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Genes of Microorganisms: Paving Way to Tailor Next Generation Fungal Disease Resistant Crop Plants

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Abstract

The automation of sequencing technologies, flooding in the knowledge of plant-pathogen interactions and advancements in bioinformatics provide tools leading to better knowledge not only of the genome of plant pathogens or microorganism beneficial to plants but also of ways of incorporating genes from microbes into plants as microbial-derived resistance. The identification of various microorganism genes playing key role during pathogensis and the dissection of the signal transduction components of the hypersensitive response and systemic acquired resistance pathways have greatly increased the diversity of options available for tailoring fungus resistant crops. The genetically engineered plants carrying these genes showed spontaneous activation of different defense mechanisms, leading the plant in an elevated state of defense. This 'defense mode' greatly enhances the plant's ability to quickly react to a pathogen invasion and more successfully overcome the infection. The aim of this review is to highlight the dynamic use of genes of microorganisms in enhancing crop tolernace towards fungal intruders by examining the most relevant research in this field.

Keywords: genetic engineering, microorganisms, antifungal proteins, hypersensitive response, systemic acquired resistance

Introduction

The greatest challenge for the 21st century is to provide food security to growing population of the world. Most agricultural crop species suffer from a vast array of bacteria fungal diseases which further makes the situation more tedious and complicated by enhancing annual yield loss over 12% all over the world (Agrios, 2005; Cook, 2006). To combat losses caused by the pathogens, various crop husbandry techniques have been adopted, and the most widely used management strategy is the application of agrochemicals (Haggag, 2008; Manczinger *et al*., 2002). The agrochemical methods and conventional breeding commonly used to control these diseases have many drawbacks. Indiscriminate use of agrochemicals has a negative impact on human health and contributes to environmental pollution. Conventional plant-breeding strategies have limited scope due to the paucity of desirable genes with these traits inside the available gene pools and also due to their time-consuming nature.

This challenge has led agriculture sector towards gene revolution after green revolution with the help of advanced biotechnology. It has been proclaimed as third technological revolution following the industrial and computer revolution (Mina *et al.,* 2008). The gene revolution of agriculture involves the understanding and modifications in the organization of traits within the genome and conversion of traits of an organism by transferring individual genes from one species to another, i.e., the creation of a transgenic organism. It has now been accepted as a method of choice for directional improvement and development of disease-resistant plants. The simplest means for engineering crops resistance to fungal diseases entails the constitutive expression of antifungal genes in transgenic plants. Transgenic plants overexpressing chitinases, β-1,3 glucanase, chitinosase and polygalacturans have been shown to exhibit enhanced levels of resistance to fungal infection and delayed disease symptoms in response to fungal invasion (Huang and Wang, 2005).

The chitinase encoding genes from plants have been used for a decade to transform a number of crops, but insufficient levels of disease resistance have been obtained (Mauch *et al*., 1998). Differences in levels and specificity of the antifungal chitinase activity indicate that the type and the source of the transgene are critical factors. The recent expression of fungal chitinases in plant appears to have successfully overcome the limits of plant chitinases, both in the level and the spectrum of disease resistance to fungal

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pathogens (Awady-El *et al*., 2007). This result should not be surprising since chitinases of microorganisms, especially those from mycoparasitic fungi, are optimized to break down fungal cell walls. Therefore, an approach based on constitutive over-expression of antimycolytic genes, hydrolytic enzymes and components of signaling pathways related to the defense response from microorganisms and involved in plant defence mechanisms represent a powerful and most promising strategy for conferring genetic resistance against a broad range of plant pathogenic fungi.

To date, two main strategies have been followed to express genes of microorganisms in plants to improve resistance against fungal pathogens. Disease resistant plants have been achieved by the (i) production of transgenic plants with antimycolytic compounds like proteins and toxins; (ii) generation of a hypersensitive response through *avr* (*avirulence*) genes; (iii) manipulation of genes of the SAR pathway (Systemic Acquired Resistance). This paper reviews some of the most successful instances in which genes from various microbes have been transferred to plants to improve fungal disease resistance. The potential and the use of genomes of microorganisms to genetically engineer new crop varieties with enhanced resistance traits are discussed, and some of the most promising research and microbial gene sources are presented.

Engineering plants with antimycolytic molecules

Within the scope of developing new strategies for the management of fungal infections, antifungal compounds that target essential fungal cell wall components are highly preferable. Ideally, newly developed antimycotic plants should also combine major aspects such as sustainability, high resistance, limited toxicity and low costs of production. The following sections described how various antifungal compounds of microorganism origin have been used as basic molecules to ingeneer plant crops to protect them from various fungal intruders (Tab. 1).

Microorganism derived hydrolytic enzymes

The most widely used approach of developing fungus resistant plants has been over-expression of chitinases and glucanases in transgenic plants. This is because chitin and glucan comprise major components of the cell wall of most of the fungi. Over-expression of these compounds in the plant cells is postulated to cause hyphal lysis, thereby inhibiting fungal growth (Park *et al*., 1996). The effectiveness of the chitinase gene of fungal origin in transgenic plants has been demonstrated by the reduced rate of lesion development and reduction of overall size and number of lesions upon challenge with pathogenic fungi (Awady-El *et al*., 2007). Terakawa *et al*. (1997), for instance, transferred chitinase encoding gene (*chi1*) of *Rhizopus oligosporus*, an enzyme responsible for causing cell autolysis of the filamentous fungus to tobacco and obtained a significant level in the reduction of the foliar symptoms imposed by *Sclerotinia sclerotiorum* and *Botrytis cinerea*. Another approach was obtained by Lorito and Scala (1999) who introduced endochitinase-encoding gene of *Trichoderma harzianum* in tobacco and potato to achieve tolerance against a variety of phytopathogenic fungi. The transgenic expression of the *T. harzianum chit42* gene in tobacco and potato conferred almost complete resistance to *Alternaria alternata*, *A. solani, B. cinerea, Rhizoctonia solani* and *S. sclerotiorum* (Lorito *et al*., 1998). Interestingly, biochemical and molecular characterization of the progeny indicated that in addition to the direct antifungal effects of the transgenic chitinase, other mechanisms of the plant defence system were also activated which helped transgenics to attain high and continuous level of disease resistance. Following this successful approach, *T. harzianum* genes have been transferred into a variety of crops, including, apple (Bolar *et al.,* 2000), petunia (Esposito *et al.,* 2000), grape (Kikkert *et al.,* 2000), broccoli (Mora and Earle, 2001), cotton (Emani *et al.,* 2003), citrus (Distefano *et al.,* 2008) and rice (Zhang *et al.,* 2009). The antifungal effect against *Venturia inaequalis* was further enhanced by the synergistic action of a co-expressed exochitinase (N-acetylhexosaminidase) from *T. harzianum* in transgenic apple (Bolar *et al.,* 2001). No effect of the fungal endochitinase was observed on the life cycle of the agriculturally beneficial micro-organisms including symbiotic mycorrhiza *Gigantus margaritus* (Lorito *et al.,* 1993). Recently, enhanced resistance to *Phoma tracheiphila* and *Botrytis cinerea* in transgenic lemon plants expressing a *Trichoderma harzianum* chitinase gene (*Chit 42*) was obtained (Gentile *et al.,* 2007). Similarly, transgenic carrot plants were generated by expressing *CHIT36* endochitinase gene of *T. harzianum* in carrot plants, showing tolerance to *Alternaria dauci*, *A. radicina* and *B. cinerea* (Baranski *et al.,* 2007). Later, *chit1* gene encoding endochitinase *CHIT42* from the entomopathogenic fungus *Metarhizium anisopliae* was engineered in tobacco plants to achieve resistance against *Rhizoctonia solan*i (Kern *et al.,* 2010).

Although, chitinase genes of bacterial origin have not been used extensively to engineer plants. This may be because they are exochitanases and presumed to be less active against fungi (Roberts and Selitrennikoff, 1998). The first attempts to use a bacterial origin chitinase gene to enhance plant defence were made by using *chiA* from *Serratia marcescens*. Jones *et al*. (1988) and Suslow *et al.* (1988) transferred the chitinase gene of *S. marcescens* into tobacco; the transgenic plants exhibited elevated chitinase activity and increased resistance to *Aspergillus longipes*. Later, Howie *et al.* (1994) found that the same gene provided tolerance to *R. solani* in the field. The protective role of bacterial chitinases was also confirmed by Toyoda *et al.* (1991) who injected chitinase from *Streptomyces griseus* into barley epidermis cells, and found that the enzyme digested the haustoria of *Erysiphe graminis*, the powdery mildew pathogen. Moreover, a bacterial family (Chitinase *ChiC*) from *Streptomyces griseus* showed evident inhibi-

Tab. 1. Exploitation of microorganism genes for the enhancement of resistance against fungal pathogens

Microorganism	Gene/gene product	Target pathogen	Recipient host	References
		Hydrolytic enzymes		
Metarhizium anisopliae	*chit1* / endochitinase *CHIT42*	*Rhizoctonia solani*	Tobacco	Kern *et al*., 2010
Trichoderma harzianum	*Th-Chit* /Trichoderma chitinase	*Alternaria alternata*	Tobacco	Saiprasad *et al.,* 2009
	CHIT36/ endochitinase CHIT36	*Alternaria dauci*	Carrot	Baranski *et al.,* 2008
	endochitinase gene	*Rhizctonia solani*	Cotton	Emani, *et al.,* 2003
	CHIT33 and *CHIT42*/ endochitinases CHIT33 and CHIT42	*Pseudomonas syringae* pv. *tabaci* and *Rhizoctonia solani* pv. *tabaci*	Tobacco	Dana *et al*., 2006
	Endochitinase, Exochitinase	*Venturia inequalis*	Apple	Bolar *et al*.,2001
	Endochitinase	*Botrytis cinerea*	Grape	Kikkert *et al*., 2000
	Endochitinase	Foliar and soil borne fungal pathogen	Potato	Lorito *et al*., 1998
T. atroviridae	*ech42, nag 70, gluc 78*	*Magnaporthe grisea*	Rice	Liu *et al*., 2004
Streptomyces griseus	*Chitinase C (Chi C)*	*Magnaporthe grisea*	Rice	Itoh *et al*., 2003
		Polygalacturonase inhibitor proteins		
Aspergillus niger	PGII	*B. cinerea*	Tobacco and *Arabidopsis thaliana*	Ferrari *et al*., 2008
		Antifungal peptides		
Aspergillus giganteus	*Afp*	Rust and Downy mildew	Pearl millet	Girgji *et al*., 2006
A. giganteus	*AFP*	*Magnaporthe grisea*	Rice	Coca *et al*., 2004, Moreno *et al*., 2005.
		Mycotoxins		
B. thuringiensis	*Cry1Ab*	*Fusarium spp.*	*Zea mays*	Hammond *et al*., 2004
Fusarium spp.	*Fusarium* specific antibody linked to antifungal peptides	*Fusarium oxysporum* f.sp. *matthiolae*	Arabidopsis	Peschen *et al*., 2004
		Elicitors		
Phytophthora cryptogea	β-cryptogein elicitor	*Phytophthora parasitica*	Tobacco	Keller *et al*.,1999
A. niger	*glucose oxidase* gene	*Phytophthora infestans Alternaria solani and Verticillium dahliae*	Potato	Kachroo *et al*., 2003
Pseudomonas pyrrocinia	*cpo-p*/ chloroperoxidase	*Aspergillus flavus*	Peanut	Tepfer *et al*., 1998
Pseudomonas fluorescence	Microbial factor 3 (*MF3*)	*Alternaria dauci, Alternaria radicina and Botrytis cinerea*	Carrot	Baranski *et al*., 2007
Pseudomonas aeruginosa	*katE* /Catalase	*Peronospora parasitica and Erysiphe polygoni*	Canola	Awady-El *et al*., 2007
Pseudomonas spp.	Chloroperoxidase	*Colletotrichum destructivum*	Tobacco	Niu *et al*., 2009
Bacterial origin	Salicylic acid synthase	*Oidium lycopersicon*	Tobacco	Rajasekaran *et al*., 2000
		Harpin proteins		
Erwinia pyrifoliae	$hrpN_{EP}$/ Harpin	*Botrytis cinerea*	Tobacco	Takakura *et al*., 2008
Ralstonia solanacearum	*popA*	*Phytophthora parasitica* var. *nicotianae*	Tobacco	Miao *et al*., 2010
Xanthomonas oryzae pv. *oryzae*	$hpa1_{Xoo}$	*Magnaporthe grisea*	Rice	Malnoy *et al*.,2005
X. oryzae pv. *oryzae*	$hpa1_{Xoo}$	*Verticillium dahlia*	Cotton	Shao *et al*.,2008
		Ion fluxes		
Halobacterium halobium	bO	*P. infestans*	Tobacco	Mourgues *et al*.,1998
		Barnase- barstar system		
Bacillus amyloliquefaciens	RNAse	*P. infestans*	Potato	Strittmatter *et al*.,1995
B. amyloliquefaciens	RNAse	Magnaporthe grisea	Rice	Esfahani *et al*.,2010
		Antimycolytic gene pyramiding		
Trichoderma atroviride and *T. virens*	*chit42* + *bgn13.1*	*Rhizoctonia solani*	Potato	Esfahani *et al*., 2010

tion on fungal hyphal extension *in vitro*. Ninety percent of transgenic rice plants expressing *ChiC* had higher resistance against *Magnaporthe grisea* than non-transgenic plants (Itoh *et al.,* 2003).

Antifungal peptides (AFP)

Antifungal peptides (AFP) are cationic molecules, with a net of positive charge, and ubiquitously switched-on the plant defense system by binding to pathogen membranes, which are generally negatively charged (Zasloff, 2002). A naturally derived molecule that possesses all these desired characteristics is the antifungal protein (AFP) secreted by the filamentous ascomycete *Aspergillus giganteus* which is a powerful antimycotic against various plant pathogens including *Magnaporthe grisea*, *Botrytis cinerea*, *Fusarium oxysporum* and several others which have been well documented in literature (Meyer, 2008). Caco *et al.* (2004) showed that rice plants can be engineered for resistance against the blast fungus (*M. grisea*) by expression of the *A. giganteus afp* gene under the control of the constitutive maize ubiquitin promoter. Transgenic rice constitutively expressing AFP protein showed inheritance of the transgene without any effect on plant morphology, growth and development (Moreno *et al.*, 2005). Recently, rust and downy mildew resistance in pearl millet (*Pennisetum glaucum*) was achieved by heterologous expression of the *afp* gene from *Aspergillus giganteus* through particle bombardment of immature zygotic embryos (Girgji *et al.,* 2006). However, examples of antifungal peptides from microorganism origin expressed in plants are still scarces and one of the unexplored niches.

Polygalacturonase inhibitor proteins (PGIPs)

Polygalacturonase-inhibiting proteins (PGIPs) are plant cell wall proteins that protect plants from fungal invasion. They interact with endo-polygalacturonases secreted by phytopathogenic fungi through the inhibition of their enzymatic activity, and favor the accumulation of oligogalacturonides, which activate plant defense responses (Federici *et al.,* 2006). Partial degradation of homogalacturonan (HGA) by fungal endo-polygalacturonases (PGs), for instance, releases oligogalacturonides (OGs) which are regarded as host-associated molecular patterns for regulation of plant innate immunity (Stern *et al.,* 2006). Working on these lines Ferrari *et al.* (2008) generated transgenic plants by expressing *Aspergillus niger* PGII into tobacco and *Arabidopsis thaliana* to enhance the level of protection of plants against *B. cinerea*. We believe that this line of research will provide new insights in the regulation of plant defense responses during plant-pathogen interactions.

Mycotoxins

Production of secondary metabolites, such as mycotoxins and oxalic acid by fungi has been shown to facilitate infections in host tissues followed by programmed cell death. Degradation of these compounds by enzymes expressed in the transgenic plants could provide an opportunity to enhance resistance against diseases. A limited number of toxins derived from fungi or bacteria have been transgenically expressed in plants increasing disease resistance. The most successful case is the expression of trichothecene-degrading enzymes from *Fusarium sporotrichioides* in tobacco. The generated plants showed reduced plant tissue damages and enhanced seedling emergence in presence of trichothecene (Muhitch and McCormick, 2000). Later on, transgenic maize was developed by using this strategy and it was found a 10% of reduction in the levels of the Fusarium-toxin zearalerone as compared to wild-type plants (Igawa *et al.,* 2007). Transgenic tobacco plants carrying and expressing the toxin of *Ustilago maydis* KP4 killer gene had already been reported by Park *et al.* (1996). These toxins are actually encoded by the dsRNA of the *U. maydis* virus (*UmV*) which persistently infects the fungus, and provides a selective advantage to the host cell by killing other *U. maydis* strains. Afterward, Clausen *et al*. (2000) demonstrated the over expression of this toxin in transgenic wheat associated with antifungal activity against *U. maydis* and *Tilletia tritici*. In a greenhouse test, the transgenic wheat plants did not exert any measurable injurious effect on soil microarthropod populations after evaluation of different parameters in a feeding bioassay (Romeis *et al.,* 2003). If these letal toxins are efficient against a broader range of plant pathogenic fungi, they could be a novel means for disease control.

Another mycotoxin is the oxalic acid which has an important role as a toxic pathogenicity factor in several species of necrotrophs, of which *Sclerotinia sclerotiorum* is a particular problem in many dicotyledonous species, for example, oilseed rape (*Brassica napus*) and sunflower (*Helianthus annuus*). Several studies have therefore taken the approach of constitutively expressing a heterologous (usually wheat) oxalate oxidase gene in a target crop in order to neutralize the oxalic acid produced by the pathogen. The products of the enzyme include the reactive oxygen species hydrogen peroxide, which itself has an important role in disease resistance (Shetty *et al.,* 2007). Examples where partial resistance has been obtained in the laboratory include sunflower and soybean (*Glycine max*) against *Sclerotinia sclerotiorum* (Cober *et al*., 2003; Donaldson *et al*., 2001; Hu *et al.,* 2003) as well as poplar (*Populus* × *euramericana*) against *Septoria musiva* (Liang *et al.,* 2004).

Transgenics engineered for hypersensitive response (HR)

After perceiving the *avr* gene product, the elicitors, signal cascade is trigger by activation of host activator molecules. These activator molecules then interact with signal molecules such as H_2O_2, salicylic acid (SA), jasmonic acid (JA) and ethylene thereby triggering defense responses in plants, exemplified by HR, PR proteins and phytoalexin (Hain *et al*., 1993).

Ion fluxes

Ion fluxes are one of the early events in incompatible plant pathogen interactions. Therefore, changes in proton translocation by altered expression of proton pumps can lead to SAR-like defense responses even without pathogen infection. The successful example that exploits such type of approach to engineer plants is based on the expression of a bacterial gene (*bO*) encoding a proton pump, the bacterio-opsin, derived from *Halobacterium halobium*. The constitutive expression of *bO* gene in potato plants produces a lesion mimic phenotype in which necrotic lesions are displayed and local and systemic defence responses are activated in the absence of pathogens. Tobacco plants expressing *bO* had increased levels of salicylic acid and were more resistant to *P. infestans* isolates showing A1 mating type (Mourgues *et al.,* 1998).

Hydrogen peroxide

Hydrogen peroxide (H_2O_2) is one of the activator molecules which interacts with a signal cascade exerting direct antimicrobial activity, diffusing the signal for activation of cellular defence genes and reinforcing the plant cell wall (Shetty *et al.,* 2008). The direct evidence that ROS (Reactive Oxygen Species) are involved conferring plant disease resistance was provided by the constitutive expression of glucose-oxidase gene (*GOX*) from *Aspergillus niger* in transgenic potato. Wu *et al*. (1995) reported that the introduction of *GOX* gene in potato caused delayed lesion development by *Phytophthora infestans* and reduced disease development by *Alternaria solani* and *Verticillium dahliae*. Enhanced resistance of transgenic rice plants to *M. grisea* was also demonstrated and it is correlated with constitutive and pathogen-induced expression of an *Aspergillus niger GOX* gene, responsible for elevating the endogenous levels of H_2O_2 which in turn caused typical cell death and activated the expression of several defense genes (Kachroo *et al.,* 2003). However, it should be done with caution since high levels of H_2O_2 in transgenic plants may cause metabolic disturbances interfering with normal growth and development. Using pathogen-inducible expression of H_2O_2 generating genes may be an effective way to confer broad-spectrum resistance in rice without the penalty of causing any developmental abnormalities or metabolic errors. Thus, the expression of H_2O_2 generating enzymes of fungal origin in transgenic plants represents a novel strategy for engineering broad-spectrum resistance to fungal pathogens.

Certain bacteria, such as *Escherichia coli* and *Pseudomonas fluorescence*, have well-characterized pathways for the production of salicylic acid. In *E. coli*, the *entC* gene encodes the enzyme isochorismate isomerase, which converts chorismic acid to salicylic acid, while in *P. fluorescence*, the *pmsB* gene encodes pyruvate lyase, which converts isochorismic acid into salicylic acid. Verberne *et al*. (2000) developed transgenic tobacco plants that constitutively expressed both *entC* and *pmsB* in the chloroplast. The transgenic tobacco plants expressing these genes showed accumulation of salicylic acid that were up to 1000 times higher than in wild-type tobacco. When exposed to the fungus *Oidium lycopersicon*, the transgenic tobacco plants showed increased levels of resistance when compared to the wild-type plants. The transgenic plants did not show any adverse effects due to the high level expression of salicylic acid. Since the role of salicylic acid in triggering SAR against a variety of pathogens is believed to be similar for all higher plants, the same strategy used in this research can be applied to other commercially important crops to enhance their resistance to different pathogens. Rajasekaran *et al.* (2000) reported that the expression of a chloroperoxidase gene from *Pseudomonas pyrrocinia* (*cpo-p*) in transgenic tobacco resulted in significant inhibition of *Aspergillus flavus* hyphal growth and reduced leaf anthracnose lesions caused by *Colletotrichum destructivum*. Recently, Niu *et al.* (2009) also demonstrated the successful insertion and expression of the *cpo-p* gene derived from *Pseudomonas pyrrocinia* into the groundnut *Arachis hypogaea*. The developed transgenic groundnut plants showed inhibition of *Aspergillus flavus* hyphal growth, which resulted in the reduction of aflatoxin contamination in peanut seeds.

Elicitors

Disease resistance responses caused by elicitors other than the products of avirulence genes have been also used to increase plant resistance to pathogens. Tobacco plants were transformed by a *PHytophthora cryptogea* gene encoding the elicitor protein cryptogein under the control of the constitutive 35S cauliflower mosaic virus (CaMV) promoter and the nos terminator (Tepfer *et al.,* 1998). The transformed plants exhibited resistance to *P. parasitica* var. *nicotianae* which does not secrete elicitins and is normally pathogenic to tobacco. Cryptogein accumulates into the cells and may be liberated upon fungal infection. Tobacco also has been transformed with cryptogein under the control of the pathogen inducible tobacco hsr 203J gene promoter (Keller *et al.,* 1999). The transgene which was silent in the absence of the pathogen, was activated upon infection by *P. parasitica* var. *nicotianae*, producing cryptogein around the infection sites and subsequently elicitating a hypersensitive response (HR), leading to resistance. Such transgenic plants were also more resistant to other fungal pathogens such as *Thielaviopsis basicola*, *Erysiphe cichoracearum*, and *B. cinerea*.

Elicitor produced by *Cladosporium fulvum* is a product of virulence gene '*avr9*' and De Wit (1992) proposed that if '*avr9*' gene can be introduced, then a broad spectrum resistance could be obtained. Hammond *et al.* (2004) showed HR response in tomato seedlings by expression of the 'avr9' transgene. Transgenic tobacco plants were made harboring a fusion between the pathogen-inducible tobacco 'hsr203J' gene promoter and *Phytophthora cryptogea* gene encoding elicitor cryptogene in order to control

the expression of 'avr9' gene only in presence of pathogen (Keller *et al.,* 1999). They reported a strict cryptogene expression, i.e., HR response was tightly controlled and observed only in presence of *P. parasitica* var. *nicotianae*. Moreover, the transgenic plants displayed enhanced resistance to few other unrelated species, such as *Thielaviopsis basicola*, *Erysiphe cichoracearum* and *Botrytis cinerea*. This report not only demonstrated that single gene could produce a broad spectrum resistance, but also implied that when a gene was introduced into heterologous system the effectiveness of the gene had to be assessed very cautiously since the introduced gene may cause resistance towards many more untargeted non-related species. Recently, Qiu *et al*. (2009) isolated an elicitor-encoding gene (*pemG1*) from *Magnaporthe grisea*, the rice blast fungus, and introduced into rice (*Oryza sativa* L. cv. 'Nipponbare') under the constitutive control of maize ubiquitin promoter. The *pemG1*-expressing plants showed enhanced resistance against the fungus suggesting that the elicitor-encoding gene could be a novel approach to enhance disease resistance in various crops.

Harpin proteins

Harpin group of proteins stimulate plant growth and pathogen defense. The proteins contain a characteristic hairpin and are are glycine-rich, protease-sensitive, heat-stable, and very often are produced by Gram negative plant pathogenic bacteria. They also induce hypersensitive cell death (HCD) in non-host plants of bacteria by activating SAR pathway. To date, a few harpins or harpin-like proteins, such as $hrpZ_{Pss}$ from *Pseudomonas syringae* pv. *syringae* (He *et al.,* 1993), $hrpN_{Ea}$ from *Erwinia amylovora* (Kim and Beer, 1998) and $hpa1_{Xoo}$ from *Xanthomonas oryzae* pv. *oryzae* (Peng *et al.,* 2004) have been characterized. The feasible use of these harpin protein genes from various infection-induced HR, as a new strategy of engineering fungal disease resistance was explored (Li *et al.,* 1999) and a transgenic potato showing enhanced level pf resistance towards *Phytophthora infestans* was first developed using a harpin protein gene from *Erwinia amylovora* and potato *prp1-1* promoter as main DNA elements. Belbahri *et al.* (2001) used the *popA* gene from the phytopathogenic bacterium *Ralstonia solanacearum* showing sequence similarity with $Hpa1_{Xoo}$ to develop transgenic tobacco plants resistant to *Phytophthora parasitica* var. *nicotianae* under the control of the hypersensitivity-related plant promoter hsr203J. The developed plants accumulating the PopA protein showed localized HR and increased resistance to *P. parasitica*. Recently, Miao *et al*. (2010) showed that the transformation of $hpa1_{Xoo}$ into cotton conferred the improved resistance to *Verticillium dahlia* in the transgenic cotton. Similarly, a high level of resistance to all predominant races of *Magnaporthe grisea* in China was obtained in the rice line transformed with $hpa1_{Xoo}$ from *Xanthomonas oryzae* pv. *oryzae* (Shao *et al.,* 2008). Several other *hrp* genes isolated have also been successfully transformed into different plant species including pear (Malnoy *et al.,* 2005) and tobacco (Sohn *et al.,* 2007). These results highlighted that engineered harpins of phytopathogenic bacteria in plants was a very promising approach to produce fungal disease resistant genotype. Unfortunately, the defense responses elicited by harpins and their active sites in hosts have not been fully understood yet and need to be dissected in future to harness their real potential in plant engineering.

Flagellin proteins

Microbe-derived molecules such as bacterial flagellin and lipopolysaccharides, collectively called pathogen associated molecular patterns (PAMPs) or microbe-associated molecular patterns (MAMPs) trigger immunity in plants. The flagellin gene from a phytopathogenic bacterium, *Acidovorax avenae* strain N1141, was introduced into rice and the resultant transgenic rice plants accumulated flagellin at various levels. The transgenic plants showed increased expression of defense genes, H_2O_2 production and cell death, suggesting that the flagellin triggers innate immune responses in the transgenic rice. When rice plants were inoculated with *M. grisea*, the transgenics exhibited enhanced resistance, suggesting that the flagellin approach might provide a new strategy for developing genetically engineered disease-resistant rice varieties (Takakura *et al.,* 2008).

Two component pathogen sensory system

Resistance gene-avirulence gene system

Genes that may protect plants by activating defence responses or inhibiting pathogen virulence factors have also been exploited in engineering resistance. Avirulence genes (*Avr*) in pathogens and their matching resistance (*R*) genes in host plants have received considerable attention (Kiraly and Hornok, 1997). In plant-pathogen systems following the gene for gene relationship, the interactions between the product of an *Avr* gene (elicitor) and the product of the corresponding *R* gene (receptor) activate a signal transduction pathway which leads to resistance, often through a hypersensitive response (HR). Several *Avr* and *R* genes have been cloned and the Avr/R gene pair has been transferred in plants to engineer resistance (Honée *et al.,* 1997), particularly, following De Wit's introduction of the two component sensor system method (Hammond *et al.,* 2004). The avirulence gene *Avr9* of *Cladosporium fulvum* and its matching resistance gene *Cf9* were the first pair to be used in this strategy. Constructs were made fusing either the *Avr9* or the *Cf9* gene with the infection site specific promoter *Pgst1* of a potato defence gene (Strittmatter *et al.,* 1995), then transferred to tomato. Several tomato transgenic lines were identified which showed resistance to a wild type *Avr9*- strain of *C. fulvum* by inducing the HR at the infection sites. In one case ECP2, a virulence factor secreted by *C. cladosporium* which is required for full virulence on tomato, proved to be useful for re-

sistance engineering. These plants recognized specifically *C. fulvum* strains producing *ECP2* and became resistant through induction of HR (Lauge *et al.,* 1998). However, since *Cf-ECP2* induces HR by recognizing an important virulence factor, it is conceivable that it may represent a durable source of resistance. This result suggests that a targeted search for HR may be of great value to discover new genes able to confer resistance, also against pathogens involved in other plant/pathogen systems. Moreover, to avoid unwanted expression of Avr/R, which leads to generalized HR and consequently plant death, one of the genes must be regulated by a pathogen inducible promoter.

Barnase-barstar system

Several systems have been tested to mimic HR in transgenic plants as a mechanism of localized cell death and resistance to pathogen. Although this approach has the potential to provide a broad spectrum resistance to bacteria, fungi and viruses, it requires the use of specific promoters to restrict the effect of the transgene at the site of infection in order to avoid a deleterious generalized HR response and plant death. An interesting strategy has been applied by Strittmatter *et al*. (1995), who expressed in plant a bacterial ribonuclease gene (*barnase*) driven by a pathogen inducible promoter from potato (*prp1-1*). They also transferred to the transgenic potato a gene coding for an inhibitor of barnase activity (*barnstar*) to reduce detrimental effects of background activity of ribonuclease (RNase) on the non-infected tissues. When the potato progenies were challenged with *P. infestans* a strong barnase activity was induced only at the infection site, with a significantly decrease. This strategy, however, was not tested at the field level. The strategy of the two-component system, was further adopted by Shengji *et al*. (2003) to obtain transgenic rice resistant to *Magnaporthe grisea*. In this study, two chimeric promoters, induced by rice blast fungus pathogen (*Magnaporthe grisea*), are fused with Barnase respectively to construct two plant expression vectors, pWBNBS and pPBNBS together with the Barstar driven by CaMV 35S promoter. The expression of Barnase is induced in rice leaves when inoculated with the spores of *Magnaporthe grisea* and the transgenic plant shows high levels of resistance to the rice fungal blast disease. These results suggest that transgenic plants harboring this two-component system may be exploited to acquire relatively broad spectrum and elevated resistance against fungal pathogen in various other agriculturally important crops.

Antimycolytic gene pyramiding

The resistance in newly released varieties can be lost quickly due to the high level of instability in the pathogen population. The best approach to circumvent this problem is to re-engenniering plants with multiple resistant microbial transgenes showing different mechanisms which could enlarge the spectrum and enhance the level of resistance in transgenic crops. This approach was first time adopted by Lorito *et al*. (1998), where they constructed a transgene containing chitinases and β-1,3-glucanases from *Trichoderma* sp. and achieved strong and highly synergistic in combination antifungal activities against various fungal pathogens. Recently, Esfahani *et al*. (2010), used chitinase gene (*chit42*) from *Trichoderma atroviride* and the β-1, 3-glucanase gene (*bgn13.1*) from *Trichoderma virens* to prepare a double gene construct containing these two genes. The transformed potato (*Solanum tuberosum* cv. Savalan) showed enhanced resistance to *Rhizoctonia solani* AG-3, responsible for causing stem and root rot diseases.

Challenge ahead

All the strategies mentioned above concentrate on introduction and over expression of antimycolytic genes to make transgenic plants to combat fungal infection. In most of the cases the antifungal genes have been introduced under constitutive expression resulting in the continuous expression of transgenes. So, the ultimate success of a transgenic plant to inhibit fungal infection depends on the expression level of the transgene(s) introduced in it. However, the expression of foreign genes in plants have also recorded numerous problems and defeats often because the genetically engineered plants failed to produce the desired gene product at the expected level, even though plant promoters were used, or the transgenic protein significantly affected plant growth and development (Hain *et al*., 1993). Under constitutive promoter CaMV35S, transgenic tomato was also reported to die when transformed with elicitor gene '*avr9*' to produce fungal resistant plants (Honée *et al.,* 1997). Although T_0 generation was morphologically normal, Anand *et al*. (2003), reported necrotic lesions containing dead cells in T_2 and subsequent generations, when the wheat transgenic lines were homozygous for the transgenes (co-expression of chitinase and glucanase). In contrast, lesions were not observed in hemizygous transgenic lines or lines silenced for transgene expression, indicating a requirement for high levels of transgene expression for the development of the lesioned phenotype. It is therefore imperative that emphasis is directed to express transgenes at a level effective for fungal inhibition.

In the light of these observations, attempts are now made to develop transgenic plants with regulated or tissue-specific expression of transgenes in order to avoid such deleterious effects of putatively harmful transgene products as well as to minimize the loss of efforts due to continuous production of transgene products. The use of pathogen-inducible promoter is the best option to tightly control the expression of transgene. Keller *et al.* (1999), developed transgenic tobacco plants harbouring a fusioned product between the pathogen-inducible tobacco '*hsr203J*' gene promoter in combination with cryptogene

elicitor encoded by *Phytophthora cryptogea*. The transgenic tobacco plants regenerated with this homologous pathogen-inducible promoter were reported to have tightly controlled expression of the transgene only in presence of the pathogen.

Conclusions

Recent advances in the investigation field of microorganism genome, molecular biology and transgenic technologies have enabled the plant tailors to know more about the elegant features of genes available within microorganism which require special attention. Exploitation of these genes for the crop improvement could be considered as the 'powerful seeds' that will solve the problems of 21st century arising from various fungal infections. Further, large scale field trials are needed to test whether expression of the introduced microbial genes will affect yields, quality, or agronomic traits. Although the introduced microbial genes are well-defined, the field trials also provide the opportunity to ascertain whether any unexpected or undesirable consequences have resulted from the transformation procedure. Genetically engineered crops by using microbial genes are just beginning to make their way into the hands of crop engineers. When carefully set up microbe genome will become an integral supplement to modern biotechnology and its enormous potential should be harnessed to the best advantage of the entire human beings. Moreover, several of the biosafety and related environmental and food safety concerns associated with the cultivation of and trade in transgenic crops remain relevant and thus appropriate policies and strategies to safeguard against potential adverse effects are require.

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