Strategies for development of fungus-resistant transgenic plants

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AFTER centuries of improving crop plants by breeding for desirable traits, agricultural scientists are now using the tools of molecular biology and genetic engineering to develop transgenic plants with the desired genes. Enormous progress has been made over the past decade in our understanding of the highly complex molecular events that occur in plant–pathogen interactions. This knowledge in turn has provided a number of options and strategies which can be and have been used to make transgenic plants resistant to pathogens. This review deals with fungal pathogens of crop plants. Starting from the first step of mutual recognition of host and pathogen which involves resistance gene–avirulence gene interaction, moving onto immediate response of the plant in terms of hypersensitive response, production of active oxygen species, followed by local resistance response in terms of production of pathogenesis-related proteins and other antifungal proteins, then to the final step of systemic acquired resistance (SAR), all this information has been/or is being used to produce fungus-resistant transgenic plants in different crop species. In this review we discuss strategies that have been used to produce fungus-resistant transgenic plants and also discuss some of the emerging possibilities in the wake of large scale genome sequencing projects being undertaken in crop plants.

Significant yield losses due to fungal attacks occur in most of the agricultural and horticultural species. In Indian context, fungal diseases are rated either the most important or second most important factor contributing to yield losses in our major cereal, pulse and oilseed crops. On the basis of a recent survey[1], contribution of fungal diseases towards total yield loss in some important crops in India has been summarized in Table 1. Incidence of plant diseases has been controlled by agronomic practices that include crop rotation and use of agrochemicals and by breeding new strains and varieties that contain new resistance conferring genes. The use of agrochemicals poses many dangers that include harmful effects on the ecosystem and an increase in the input cost of the farmers. The breeding of resistant crops is time consuming and has to be a continuous process as often new races of pathogens evolve and crops become susceptible. Despite the boom and bust cycles, breeders have been successful in protecting some of the major crops grown around the world from fungal diseases. A major success story is wheat in which systematic breeding has been done to develop varieties resistant to wheat rust by first incorporating genes from the primary gene pool and when this option ran out, from the secondary and tertiary gene pools of alien species and genera. Although shown to be possible, wide hybridization programmes face numerous difficulties. Often sexual crosses are difficult to make and genetic exchange in the hybrids is poor due to low frequency of pairing between chromosomes of crop species and alien species. Problems can also arise due to linkage drag (gene/s for resistance are linked to some deleterious genes which lower the yield of the crop variety).

Novel alternative strategies that would circumvent the problems faced in wide hybridization are required to produce fungus-resistant crop varieties. Such strategies will be particularly important in cases where source of resistance is not available in taxonomically related species. 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 transgenics resistant 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. The estimated global area of transgenic or genetically modified (GM) crops in the year 2001 was 526 million hectares[2]. To identify the important genes which need to be introduced in the plants to improve their resistance to fungal pathogens, lot of basic work has been done in the area of host–pathogen recognition[3–5]. During the last decade, many resistance genes whose products are involved in recognizing invading pathogens have been identified and cloned[6]. A number of signalling pathways which follow the pathogen infection have been dissected[7]. Many of the antifungal compounds which are synthesized by plants to combat fungal infections have been identified[8]. The complete sequencing of *Arabidopsis* genome has led to identification of a number of tentative resistance gene clusters[9]. All this knowledge would greatly advance development of different strategies for producing fungus-resistant transgenic plants.

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We review here strategies for the production of fungus-resistant transgenics. These can be basically classified into two categories namely (i) production of transgenic plants with antifungal molecules like proteins and toxins, and (ii) generation of a hypersensitive response through *R* genes or by manipulating genes of the SAR pathway. Diseases caused by bacterial pathogens are also covered wherever appropriate as there is considerable commonality in modes of pathogenesis and plant responses in fungal and bacterial diseases.

Some of the terms of molecular plant pathology relevant for this review have been explained in Box 1.

Transgenics with antifungal molecules

Antifungal compounds include antifungal proteins from plants and lower organisms and metabolites like phytoalexins.

Antifungal proteins

Till date, genes encoding many antifungal proteins which can inhibit fungal growth *in vitro* have been exploited to make fungus-resistant transgenic plants although, it is not

Table 1. Contribution of fungal diseases toward yield loss in some major crops of India

Crop	Pathogen	Disease	Total yield loss (%)
Rice	*Pyricularia ozyzae*	Blast	21
Wheat	*Puccinai recondiata*	Leaf rust (Brown rust)	30
Maize	*Helminthosporium maydis* and *H. turcicum*	Leaf blight	30
Sorghum	*Sphacelotheca reiliaria*	Grain mould	18
Pigeonpea	*Fusarium udum*	Wilt	24
Chickpea	*Fusarium oxysporum*	Wilt	23
Brassica	*Alternaria brassiceae*	Blight	30
Soybean	*Phakospora packyrhizi*	Rust	23
Potato	*Phytophthora infestans*	Late blight	31

Box 1. Some common terms used by molecular plant pathologists

Elicitors	– Molecules (generally from pathogen cell wall), which can trigger defense reactions in the host plant.
Compatible interaction	– Interaction between susceptible host and virulent pathogen.
Incompatible interaction	– Interaction between resistant host and avirulent pathogen.
Gene for gene relationship	– Proposed by Flor[81] in flax-rust system. For every resistance gene in the host there is a corresponding avirulence gene in the pathogen.
Hypersensitive response (HR)	– Small brown necrotic lesions produced by the host plant during incompatible interaction, because of localized cell death. Associated with resistance.
Oxidative burst	– Rapid generation of active oxygen species like superoxide anion (O_2^-), hydroxide radical (OH), H_2O_2. It is one of the very early defense mechanisms triggered by infection.
Systemic acquired resistance	– After a plant has been infected by one pathogen and recovered, it can show remarkable resistance to future infections by the same or other pathogens for days. Something similar to immunity in animals.
Defense signalling pathways	– The entry of pathogen is sensed by the plant. This signal is then transduced into activation of defense mechanisms through different signalling pathways mediated by small molecules such as salicylate, jasmonate and ethylene. These different pathways are under both positive and negative control and are interconnected also.
Pathogenesis-related proteins	– A class of proteins, which are induced by many biotic and abiotic stresses. First discovered in tobacco after TMV infection, hence called PR proteins. Have a role in defense.
Resistance gene clusters	– From *Arabidopsis* genome sequence we know that resistance genes are not spread throughout the genome but are clustered at specific loci.

known whether they are also involved in defense responses against fungi *in vivo*. Some of these proteins are: Pathogenesis-related proteins, Ribosome-inactivating proteins, Small cystein-rich proteins, Lipid transfer proteins, Storage albumins, Polygalacturonase inhibitor proteins (PGIPS), Antiviral proteins, and Non-plant antifungal proteins.

Pathogenesis-related proteins: In a seminal work, Van Loon and Van Kammen showed that a set of proteins is induced in tobacco plants after tobacco mosaic virus infection[10]. These proteins were described as pathogenesis-related (PR) proteins. Later, 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. PR proteins have been grouped into five families based on primary structure, serological relatedness and enzymatic and biological activities. Members of all the five PR families (PR-1 to PR-5) have been shown to have antifungal activity[11]. The family of PR-1 proteins consists of low molecular weight (15–17 kDa) proteins. Their biological function is not known, nevertheless, constitutive expression of *PR1A* gene in tobacco enhances resistance of the plant to *Peronospora tabacina*[12]. PR2 and PR3 type proteins are the fungal cell wall hydrolysing enzymes, glucanase and chitinase respectively[13,14]. These proteins can inhibit the fungal growth *in vitro* by causing lysis of hyphal tips[15]. Proteins of PR4 families are also low molecular weight and similar to potato win proteins. They show *in vitro* antifungal activity particularly in combination with other antifungal proteins[16]. PR5 proteins (thaumatin-like or AP24 or osmotin), in all probability, cause lysis of the pathogen by permeabilizing the fungal cell wall[17].

The first report on developing fungus-resistant transgenics came in 1991. Broglie *et al*. constitutively expressed bean chitinase gene in tobacco and *Brassica napus* and the plants showed enhanced resistance to *Rhizoctonia solani*[18]. Since then there have been a number of reports on transgenics developed by constitutively expressing PR-protein genes (Table 2)[18–38]. Although many such plants showed a degree of resistance to fungal pathogens, some did not, even though PR proteins were found to inhibit fungal growth *in vitro*. Since many of the PR proteins may be acting synergistically *in vivo* and also show enhanced inhibition of fungal growth when tested in combinations *in vitro*[39], transgenic plants expressing more than one PR protein genes in a constitutive manner were developed (Table 3). Such transgenics showed better resistance levels than transgenics having a single gene.

At this stage it is important to mention that the introduction of a desired gene in the host plant under constitutively high expressing promoter can cause silencing of the transgene as well as its endogenous homologue leading to a high proportion of progeny losing its enhanced resistance[40,41]. Therefore, studies on gene silencing will have important implications in the use of transgenic plants for combating fungal diseases.

Plant ribosome-inactivating proteins: Plant ribosome-inactivating proteins (RIPs) have *N*-glycosidase activity and they remove an adenine residue from 28S rRNA. As a consequence, the 60S ribosomal subunit is not able to bind to elongation factor 2, resulting in inhibition of protein elongation. 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*[42]. Tobacco plants constitutively expressing a RIP encoding DNA sequence of barley showed better resistance to *R. solani*[43]. Resistance levels improved when RIP was used in combination with either PR2 or PR3 (ref. 31). However transgenic wheat plants expressing barley RIP showed only moderate or no resistance to *Erysiphe graminis*[44].

Small cystein-rich proteins: In addition to PR proteins, there are other plant proteins which have antifungal activities. A number of small cystein-rich proteins form a separate group of antifungal polypeptides. Some of these are chitin-binding proteins, plant defensins and thionins. Hevein, a non-enzymatic chitin-binding protein of 43 amino acids from latex of rubber trees, is cystein-rich and its precursor, a preprotein is homologous to tobacco PR4 protein[39]. An agglutinin (UDA) isolated and characterized from *Urtica dioica* (stinging nettle) is another chitin-binding protein homologous to hevein and has two chitin-binding domains. Hevein and UDA are the only two chitin-binding plant lectins which have been shown to inhibit fungal growth *in vitro*. Transgenic tomato plants expressing hevein gene showed fewer symptoms on slices of transgenic tomato fruits compared to controls when infected with *Trichoderma hamatum*[45]. Partial protection may have been due to poor processing of the preprotein. In transgenic tobacco expressing *UDA* gene, the agglutinin was processed properly and showed antifungal activity[46].

Thionins are other cystein-rich low molecular weight proteins (about 5 kDa) and have been identified in various organs of a number of plant species. They show antimicrobial activity when tested *in vitro* against various bacteria and fungi[47]. The antimicrobial action is thought to be based on the ability of thionins to form pores in cell membrane resulting in membrane disruption and cell death. Expression of α-thionin gene from barley in tobacco confers enhanced resistance to bacterial pathogens[48]. Over expression of an endogenous thionin enhances resistance in *Arabidopsis* against *Fusarium oxysporum*[49]. Plant defensins are another class of small cystein-rich

Table 2. PR protein genes used for making fungus-resistant transgenic plants

Plant species	PR protein	Donor	Fungus tested	Resistance	Ref.
Alfalfa (*Medicago sativa*)	PR2 (class II glucanase)	*Alfalfa* (*M. sativa*)	*Phytophthora megasperma*	+	19
Canola (*Brassica napus)*	PR3 (class I chitinase)	Bean (*Phaseolus vulgaris*)	*Rhizoctonia solani* *Pythium aphanidermatum*	+	18
Carrot (*Daucus carota)*	PR5	Tobacco (*Nicotiana tabacum*)	*Erysiphe heraclei*	+	20
Grapevine (*Vitis vinifera*)	PR3 (class I chitinase)	Rice (*Oryza sativa*)	*Elisinoe ampelina*	+	21
Kiwifruit (*Actinidia chinensis*)	PR2 (class I glucanase)	Soybean (*Glycine max*)	*Botrytis cinerea*	+	22
Potato (*Solanum tuberosum*)	PR5	Potato (*S. commersonii*)	*Phytophthora infestans*	+	23
	PR5	Tobacco	*P. infestans*	+	17
Rapeseed (*B. napus)*	PR3 (class I chitinase)	Tobacco–tomato (chimeric)	*Cylindrosporium concentricum* *Phoma lingam* *Sclerotinia sclerotiorum*	+ + +	24
Rice	PR3 (class l chitinase)	Rice	*Rhizoctonia solani*	+	25
		Rice	*Magnaporthe grisea*	+	26
	PR5	Rice	*Rhizoctonia solani*	+	27
Tobacco (*N. tabacum)*	PR1a	Tobacco	*Pernospora tabacina*	+	12, 28
			Phytophthora parasitica var. *nicotianae*	+	12
			Cercospora nicotianae	–	
	PR2 (class I glucanase)	Soybean	*P. infestans*	+	29
	PR2 (class II glucanase)	Alfalfa	*C. nicotianae*	+	30
		Barley (*Hordeum vulgare*)	*R. solani*	+	31
	PR3 (class I) chitinase	Bean	*R. solani*	+	18
		Rice	*C. nicotianae*	+	30
		Tobacco	*R. solani*	+	28, 32
	PR3 (class II chitinase)	Barley	*R. solani*	+	31
	PR3 (class III chitinase)	Sugarbeet (*Beta vulgaris*)	*C. nicotianae*	+	33
		Cucumber	*R. solani*	+	28
		Tobacco	*R. solani*	+	28
	PR5	Tobacco	*P. parasitica* var *nicotianae*	–	17
	SAR 8.2(d)	Tobacco	*Phytophthora parasitica*	+	28, 34
	SAR 8.2d	Tobacco	*Pythium torulosum*	+	34
Tobacco (*N. sylvestris)*	PR3 (class I) chitinase	Tobacco	*Cercospora nicotianae*	–	35
Tomato (*Lycopersicon esculentum*)	PR2 (class I glucanase)	Tobacco	*Fusarium oxysporum* f.sp. *lycopersici*	+	36
	PR3 (class I chitinase)	Tobacco	*Fusarium oxysporum* f.sp. *lycopersici*	–	36
	PR3 (class II chitinase)	Tomato	*Verticillium dahliae*	+	37
Wheat (*Triticum aestivum*)	PR3 (class II chitinase)	Barley	*Erysiphe graminis*	+	38

proteins and they are structural and functional homologues of insect and mammalian proteins that have well established roles in host defense[50]. Plant defensins can be classified into at least three groups. The groups show *in vitro* antifungal activities against various fungi either without morphological changes of the fungi ('nonmorphogenic' plant defensins) or with an increase in hyphal branching ('morphogenic' plant defensins). The third group amongst defensins belongs to α-amylase inhibitors and these do not show inhibitory effects on fungal growth. One of the best studied plant defensins is Rs-AFP2 (*Raphanus sativus* antifungal protein-2). Transgenic tobacco plants producing RS-AFP2 show enhanced resistance to the foliar pathogen *Alternaria longipes*[50]. Gene encoding cysteine-rich antimicrobial protein (Ace-Amp-1) from onion overexpressed in geranium leads to increased resistance to *Botrytis cinerea*[51]. Similarly, a gene for cysteine-rich defensin from alfalfa seeds alfAFP (alfalfa antifungal peptide) when expressed under the control of 35S promoter in transgenic potato imparted resistance to *Verticillium dahliae*, *Alternaria solani* and *Fusarium culmorum* but not to *Phytophthora infestans*[52].

Rirlb gene belongs to a family of defense-related genes (WIRI family) that have so far only been described in

cereals[53]. Constitutive expression of defense-related *Rirlb* gene of rice in transgenic rice plants confers 40–50% enhanced resistance to the rice blast fungus *Magnaporthe grisea*[54].

Lipid transfer proteins: The proteins are so named because of their ability to stimulate the transfer of a broad range of lipids through the membrane *in vitro* and might be involved in secretion of or deposition of extracellular lipophillic materials such as cutin or wax. Defensive role for LTPs has been reviewed by Garcia-Olmedo *et al*.[55]. The same group developed transgenics in tobacco and *Arabidopsis* with constitutively expressing barley LTP2 protein and reported enhanced tolerance to *Pseudomonas syringae*[56].

2S storage albumins: Although 2S albumins are generally considered storage proteins, these proteins are known to inhibit the growth of pathogenic fungi. Terras *et al*.[57] showed that a 14 kDa heterodimeric 2S albumin from Brassicaceae seeds are inhibitors of fungal growth *in vitro*. Furthermore, thionin antifungal activity was synergistically enhanced by either small submit (4 kDa) or large subunit (10 kDa) of the radish 2S-albumin and also by three other 2S-albumin like proteins. These results suggest a dual role for 2S albumins, one as a storage protein and the other in plant defense although definite evidence for this can be only obtained by production of transgenic plants with such genes.

Polygalacturonase inhibitor proteins (PGIPS): Proteinaceous inhibitors of fungal polygalacturonase have been identified in extracts of several plants like pear, tomato and bean[58,59]. It is presumed that polygalacturonases function in pathogen infection by facilitating host cell wall degradation and PGIPs interfere with this process. Transgenic tomato fruits constitutively expressing pear PGIPs showed reduced colonization by *Botrytis cinerea*, which was observed as reduced lesion number and reduction in the size of lesions by 25% and also reduced post-harvest infection on fruits[60].

Antiviral protein: High level constitutive expression of pokeweed antiviral protein II (PAP II) cDNA in tobacco plants conferred resistance to the host plant against tobacco mosaic virus, potato virus X and the fungal pathogen *R. solani*[61]. TMV lesions were reduced by 60–80% in transgenic plants. During fungal infection, seedling mortality was reduced by 30 to 40% compared to 90% in controls.

Non-plant antifungal proteins: Fungal growth is inhibited *in vitro* by cell wall degrading enzymes, mostly chitinases, from various fungi. Some of these chitinases show synergy with PR5 proteins or other membrane affecting compounds and other fungal cell wall hydrolases[62,63]. An exochitinase gene from bacterium *Serratia marcescens*, when expressed in transgenic tobacco, renders the host plants less susceptible to *R. solani*[64,65]. Plants which co-express *Trichoderna harzianum* endochitinase gene and a tobacco *PR5* gene[63] and plants over expressing *Streptomyces* chitosanane gene[66] have been produced. Chitosanase enzyme isolated from these transgenic plants was found to be as effective as native *Streptomyces* chitosanase in inhibiting fungal growth *in vitro*. A fungal chitinase gene from *Rizopus oligosporus* confers antifungal activity to transgenic tobacco[67].

Different strains of *Ustilago maydis*, a fungal pathogen of *Zea mays*, harbour different double-stranded RNA viruses encoding antifungal proteinaceous killer toxins, e.g. three subtypes P_1, P_4 and P_6 of *U. maydis* produce KP_1, KP_4 and KP_6 killer toxins respectively. *U. maydis* strains are resistant to the toxin produced within themselves but sensitive to the killer toxins of other strains. High level secretion of KP_4 or KP_6 killer toxin in transgenic tobacco plants rendered them resistant to fungal pathogens[68].

Table 3. Two genes used in combination for making fungus-resistant transgenic plants

Plant species	Gene 1	Donor	Gene 2	Donor	Fungus tested	Resistance	Ref.
Carrot	PR3 (class I chitinase)	Tobacco	PR2 (class I glucanase)	Tobacco	*Alternaria dauci* *Alternaria radicina* *Cercospora carotae* *Erisyphe heraclei*	+ + + +	20
Tobacco	PR3 (class I chitinase)	Rice	PR2 (class II glucanase)	Alfalfa	*C. nicotianae*	+	30
	PR3 (class II chitinase)	Barley	PR2 (class II glucanase)	Barley	*R. solani* *Alternaria alternata* *B. cinerea*	+ + +	31
	PR3 (class II chitinase)	Barley	type I RIP	Barley	*R.solani* *A. alternata* *B. cinerea*	+ + +	31
Tomato	PR3 (class I chitinase)	Tobacco	PR2 (class I glucanase)	Tobacco	*Fusarium oxysporum* f. sp. *lycopersici*	+	36

Induced expression of sarcotoxin IA, a bactericidal peptide from *Sarcophaga peregrina* enhanced the resistance of transgenic tobacco plants to *R. solani* and *Pythium aphanidermatum*[69].

A hen egg white lysozyme (HEWL) gene has been expressed in transgenic potato and tobacco plants. The HEWL recovered from transgenic tobacco plants exhibited antimicrobial activity towards several bacteria and chitin containing fungi like *Botrytis cinerea*, *Verticillium albo-atrum* and *R. solani*[70]. Fungi containing mainly chitosan or cellulose in their cell wall were not inhibited in their growth by HEWL.

A modified synthetic gene encoding for chimeric cationic antimicrobial peptide (CAP) which contains sequences of cercosporin A at N terminus and modified mellttin sequence at C terminus, when expressed constitutively in transgenic potato conferred high level of resistance against *Erwinia carotovora*, *Phytophthora cactorum* and *Fusarium solani*[71].

Four synthetic cationic peptides pep6, pep7, pep11 and pep20 have been found to inhibit *Phytophthora infestans* and *Alternaria solani in vitro*[72]. One synthetic hexapeptide inhibits growth of *Penicillium italicum*, *P. digitatum* and *Botrytis cinerea* during post-harvest infection of fruits[73]. These peptides can be expressed in transgenic plants for improving their resistance to fungal pathogens.

Plants can also be engineered to produce antibodies against fungal molecules necessary for pathogen to successfully infect plants. The possibility to produce functional plantibodies against fungal antigens is being explored by different groups[74].

Phytoalexins

Phytoalexins are antimicrobial low molecular weight secondary metabolites produced in plants following pathogen attack and are believed to have a role in plant defense[75]. Biosynthesis of phytoalexins is often complex involving many pathways and hence, several substrates and enzymes. Nevertheless, there has been success in developing transgenics which synthesize new phytoalexins by simply introducing the gene for the last enzyme of the pathway. As an example, Hain and coworkers introduced the gene encoding stilbene synthase from grape vine (*Vitis vinifera*) into tobacco plants[76]. In tobacco the substrate for stilbene synthase is available, but the enzyme per se is absent. The expression of stilbene synthase (or resveratrol synthase) gene resulted in the production of resveratrol, a stilbene-type phytoalexin. Such transgenics showed enhanced resistance to *B. cinerea*. Similar transgenic plants were developed in rice, tomato, barley and wheat and were shown to have increased resistance to *Magnaporthe grisea*, *P. infestans* and *B. cinerea* respectively[77–79]. *Arachis hypogea* resveratrol synthase cDNA when expressed under 35S CaMV promoter in transgenic alfalfa confers resistance to *Phoma medicaginis*[80].

Transgenics engineered for hypersensitive-response

In the above section, we have reviewed several reports concerned with the development of transgenics using genes which encode for antifungal compounds like PR proteins, phytoalexins, toxins, etc. However, it appears that genes encoding these antifungal proteins provide resistance to only a limited level and to only a limited number of fungi. For example, over-expressing the chitinase gene did not provide resistance against fungi lacking chitin. Moreover, a fungus can modify its cell wall by biosynthesis of more chitosan or glucan in place of chitin and, therefore, may become pathogenic again or it can evolve mechanisms to detoxify certain phytoalexins. Sexually reproducing fungi may develop resistance much faster. Furthermore, since plants are attacked by different microorganisms during their life cycle, absence of one kind of pathogen (e.g. chitinase sensitive) will benefit other pathogens. Currently strategies that will lead to more durable and broad spectrum resistance in transgenic plants are being investigated. These strategies depend upon pathogen-induced cell death and general defense responses occurring in plants during incompatible plant–pathogen interactions.

Resistance genes from plants

All plants have passive defense lines such as cell walls, wax layers and chemical barriers against pathogens. If the pathogen overcomes this first line of defense, there is a second line of defense, which is mounted by proteins encoded by specific resistance *(R)* genes. This line of defense is best described genetically by the gene for gene model[81]. It requires a pathogen protein encoded by an avirulence (*Avr*) gene to be recognized by a plant protein encoded by a resistance (*R*) gene. This activates an array of defense mechanisms, including the hypersensitive response. The gene-for-gene model although first proposed in flax-rust system, explained the genetics of resistance in other pathogens as well whether obligate or facultative. Evolutionary pressure to combat a pathogen with the evolution of new *R* genes in the host plant is more for obligate parasites. During the last decade more than 30 resistance genes which confer resistance against a wide range of pathogens, including viruses, bacteria, fungi, nematode and even aphids have been cloned from both monocots and dicots[6,9,82–84]. Interestingly, different resistance genes are highly homologous to each other and their products are remarkably similar. All the R proteins contain leucine-rich repeat (LRR) domain, with only one exception of R protein of tomato, Pto. Besides the LRR domain, some R proteins contain nucleotide-binding site (NBS) and/or leucine zipper (LZ) or a domain with homology to toll receptor or interleukine I receptor (TIR) (see Table 4). The structure of R protein does not reflect

much about the kind of pathogen it works against. As an example, *Sw-5* gene conferring resistance to topsovirus in tomato is a homolog of root-knot nematode resistance gene *Mi* in tomato[85]. The *R* genes in *Arabidopsis* conferring resistance against viruses and oomycete fungi belong to the same HRT/RP8 family of *R* genes[86].

Although the gene-for-gene model assumes that the resistance gene product of a plant binds to corresponding avirulence gene product of a pathogen to trigger HR, it should be kept in mind that till date only three cases of direct *R-Avr* product interactions have been demonstrated, i.e. *Pto–AvrPto* system in tomato–*Pseudomonas* interaction[87], *Pi-ta–AvrPITA* in rice–*Magnaporthe* interaction[88] and *TIP–TCV* coat protein in *Arabidopsis*–turnip crinkle virus interaction[89]. Different plants have different spectrum of *R* genes which work against different *Avr* genes present differentially in pathogens thereby explaining, at least to an extent, their different resistance-conferring behaviour. Hence incorporation of *R* gene from resistant plant to susceptible plant should logically lead to resistance to pathogen carrying corresponding *Avr* gene. Tobacco plants transgenic for *pto*, the *R* gene in tomato against *Psuedomonas*, were resistant to *Pseudomans syringe* pv *tabaci* expressing *avr pto*[90]. But it is interesting to note that a different region (C-terminus) of avr–pto protein of *Psuedomonas* is recognized by the *R* gene when it is in tobacco compared to when it was in tomato where the central region of *avr–pto* is recognized[91]. A constitutive mutant of *Pto* induces a hypersensitive response in the absence of *avrPto*[92]. Expression of such mutants under the control of defined inducible promoters would be a useful strategy for expressing disease resistance. Also overexpression of *Pto* in tomato activates defense responses and confers broad resistance, not only to *Psuedomonas syringae* but also to *Xanthomonas campestris* and *Cladosporium fulvum*[93]. In rice, the *Xa21* gene (conferring resistance to bacterial blight caused by *Xanthomonas oryzae*) isolated from indica rice strain IRBB21 when introduced into a susceptible variety IR72, resulted in excellent field resistance against the pathogenic bacteria[94]. Three alleles of flax rust resistance genes namely *L2*, *L6* and *L10* were incorporated into flax lines that were highly susceptible to different rust strains. The transgenic plants were shown to be resistant to strains of flax rust which had corresponding *avr* genes[95]. However, the plant breeder often faces the problem of lack of effective resistance genes for a particular plant disease in the related germplasm of a crop species which could be readily intercrossed.

The intensive efforts in the past few years to completely sequence ~ 130 Mb *A. thaliana* genome are beginning to make an impact on finding more and more resistance genes and their structural homologues. More than 160 genes belonging to NB-LRR gene family have been annotated in the completed DNA sequence. Most of these genes are clustered at about 15 loci[9]. Efforts are being made to establish a functional role for numerous *R* genes. Although the transformation technology has circumvented problems like linkage drag associated with wide hybridization, the use of *Arabidopsis R* genes or some of its defense pathways in other crops will be possible only if the underlying defense mechanisms are conserved between *Arabidopsis* and other crops. One can keep in mind the examples of *RPS2* gene of *Arabidopsis* being non-functional in tobacco and *Bs2* gene of pepper against *Xanthomonas* being functional in tomato but not in non-solanaceous plants[96]. This suggests that there could be difficulties in interfamily transfer. For example, *R* genes against *Fusarium oxysporum* occur in tomato[97] but not in cotton. Can these genes be transferred from tomato to cotton and will they work in cotton or they will show restricted taxonomic functionality[96]? Even transfer of *R* genes within a family might be rewarding. Alternatively, naturally occurring resistance genes could be modified and designed *in vitro* for altered specificity. Isolation of *R* genes from *Arabidopsis* will also facilitate isolation of *R* genes from other crops keeping in view the extent of parallelism in gene order among genera. Another issue in

Table 4. Different classes of R genes and their examples

Structure of the gene	Example	Plant	*Pathogen*
LZ–NBS–LRR	*Prf*	Tomato	*Pseudomonas syringae*
	RPP8	*Arabidopsis*	*Peronospora parasitica*
NBS–LRR	*Xa1*	Rice	*Xanthomonas oryzae*
	Mla	Barley	*Erysiphe graminis*
TIR–NBS–LRR	*N*	Tobacco	*TMV*
	RPP5	*Arabidopsis*	*Peronospora parasitica*
LRR–TM–PK	*Xa21*	Rice	*Xanthomonas oryzae*
PK	*Pto*	Tomato	*P. syringae*
LRR–TM	*Cf2*	Tomato	*Cladosporium fulvum*
	Cf9	Tomato	*C. fulvum*

LZ, Leucine zipper, NBS, Nucleotide binding site, LRR, Leucine rich repeats, TIR, Toll or interleukine 1 receptor, TM, Transmembrane, PK, Protein kinase.

transferred *R* gene functionality is the frequency with which corresponding *Avr* gene occurs in the pathogen of recipient host. Some non-host *R* genes may be more durable if they recognize general or genera-specific elicitors. Non-host *R* genes are likely to confer resistance to all races when first employed if the pathogen population has not been exposed to the *R* gene[84]. Understanding the mechanism of non-host resistance will have a great deal of applied significance in developing resistance to a broad range of pathogens.

Resistance gene-avirulence gene two-component system: De Wit[98] proposed a model of expressing both the resistance gene (*R*) and avirulence gene (*Avr*) in the plant. When this *R–Avr* gene cassette is put under strict pathogen-inducible promoter, resistance reactions like HR will be activated upon pathogen infection. Tang and coworkers[87] and Scofield *et al*.[99] showed that resistance to bacterial speck disease in tomato transgenics occurs when the resistance gene *pto*, a kinase, binds to *avr–pto* transgene (from *P. syringae*) expressing in the tomato plants. Similarly, expression of avirulence gene *hrmA* from *P. syringae* in transgenic tobacco plants under the control of a nematode-inducible promoter confers high level of resistance to these plants against tobacco vein mottling virus, tobacco etch virus, *Phytophthora parasitica* and *P. syringae*[100]. On similar lines, resistance gene of plants against fungal pathogens can be used in combination with fungal *avr* genes to produce fungus-resistant transgenic plants. *R–Avr* two component system is more advanced, sophisticated and broad spectrum in action in the sense that it will provide resistance to any pathogen which can activate the promoter of *R–Avr* cassette provided the promoter used is tightly pathogen-inducible and non-leaky.

Barnase–barstar two component system: Barnase, a cytotoxic protein with RNAse activity and barstar, its inactivator, are two proteins present in *Bacillus amyloliquefaciens*. Stritmatter *et al*.[101] placed the *barnase* gene under the control of pathogen-inducible potato *prp-1-1* promoter so that *barnase* activity kills the cells at the site of infection. To avoid cell death due to unwanted expression of the *barnase* gene, the *barstar* gene was constitutively expressed in all tissues. Cells are killed only if barnase activity is higher than barstar activity. Transgenic potato plants showed severe local necrosis of leaf tissue upon inoculation with *Phytophthora infestans* spores. Symptom development was greatly reduced. This strategy, however, was not tested at the field level.

Broad spectrum disease resistance using SAR

One of the effective strategies for broad spectrum plant disease resistance has been to exploit SAR pathway. Several plant mutants have been obtained that constitutively induce SAR[102–105]. Such lesion-mimic mutations have been effective in designing resistance to powdery mildew in barley. However the cell death lesions were not tightly regulated and plants were dwarfed[106,107]. A major challenge is to develop transgenics that can express SAR pathway without such deleterious side effects.

Oldroyd and Staskawicz developed transgenic tomato plants showing resistance to a number of bacterial and viral pathogens by overexpressing a *Prf* gene which works downstream of or along with *Pto* (both *Prf* and *Pto* are resistance genes in tomato against *P. syringae*)[108]. Overexpression of *Prf* gene induces SAR in tomato in a pathogen independent manner and interestingly low levels of *Prf* mRNA overexpression are sufficient for the induction of SAR but insufficient for HR. The induction of SAR without deleterious side effects makes *Prf*-mediated transgenic SAR a target for production of broad spectrum-enhanced resistance in agricultural crops. This strategy can be extended to fungal pathogens as well.

An *RDR* (required for disease resistance) gene *Npr1* of *Arabidopsis* whose function in signal transduction pathway is not known, confers broad spectrum resistance to bacterium *P. syringae* and fungus *Peronospora parasitica*[109] when constitutively expressed in *Arabidopsis*. Resistance responses in such transgenic plants are not constitutively activated when plants are grown under non-inducing conditions. However, upon infection by pathogen like *P. syringae* and *Peronospora parasitica* the responses are induced at higher levels. Negative regulation or mutation in genes like *MAP4* kinase of *Arabidopsis* has been shown to induce constitutive SAR response but without lesions[110]. This opens up yet another avenue for induction of broad spectrum resistance in plants. However finer understanding of regulation of genes involved in SAR will help us to develop resistant transgenic plants without undesired side effects like dwarfism and sterility.

A significant challenge is to understand the means by which plants sense pathogens in the absence of the *R* genes. Various defense pathways can be activated by virulent pathogens which are not recognized by *R* genes, suggesting that other pathogen surveillance mechanisms exist which attenuate the severity of disease[111]. Understanding these mechanisms will provide us with more options for developing fungus resistance in crop plants.

Other approaches to induce cell death

One of the earliest events in incompatible plant pathogen interaction is oxidative burst during which active oxygen species such as H_2O_2 are produced[112]. H_2O_2 triggers production of phytoalexins, PR proteins and other HR-related processes.

H_2O_2 also has a direct inhibitory effect on microbial growth[113]. Glucose oxidase (GO), an enzyme occurring in some bacteria and fungi, brings about the oxidation of *b*-D-glucose, yielding gluconic acid and H_2O_2. *GO* has not

been found in animals and plants. Expressing a *GO* gene from a fungus *Aspergillus niger* in potato showed increased level of H_2O_2. Such transgenics had reduced susceptibility to *E. carotovora* subspecies *carotovora*, *P. infestans* and *Verticillim dahliae*[113].

Animals contain myeloperoxidase (MPO) and haloperoxidase (HPO) which convert H_2O_2 to a much stronger antimicrobial compound hypochlorous acid (HoCl). But plants transformed with MPO do not produce HoCl because of species–specific requirement of heme-containing prosthetic groups to catalyse the redox reaction. Certain bacterial HPOs do not require heme prosthetic groups or even metal ion cofactors. Such a chloroperoxidase (CPO-P) gene from *Pseudomonas pyrrocinia* when over expressed in tobacco conferred resistance to fungal pathogen *Colletotrichum destructivum*[114].

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. When Mittler *et al.*[115] expressed a gene for light driven proton pump bacterio-opsin (bO) from *Halobacterium holobium* in transgenic tobacco, responses such as HR-type lesions, accumulation of PR gene transcripts, phenylammonia lyase and some other compounds typically associated with SAR were observed. Transgenic plants showed enhanced resistance towards tobacco mosaic virus and *P. syringae*. It is not clear how bO activates cell death but can be one of the tools to produce broad spectrum resistance in plants.

Concluding remarks

Our knowledge of molecular events occurring during plant–pathogen interactions has expanded significantly in the last ten years. Based on this knowledge, 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. For the antifungal compounds strategy to be successful in the long term, level of resistance in transgenic plants should be increased and its range should be broadened by isolating new genes and by testing new combinations of genes. 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 pathways and in isolation of more and more *R* genes in *Arabidopsis* and their orthologous counterparts in other crop species. With the publication of draft sequences of the rice genome by two groups[116, 117], in April 2002 the work on the isolation of *R* genes from rice, wild relatives of rice and other cereal crops would get a major boost. Two component systems like '*barnase–barstar*' system or '*R–Avr*' system are being developed but such a strategy must rely on tightly regulated plant promoters which express specifically and are exclusively limited to infection sites. Genetic manipulation of the regulatory mechanisms and signalling processes controlling the coordinate activation of multiple defense responses like SAR might be the ultimate approach to modify plant resistance. However, this requires precise knowledge of both the signalling pathways involved and subsequent metabolic pathways that get triggered. While exploiting the genes in signalling pathway for making fungus-resistant transgenic plants one needs to be cautious about the role of the signalling gene in various other pathways which would lead to undesirable side effects in transgenic plants. The earlier the gene function in the pathway, the greater the intricacies of regulation that will have to be addressed. Correct temporal and spatial expression of the transgene will be of critical importance and will require the availability of well-defined, pathogen-inducible promoters with the desired properties.

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