European Journal of Plant Pathology **105:** 319–326, 1999.

Mini review

Engineered resistance against fungal plant pathogens

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Accepted 5 February 1999

Key words: avirulence genes, defensins, hypersensitive response, phytoalexins, PR proteins, resistance genes

Abstract

Development of genetic engineering technology and molecular characterization of plant defense responses have provided strategies for controlling plant diseases additional to those based on chemical control or classical breeding programs. Most of these alternative strategies are based on the overproduction of one component of the plant's own defense response. Some strategies exploit the hypersensitive response, a rapid, localized death of tissue surrounding the infection site, which is observed in many resistant plants upon unsuccessful pathogen attack. Most approaches to increase resistance to fungi have been described to be successful under laboratory conditions. Incorporation of these successful, alternative strategies in resistance breeding programs of agriculturally important crops will depend on the results obtained from field experiments.

Abbreviations: AOS – active oxygen species; HR – hypersensitive response; IDPM – Integrated Disease and Pest Management; PR – pathogenesis-related; RIP – ribosome inactivating protein.

Introduction

As long as agriculture exists, farmers have to battle against pathogens which infect crop plants and thereby cause considerable losses of yield and quality. To control plant diseases and pests, several strategies have been developed. These strategies are based on genetic, chemical, biological and cultural methods which, nowadays, are combined in Integrated Disease and Pest Management (IDPM). IDPM aims at maximum productivity with the least possible negative environmental and ecological consequences.

Introduction of pathogen-resistant varieties is one of the strategies to protect crops against diseases. Breeding programs have resulted in many new varieties with desirable resistance traits. However, breeding for disease resistance has been complicated by the rapid evolution of pathogens which results in new strains overcoming the resistant plant variety. Genetic engineering accelerates the breeder's efforts and opens possibilities for alternative strategies to control plant diseases. Elucidation of the cytological, physiological and molecular basis of plant–pathogen interactions and plant resistance provides tools and methods for genetic engineering to produce new, disease-resistant crop varieties.

This review focuses on the different genetic engineering strategies that have been used to obtain transgenic plants resistant to fungal diseases. Resistance is being defined as the range of protection from delayed to complete inhibition of fungal disease development. Current approaches towards molecular resistance breeding can be classified into two major categories. The first approach involves constitutive overproduction of one antifungal compound of the plant's own defense machinery, e.g. PR proteins or phytoalexins, thereby having the plant in a constant state of alert. A variation of this strategy is the constitutive expression of genes from organisms other than plants encoding antifungal compounds, like the gene encoding a human lysozyme. The second approach for obtaining fungus-resistant plants relies on the activation of the whole

array of defense responses by, for instance, activating a hypersensitive response (HR): a rapid, localized collapse of host tissue which prevents the pathogen from further infection. In this review, molecular aspects of defense mechanisms will be briefly discussed as well as the various strategies exemplifying the two major approaches mentioned above to obtain plants resistant to fungal pathogens.

Defense responses

Plants are constantly being challenged by pathogenic microorganisms. However, a certain plant species can only be successfully infected by a rather limited number of pathogens. Failure of infection (incompatible interaction) is mostly based on so-called nonhost resistance. This type of resistance is pathogen-nonspecific and might rely on various complex causes (Heath, 1991). Nonhost resistance can be based on preformed defenses, such as preformed structural barriers (Schlösser, 1997) or toxic compounds (Osbourn, 1996) that arrest the infection process of potential pathogens.

An incompatible interaction can also be the result of induced defense responses: upon pathogen recognition several defense responses are activated and pathogen invasion remains localized. Plant defense responses include reinforcement of cell walls, and accumulation of antimicrobial compounds such as active oxygen species (AOS), phytoalexins and pathogenesis-related (PR) proteins (Dixon et al., 1994). Often, these responses are accompanied by an HR resulting in pathogen containment. At present, a causal link between HR and other defense responses is lacking (Dangl et al., 1996; Heath, 1998). However, the HR may withhold the invading pathogen of nutrients or it may promote the release of antimicrobial compounds from the affected host cells that stop pathogen growth.

Active defense responses are induced by elicitor molecules which originate from the pathogen (De Wit, 1995) or are released from the plant during the infection process (Boller, 1995). Pathogen-derived elicitor molecules can be nonspecific and enzymatically released from the surface of the pathogen during the infection process, or the molecules can be genus- or race-specific and produced by certain strains of the pathogen. The race-specific elicitor molecules are products of avirulence genes. Perception of elicitors by the plant leads to the induction of a large array of biochemical changes, including electrolyte leakage, oxidative burst and changes in protein phosphorylation, as part of the resistance response (Ebel et al., 1994; Hahlbrock et al., 1995; Hammond-Kosack et al., 1996; Ligterink et al., 1997; May et al., 1996; Vera-Estrella et al., 1994).

Constitutive production of defense components with antifungal activity

PR proteins

In many plant species PR proteins accumulate upon infection by pathogenic organisms such as viruses, bacteria and fungi. The PR proteins have been classified into twelve major families (Gamas et al., 1998; Van Loon et al., 1994; Van Loon, 1998). *In vitro* antifungal activity has been described for PR proteins of some families (Van Loon et al., 1994; Wubben et al., 1997). For instance, proteins of the PR-2 and PR-3 families represent β-1,3-glucanases and chitinases, respectively, that are able to degrade fungal cell walls (Kauffmann et al., 1987; Legrand and Fritig, 1987). Proteins of the PR-5 family probably permeabilize the plasma membrane of fungi (Vigers et al., 1992).

Many of the initial approaches to reduce plant susceptibility to infection by fungal pathogens aimed at the constitutive production of one component of the plant's own defense, e.g. PR proteins. The first success was reported in 1991: constitutive production of a vacuolar isoform of chitinase (PR-3(I) family) from bean in tobacco and canola plants resulted in reduced susceptibility to *Rhizoctonia solani* (Broglie et al., 1991). Expression of the transgene provided a reduction in the mortality of infected seedlings as compared to controls (by 20%; from 50% to 30%), but not a complete protection. In contrast, no difference in susceptibility compared to wild-type plants was observed after inoculation with the pathogen *Pythium aphanidermatum*, an oomycete lacking chitin in its cell wall. Transgenic plants that express chitinase genes and show increased resistance to fungal pathogens have also been reported by others. Constitutive expression of PR-3(I) chitinase genes from rice in transgenic rice and cucumber plants resulted in increased protection to *R. solani* and *Botrytis cinerea* infection, respectively (Lin et al., 1995, Tabei et al., 1998).

Mixtures of purified PR-3(I) chitinase and β-1,3-glucanase (PR-2 family) from tobacco showed synergistic antifungal activity *in vitro* (Sela-Buurlage et al., 1993). Accordingly, in transgenic plants co-expressing chitinase and β-1,3-glucanase genes,

enhanced protection to fungal pathogen attack has been observed (Jach et al., 1995; Jongedijk et al., 1995; Zhu et al., 1994). In transgenic tobacco, co-production of chitinase and β-1,3-glucanase resulted in a substantially higher protection to *Cercospora nicotianae* than production of any one of the PR proteins (Zhu et al., 1994). Similarly, combined expression of a PR-3(II) chitinase gene (encoding an extracellular isoform of chitinase) and a PR-2(II) β-1,3-glucanase gene (encoding an extracellular isoform of β-1,3-glucanase) from bean in transgenic tobacco plants revealed enhanced protection to *R. solani* infection as compared to protection levels obtained with transgenic tobacco lines expressing the single transgene to a similar level (Jach et al., 1995). Transgenic tomato plants expressing either one of the tobacco PR-3(I) chitinase or PR-2(I) β-1,3-glucanase genes, showed no protection to infection with *Fusarium oxysporum* f.sp. *lycopersici* (Jongedijk et al., 1995). However, in tomato plants that simultaneously express both genes, the disease severity decreased by 40–60% (Jongedijk et al., 1995).

Organisms other than plants have also been used as a source for genes encoding hydrolytic enzymes such as chitinase and glucanase to produce fungus-resistant plants. A chitinase gene from the soil pathogen *Seratia marcescens*, constitutively expressed in transgenic tobacco plants, resulted in improved resistance to *R. solani* infection (Jach et al., 1992). Mortality was reduced by 60% in transgenic seedlings grown in soil infested with *R. solani* as compared to untransformed tobacco seedlings. Similarly, production of the human lysozyme, an enzyme that cleaves the β-1,4-glycosidic bond of chitin in the fungal cell wall, in transgenic tobacco plants, resulted in enhanced resistance to *Erysiphe cichoracearum* (Nakajima et al., 1994). Both conidia formation and mycelial growth were reduced in the resistant plants.

Constitutive production of PR proteins not belonging to the PR-2 and PR-3 families can also improve disease resistance. Constitutive expression of the PR-1a gene in transgenic tobacco plants resulted in increased tolerance to two oomycete pathogens, *Peronospora tabacina* and *Phytophthora parasitica* var. *nicotianae*, respectively (Alexander et al., 1993). These transgenic plants showed no reduced susceptibility to diseases caused by tobacco mosaic virus, potato virus Y, *Pseudomonas syringae* pv. *tabaci* or *C. nicotianae*. Overexpression of PR-5 in potato resulted in a delay in disease progression upon infection by *Phytophthora infestans* (Liu et al., 1994).

Recently, defensins were classified as PR-12 family members (Van Loon, 1998). Plant defensins are small, cysteine-rich, defense-related antimicrobial peptides and are present in most plant species studied (Broekaert et al., 1995, 1997). Defensins exert their antimicrobial activity at the level of the plasma membrane of the pathogen, but the different types of defensins probably act via different mechanisms. The ability of defensins to play a role in plant defense was first demonstrated by transgenic tobacco plants constitutively expressing a defensin gene from radish which resulted in enhanced resistance to *Alternaria longipes* (Terras et al., 1995; Broekaert et al., 1997). Induced expression of defensins has been observed in some plant species upon pathogen attack.

In conclusion, there are now a number of reports describing constitutive production of PR proteins in transgenic plants which results in increased disease resistance. However, this approach is not always successful: constitutive production of tobacco PR-3(I) chitinase did not increase resistance to *C. nicotianae*, despite the fact that *in vitro* the fungus is sensitive to this chitinase (Neuhaus et al., 1991). Similarly, transgenic tobacco plants producing PR-5 showed no effect on disease development upon infection with *P. parasitica*, although *in vitro* antifungal assays showed that PR-5 is effective against this fungus (Liu et al., 1994). In addition, some pathogens have evolved systems to counteract defense mechanisms of plants. A glucanase inhibitor protein from *Phytophthora sojae* f.sp. *glycines* has been isolated that specifically inhibits a β-1,3-glucanase from soybean (Ham et al., 1997). Combined production of several PR proteins appears to be a promising strategy. However, even in this case, one should expect a quantitatively enhanced resistance rather than a qualitative, complete resistance.

Other antifungal compounds: ribosome-inactivating proteins, phytoalexins and active oxygen species

The plant defensive ribosome-inactivating proteins (RIPs) inhibit protein synthesis by a specific N-glycosidase modification of 28S rRNA. RIPs differ significantly in their substrate specificity, but do not inactivate self-ribosomes (Endo et al., 1988). Transgenic tobacco plants that express a barley RIP gene exhibited increased protection to *R. solani* infection (Logemann et al., 1992). *In vitro* studies showed synergism in antifungal activity between RIP and class I

chitinase when applied to *Trichoderma reesei* and *Fusarium sporotrichioides* (Leah et al., 1991). Transgenic tobacco plants simultaneously expressing the barley RIP gene and a gene encoding a barley class II chitinase showed improved protection to *R. solani* attack as compared to transgenic lines expressing only one transgene to a similar level (Jach et al., 1995). The authors suggest that the hydrolytic activity of chitinase enables an increased uptake of RIP into the fungal cells and therefore enhances inhibition of fungal growth. Transgenic tobacco plants expressing an RIP gene isolated from pokeweed, *Phytolacca americana*, which encodes an antiviral protein that does not inhibit fungal growth when applied *in vitro*, showed resistance to *R. solani* (Zoubenko et al., 1997). In the same study, plants expressing a nonfunctional antiviral protein, also showed resistance to *R. solani* infection. In these fungus-resistant plants, increased PR protein levels were detected, leaving unexplained the role of the antiviral RIP in the increased fungal resistance that was observed.

Production of a phytoalexin, another component of the plant defense response, has been reported to protect the plant from fungal attack (Hain et al., 1993). In grapevine, synthesis of the phytoalexin resveratrol is induced upon pathogen attack. Two stilbene synthase genes from grapevine, required for resveratrol synthesis, were expressed under the control of their own promoters in tobacco, a plant that normally does not have the capacity to form resveratrol. As a consequence, upon attack by *B. cinerea* the transgenic plants produced resveratrol, and disease incidence was reduced by up to 50% as compared to control plants.

The production of active oxygen species (AOS) in the oxidative burst such as superoxide anions (O_2^-), hydroxy radicals ($OH^\bullet$) and hydrogen peroxide (H_2O_2) has been observed in many plant–pathogen interactions and plays an important role in plant defense (Baker and Orlandi, 1995; Hammond-Kosack and Jones, 1996). On one hand, AOS may be directly toxic to pathogens. On the other hand, AOS may contribute to plant cell wall strengthening and play a role in the signal transduction pathway leading to resistance. Therefore, an increase in AOS levels was seen as a possible strategy to improve plant resistance. Indeed, transgenic potato plants constitutively producing an H_2O_2-generating glucose oxidase from *Aspergillus niger* in the apoplast resulted in enhanced resistance to *P. infestans*, *Verticillium dahliae* and *Alternaria solani* (Wu et al., 1995, 1997).

Induction of multiple defense responses

Expression of resistance genes

In recent years, genes conferring resistance to a variety of bacterial, fungal and viral pathogens have been isolated. The predicted products encoded by these resistance genes show a high degree of similarity and can be grouped into five classes based on the presence of conserved structural components (Bent, 1996). These resistance genes confer race-specific resistance which results from the direct or indirect interaction of the resistance gene product with a product derived from the avirulence gene matching the resistance gene (De Wit, 1995, 1997). The subsequent signal transduction events lead to the activation of an array of defense responses (discussed above). The resistance genes are members of gene families, and often occur in clusters. For some resistance gene families the presence of multiple functional homologs within one cluster has been demonstrated (Dixon et al., 1996; Parniske et al., 1997; Wang et al., 1998). Transformation of a susceptible genotype with a resistance gene confers resistance to races of the pathogen which carry the matching avirulence gene. For instance, transfer of the first race-specific resistance gene cloned, *Pto*, to a tomato genotype susceptible to the bacterial pathogen *P. syringae* (avrPto$^+$) resulted in resistance comparable to that observed in the donor genotype (Martin et al., 1993). Also successful cases of interspecies transfer of resistance genes have been described (Parniske et al., 1998; Rommens et al., 1995a; Thomas et al., 1997; Whitham et al., 1996). However, this strategy can only be used against certain strains of a particular pathogen in its natural host. For some resistance genes, developmental regulation of resistance gene-mediated defense has been observed (Hammond-Kosack et al., 1994; Honée et al., 1995, 1998; Parniske et al., 1997) suggesting that durable resistance might be reinforced by differential expression of resistance gene specificities throughout development. Recently, a resistance gene from tomato against the leaf pathogen *Cladosporium fulvum* has been identified that operates through recognition of a crucial virulence factor of the fungus (Laugé et al., 1998). Therefore, it can be expected that this resistance gene confers durable resistance to a broad range of *C. fulvum* genotypes. In the future, resistance genes conferring different resistance specificities or broad range resistance can possibly be created in the laboratory (Bent, 1996; Rommens et al., 1995b).

These artificial resistance genes will be valuable in breeding for durable resistance.

Engineered acquired resistance

The formation of necrotic lesions (HR), that is induced upon specific pathogen recognition, coincides with a number of defense responses that occur around the infection site and distally in uninfected parts of the plant. As a result, these plants show a systemic acquired resistance (SAR) against a broad spectrum of pathogens (Ryals et al., 1994, 1996). To confer pathogen resistance, transgenic plants are engineered which exhibit HR-like symptoms in the absence of pathogen infection. In tobacco and potato, expression of the gene encoding the bacterio-opsin proton pump from *Halobacterium halobium* resulted in the formation of necrotic lesions and enhanced levels of salicylic acid and mRNA levels of genes encoding PR proteins (Abad et al., 1997; Mittler et al., 1995). The transgenic tobacco plants showed enhanced resistance to tobacco mosaic virus, tobacco necrosis virus and the bacterial pathogen *P. syringae* pv. *tabaci* (Mittler et al., 1995). In potato, expression of the bacterio-opsin gene resulted in enhanced resistance to a *P. infestans* isolate (Abad et al., 1997). However, no resistance was conferred to another more aggressive *P. infestans* isolate, Potato Virus X, and the bacterial pathogen *Erwinia carotovora*.

Constitutive expression of an avirulence gene in transgenic plants which contain the matching resistance gene results in necrosis and eventually in death of the whole plant (Hammond-Kosack et al., 1994; Honée et al., 1995, 1998; Pfitzner and Pfitzner, 1992). Transgenic tomato plants that carry the resistance gene against *C. fulvum* inactivated by insertion of the maize transposable element *Ds*, the avirulence gene *Avr*9 from *C. fulvum*, and a maize *Ac* transposase gene, show somatic excision of the *Ds* element thereby restoring *Cf-9* resistance gene function. As a result, defense responses are induced and necrotic lesions appear in sectors that simultaneously express the *Avr*9 gene and the restored *Cf-9* gene. For these plants, reduced susceptibility to fungal infection has been reported as a consequence of the induction of acquired resistance (Hammond-Kosack et al., 1998).

Constitutive expression of SAR without the appearance of spontaneous lesions has been observed in the *cpr* mutants (constitutive expression of PR genes) in *Arabidopsis thaliana* (Bowling et al., 1994). In these mutants elevated levels of PR gene expression and elevated SA levels have been observed. The *cpr* mutants showed enhanced resistance to the fungal pathogen *P. parasitica* and the bacterial pathogen *P. syringae* (Bowling et al., 1994).

The examples described above show the potential of the use of engineered acquired resistance in molecular resistance breeding. This method for pathogen control is based either on the random appearance of necrotic lesions on the plant or on mutations that affect control genes of the SAR-related signaling pathway(s). However, detrimental phenotypic characteristics observed in these plants are undesired (Abad et al., 1997; Bowling et al., 1994) and need to be solved before utilizing this strategy in agronomically important crops.

Engineered HR

The HR triggered upon recognition of intruding pathogens is an efficient defense reaction that allows the plant to withstand pathogen invasion. Artificial cell death, generated by the synthesis of cytotoxic compounds in infected tissue has been used for engineering disease resistance (Strittmatter et al., 1995). Transgenic potato plants were generated, containing the barnase gene from *Bacillus amyloliquefaciens* that encodes a cytotoxic RNase, under the transcriptional control of the pathogen-inducible promoter *gst-1* (Martini et al., 1993). Besides the barnase gene, these potato plants also expressed the barstar gene isolated from *B. amyloliquefaciens*, encoding a specific barnase inhibitor to reduce detrimental effects due to background synthesis of barnase in unaffected tissue. Induction of barnase expression and thereby changing the balance between barnase and barstar expression resulted in cell death. Upon inoculation with *P. infestans*, these plants allowed reduced sporulation of the fungus, but the necrotic area was not restricted to the vicinity of the inoculation sites.

To control pathogen diseases, the use of an avirulence gene and its matching resistance gene in transgenic plants, that by simultaneous expression induce rapid and localized cell death at infection sites, has been suggested (De Wit, 1992). Expression of the avirulence transgene in plants carrying the matching resistance gene at the time and site of infection will trigger a local HR. Transgenic Cf9 tomato plants, carrying the *Avr*9 gene under the transcriptional control of the pathogen-inducible *gst-1* promoter (Martini et al., 1993) showed

resistance to infection by several fungal pathogens including *C. fulvum* and *Oidium lycopersicum* (Stuiver and Honée et al., unpubl. results). At the infection sites, host cell death, unsuccessful colonization and restricted fungal growth were observed. These responses were similar to the resistance responses naturally induced upon infection of Cf9 genotypes by *Avr*9-containing *C. fulvum* genotypes. In addition to resistance to fungal pathogens, preliminary results demonstrated resistance to a viral pathogen (Stuiver and Honée et al., unpubl. results). These results demonstrate that this strategy to obtain broad-spectrum pathogen resistance is feasable. The two key components required are the *Avr*9 gene and the *Cf-9* gene, which have been cloned and well-defined, enabling successful application of this strategy in other plant species. A prerequisite, however, is that the signaling cascade to trigger AVR9–CF9-mediated resistance be present in these plant species. Furthermore, promoters are needed which are tightly regulated and result only in an AVR9–CF9-mediated HR at the time and site of pathogen infection. For instance, the plant signal molecules auxin and salicylic acid also induce *gst-1* promoter activity and in transgenic potato expression is observed in root caps and senescing leaves (Strittmatter et al., 1996). Positioning of the *Avr*9 and *Cf-9* genes under the control of different pathogen-inducible promoters which show divergent expression patterns, but a simultaneous expression only at the infection sites, would allow induction of HR exclusively at these sites.

Concluding remarks

Research on the molecular biology of plant–pathogen interactions has provided extensive new insights in susceptibility or resistance of plants and in pathogenicity of plant pathogens. Application of these findings to engineer pathogen-resistant plants is still in the phase of development and model testing. Two approaches in molecular resistance breeding are currently being followed: enhancement of one or few elements of the plant's own defense and activation of orchestrated defense responses by induction of a hypersensitive response. A drawback of the first strategy is that the genes presently available to confer enhanced resistance to fungal pathogens have limited potential only. Furthermore, it is most likely that in transgenic crop plants several elements of the plant's own defense have to be introduced in order to enhance the durability of engineered resistance in the field. The second approach, exploitation of the hypersensitive response, needs more research on its effectiveness against the different pathogens a plant has to deal with. Furthermore, tightly regulated promoters are needed that are induced exclusively upon pathogen attack. Other stress situations or invasion of a harmless endophyte or a symbiont should not result in induction of HR.

Until now, reports on strategies to engineer transgenic plants showing enhanced resistance describe results which were obtained in the laboratory. Success of the strategies applied will eventually be determined by the outcome of field experiments and it can be expected that these results will become available in the near future.

Acknowledgements

Drs Pierre J.G.M de Wit, Matthieu.H.A.J. Joosten and Richard Laugé are gratefully acknowledged for critical reading of the manuscript. G.H. was financially supported by the Ministry of Economic Affairs, the Ministry of Education, Culture and Science, the Ministry of Agriculture, Nature Management and Fishery in the framework of an industrial relevant research program of the Netherlands Association of Biotechnology Centers in the Netherlands (ABON).

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