGT1, leading to a form of cell death termed hypersensitive response (Rowland *et al.,* 2005). *AVR* genes were isolated from *Bgh*, a representative of the powdery mildews as the third major group of obligate biotrophic parasites (Ridout *et al.,* 2006). The results indicated that the mildew fungus has a repertoire of *AVR* genes, which may function as effectors and contribute to parasite virulence. Multiple copies of related but distinct *AVR* effector paralogues might enable populations of *Bgh* to rapidly overcome host *R-genes* while maintaining virulence. A combination of several interacting genes, similar to that for the antifungal proteins, will likely to be required. An enhanced understanding of *R-gene* structure and function could, however, make it possible to modify functional domains in the future to tailor *R-genes* for use in providing broad-spectrum resistance to diseases in transgenic plants (Dempsey *et al.,* 1998).

(vii) Degradation of Phytotoxic metabolites

The plant cell wall acts as a barrier for the penetration of fungal pathogens and numerous strategies have evolved among plant pathogens to overcome this (Walton, 1994). Production of phytotoxic metabolites of fungal pathogens, such as mycotoxins and oxalic acid, have been shown to facilitate infection of host tissues following cell death. Degradation of these compounds by enzymes expressed in transgenic plants could provide an opportunity to enhance resistance to disease. Expression of a trichothecene degrading enzyme from *Fusarium sporotrichioides* in transgenic tobacco reduced plant tissue damage and enhanced seedling emergence in the presence of the trichothecene (Muhitch *et al.,* 2000). Their activity on the substrate of oxalic acid results in the production of CO_2 and H_2O_2, which latter can induce defense responses in the plant and enhance strengthening of cell walls. Expression of oxalate oxidase in transgenic hybrid poplar enhanced the resistance to *Septoria*, while oxalate decarboxylase expression enhanced resistance of tomato to *Sclerotinia sclerotiorum* (Thompson *et al.,* 1995). These results indicate that the inactivation of specific pathogen virulence factors, such as toxins, by gene products expressed in transgenic plants has the potential to reduce the development of specific fungal pathogens.

RNA silencing

RNA-mediated gene silencing is being tried as a reverse tool for gene targeting in plant diseases caused by fungal, bacterial and viral pathogens (Sanghera *et al*., 2009). Homology-based gene silencing induced by transgenes (co-suppression), antisense RNA, or dsRNA has been demonstrated in many plant pathogenic fungi, including *Cladosporium fulvum* (Hamada and Spanu, 1998), *Magnaporthae oryzae* (Kadotani *et al*., 2003), *Venturia inaequalis* (Fitzgerald *et al*., 2004), *Neurospora crassa* (Goldoni *et al*., 2004), *Aspergillus nidulans* (Hammond and Keller 2005), and *Fusarium graminearum* (Nakayashiki *et al*., 2005). Fitzgerald and colleagues (2004), using hairpin-vector technology, have been able to trigger simultaneous high frequency silencing of a green fluorescent protein (*GFP*) transgene and an endogenous trihydroxynaphthalene reductase gene (*THN*) in *V. inaequalis*. The *GFP* transgene acted as an easily detectable visible marker, while the trihydroxynaphthalene reductase gene (*THN*) played a role in melanin biosynthesis. Nakayashiki *et al*. (2005) developed a protocol for silencing the *mpg1* and polyketide synthase-like genes. The *mpg1* gene is a hydrophobin gene that is essential for pathogenicity, as it acts as a cellular relay for adhesion and trigger for the development of appressorium (Talbot *et al*., 1996). Nakayashiki *et al*. (2005) were successful in silencing the above-mentioned genes to varying degrees by pSilent-1-based vectors in 70-90% of the transformants. Ten to fifteen percent of the silenced transformants exhibited almost "null phenotype." This vector was also efficiently able to silence a *GFP* reporter in another ascomycete fungus, *Colletotrichum lagenarium*.

Conclusions and future prospects

After discussing the various mechanisms involved in the plant resistance to fungal diseases, it can be concluded that several strategies have emerged for developing crop varieties resistant to pathogens. Strategies include the manipulation of resistance by expression of PR proteins, antifungal peptides and manipulation of biosynthesis of phytoalexins. However, in these cases the observed resistance was not absolute and was restricted to a limited number of fungi. As to the antifungal compounds strategy to be successful in the long term, the level of resistance in transgenic plants should be increased and its range should be broadened by isolating new genes and by testing new gene combinations. Resistance genes involved in R- *Avr* interaction have been isolated from many crops and fungus-resistant transgenics are being produced by incorporating the *R-genes* in susceptible plants within a genus or a family, or even outside the family. *Arabidopsis*, with its whole genome sequenced, will prove to be an increasingly useful system in decoding the functions of various defense genes and become pathways for isolation of more and more *R-genes* in *Arabidopsis* and their orthologous counterparts in other crop species. Biotechnology provides new opportunities to build disease resistance into plants. Such developments may reduce the demand for fungicides. Transgenic plants with enhanced disease resistance can become a valuable component of a disease management program in the future. Thus, biotechnology in addition to traditional breeding techniques will help minimize losses due to biotic stresses and render a more sustainable agriculture.

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