

INVITED REVIEW
**MICROBIAL GENES EXPRESSED IN TRANSGENIC PLANTS
TO IMPROVE DISEASE RESISTANCE**

M. Lorito and F. Scala

Dipartimento di Arboricoltura, Botanica e Patologia Vegetale, sezione di Patologia Vegetale,

Università degli Studi di Napoli "Federico II" and

Centro CNR per lo Studio delle Tecniche di Lotta Biologica (CETELOBI), Via Università 100, I-80055 Portici, Napoli, Italy

SUMMARY

The development of plant transformation and gene cloning techniques has opened new avenues for augmenting disease and insect resistance in crops. The concept of 'Genetically Acquired Resistance' now encompasses a variety of strategies based on the transgenic expression in plant of genes from many different origins. Some of the most potentially useful transgenes capable of enhancing resistance to attack by microbes, viruses and insects have been obtained from bacteria and fungi, which are regarded as rich sources of desirable traits for plant genetic improvement. The main research directions have included: (i) the enhancement of the plant anti-microbial and insecticidal arsenal by the constitutive or inducible synthesis of various pathogen inhibiting compounds; (ii) the alteration of the plant response to pathogens by the appropriate induction of the local or systemic defence mechanisms, or by the interference with plant-pathogen signalling and (iii) the inactivation of pathogen toxins or the improvement of plant resistance to them. Although transgenic plants that are insect-resistant or bear other useful traits obtained from microbes, such as herbicide-resistance or enhanced food quality, are being commercially marketed, genetically-engineered crops exhibiting resistance to fungal or bacterial diseases have yet to reach the marketplace. The main focus of this paper addresses the application of bacterial or fungal genes to improve plant resistance to insect, fungi, bacteria and viruses, and the usefulness of these microbial genomes in the development of new transgenic technologies. In addition, perspectives dealing with environmental concerns and important biosafety questions resulting from these technologies are briefly discussed.

RIASSUNTO

GENI DI ORIGINE MICROBICA ESPRESSI IN PIANTE TRANSGENICHE PER MIGLIORARE LA RESISTENZA ALLE MALATTIE. Lo sviluppo di tecniche sempre più potenti per la trasformazione di piante e per il clonaggio di geni ha aperto nuove vie per la produzione di colture resistenti alle malattie ed agli insetti. Il concetto di "Resistenza Geneticamente Acquisita" è oggi ampiamente applicato in una varietà di strategie che si basano sull'espressione in pianta di geni di varia origine. Alcuni dei geni che si sono dimostrati potenzialmente più utili per aumentare la resistenza a microbi, virus e insetti sono stati ottenuti da batteri e funghi, i quali sono oggi considerati come una ricca sorgente di caratteristiche interessanti per il miglioramento genetico delle piante. Le principali linee di ricerca hanno riguardato finora: (i) l'incremento dell'arsenale antimicrobico e insetticida della pianta ottenuto mediante la sintesi costitutiva o inducibile di diversi composti che direttamente inibiscono il patogeno; (ii) l'alterazione della risposta della pianta ai patogeni inducendo in maniera appropriata i normali meccanismi di difesa localizzata o sistemica, o interferendo con i segnali di riconoscimento tra pianta e patogeno; (iii) l'inattivazione di tossine prodotte dal patogeno o l'aumento della tolleranza della pianta verso di esse. Anche se varietà transgeniche resistenti agli insetti o che contengono altre caratteristiche utili derivate da microrganismi (ad esempio resistenza a erbicidi o migliorata qualità del prodotto) sono state commercializzate, colture geneticamente modificate per la resistenza a malattie fungine e batteriche non sono ancora apparse sul mercato. Questo articolo riguarda principalmente le applicazioni di geni fungini o batterici che hanno lo scopo di migliorare la resistenza delle piante a insetti, funghi, batteri e virus, e discute l'utilità del genoma di questi microrganismi nello sviluppo di nuove biotecnologie transgeniche. Inoltre, si prendono in considerazione importanti problematiche che riguardano l'impatto ambientale, la sicurezza ed i rischi connessi all'uso su larga scala di piante resistenti ad agenti patogeni.

Corresponding author: M. Lorito
Fax: +39.081.7755320
E-mail: LORITO@UNINA.IT

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INTRODUCTION

One of the major challenges facing modern agriculture is to achieve a satisfactory, but environmentally friendly, control of plant diseases. Although the extensive use of chemicals remains the main strategy of disease control, a variety of alternative approaches have been considered which include the use of biocontrol agents and pathogen-resistant crop cultivars. Classical plant breeding has been very useful in producing disease resistant varieties, but has not access to resistance available in sexually incompatible species. Moreover, the rapid evolution of new virulent forms of phytopathogens demands less expensive and time-consuming processes for genetic plant improvement. In the past twenty years, the tremendous advancements in technologies for identification, isolation and transfer of genes have enabled the expression of traits for disease resistance in plants with minimal effects on their intrinsic properties.

Since the first plant expressing a transgene was obtained by *Agrobacterium* mediated transformation more than 10 years ago, a variety of desirable traits have been transferred to model and crop plants by using a few but powerful techniques (Mourgues *et al.*, 1998). In many cases endogenous plant genes have been reengineered to improve crop quality or resistance, such as the introduction of sense and antisense configurations to alter ripening and senescence processes (Fray and Grierson, 1993). Many plant genes, derived from acceptor or other species, have also been used transgenically in the attempt to improve disease resistance or to study pathogenicity processes by using both constitutive and inducible promoters (Dempsey *et al.*, 1998). In addition, much effort has been made to identify and isolate genes from a variety of other sources, including viruses, bacteria, fungi, animals and humans, useful for transfer to plants. Some of these foreign genes have been successfully expressed in plant: (i) to enhance resistance to microorganisms and insects (During, 1996; Dempsey *et al.*, 1998), herbicides and other chemicals (Botterman and Leemans, 1998), or environmental stress and nutrient deficiency (Bordas *et al.*, 1997; Samuelsen *et al.*, 1998); (ii) to study plant/pathogen interactions and other physiological or genetic processes (Salmeron and Vernooij, 1998; see references on Table 2 and 3); (iii) to improve human and animal plant food quality, *i.e.* by altering lipid or carbohydrate composition of crops (see references in Table 3); (iv) to produce enzymes, recombinant proteins, antigens etc. for industrial (technical), pharmaceutical and therapeutic purposes (see Herbers and Sonnewald, 1999 for a review); (v) to produce new compounds such as biodegradable plastics (Nawrath *et al.*, 1994; Poirier *et al.*, 1995). Resulting from these successful attempts with

non-plant genes and due to a world-wide effort to study genome function, almost any gene potentially useful for improving plant disease resistance is now or will soon be available, and many novel transgenic plants and their relative products will be accessible to the market.

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 because the transgenic protein significantly affected plant growth and development. Unfortunately, most of these unsuccessful but not uncommon cases have not been published, with the exception of the insecticidal *Bacillus thuringiensis* toxin genes, which could provide insight into problems that can limit the use of foreign genes in crop plants (Diehn *et al.*, 1996).

To date, two main strategies have been followed to express microbial genes in plants to improve resistance against pathogens. Disease resistant plants have been obtained that (i) synthesize transgenic compounds which directly improve the antimicrobial and insecticidal activity of the plant, or (ii) that express transgenes, often pathogen derived, capable of activating plant defence response or providing resistance to pathogen toxins and enzymes. This paper reviews some of the most successful cases in which genes from fungi or bacteria have been transferred to plants to improve disease resistance. The potential and the use of microbial genomes to genetically engineer new crop varieties with enhanced resistance traits are discussed, and some of the most promising research and gene sources are presented. In addition, most of the microbial genes already expressed in plants for a variety of purposes other than disease control are listed and briefly discussed.

FUNGAL AND BACTERIAL TRANSGENES WITH ANTIMICROBIAL AND INSECTICIDAL EFFECT IN PLANT (TABLE 1)

Antifungal and antibacterial proteins are key components of defence and offence mechanisms of many groups of fungi and bacteria. They are often effective on a broad range of targets and function synergistically in combinations, also with other biologically active compounds (*i.e.* antibiotics) (Lorito *et al.*, 1996c). Although a number of such genes have been identified, not many have been tested transgenically in plant to improve disease resistance. Chitinase encoding genes have been among the most used to improve plant defence against fungal pathogens. These enzymes are capable of degrading the linear homopolymer of β -1,4-N-acetyl-

Table 1. Microbial genes expressed in plants, that enhance defense response to pathogens.

Gene encoding	Source	Activity / effect	Plant	Reference
Chitinase (<i>chiA</i>)	<i>Serratia marcescens</i>	Antifungal chitinase. Resistance to <i>Alternaria longipes</i> and <i>Rhizoctonia solani</i>	Tobacco	Howie <i>et al.</i> , 1994; Jones <i>et al.</i> , 1988; Suslow <i>et al.</i> , 1988
Chitosanase	<i>Streptomyces</i> sp.	Antifungal chitosanase	Tobacco	El Quackfaoui <i>et al.</i> , 1995
Chitinase (<i>chi1</i>)	<i>Rhizopus oligosporus</i>	Chitinase involved in autolysis. Resistance to <i>Sclerotinia sclerotiorum</i> and <i>Botrytis cinerea</i>	Tobacco	Terakawa <i>et al.</i> , 1997
Endochitinase (<i>chit42</i>)	<i>Trichoderma harzianum</i>	Antifungal chitinase. Resistance to <i>A. alternata</i> , <i>A. solani</i> , <i>B. cinerea</i> , <i>R. solani</i>	Tobacco, potato, apple, petunia, grape etc.	Bolar <i>et al.</i> , 1997; Lorito <i>et al.</i> , 1998; Esposito <i>et al.</i> , 1999; B. Reisch <i>et al.</i> , unpublished
Glucose-oxidase	<i>Aspergillus niger</i>	Generate reactive oxygen and HR. Resistance to <i>Erwinia carotovora</i> and <i>Phytophthora infestans</i> . Broad-spectrum resistance to fungi and bacteria	Potato	Wu <i>et al.</i> , 1995
Bacteria ribonuclease (barnase) with inducible promoter (<i>prp1-1</i>) and barstar	<i>Bacillus amyloliquefaciens</i>	Ribonuclease localized at infection site. Reduction of <i>P. infestans</i> sporulation	Potato	Strittmatter <i>et al.</i> , 1995
Ribonuclease (<i>pac1</i>)	<i>Schizosaccharomyces pombe</i>	Degradation of dsRNA. Resistance to viruses	Tobacco	Watanabe <i>et al.</i> , 1995
Trichodiene synthase (<i>Tri5</i>)	<i>Fusarium sporotrichioides</i>	Produce sesquiterpenoid phytoalexin. Resistance to microbes and insects	Tobacco	Hohn and Ohlrogge, 1991; Zook <i>et al.</i> , 1996a, 1996b
Killer toxin	<i>Ustilago maydis</i> (mycovirus)	Toxic to closely related <i>Ustilago</i> species. Resistance to <i>U. maydis</i>	Tobacco	Park <i>et al.</i> , 1996
Cholera toxin, subunit A	<i>Vibrio cholerae</i>	Antibacterial. Resistance to <i>Pseudomonas syringae</i> pv. <i>tabaci</i>	Tobacco	Beffa <i>et al.</i> , 1995
<i>B.t.</i> toxin	<i>B. thuringensis</i>	Insecticidal. Resistance to caterpillars, looper, bollworms, hornworms, armyworm, beetles and other insects	Corn, potato, cotton, poplar, cranberry, tomato, rice, <i>Arabidopsis thaliana</i> , carrot, etc.	See Diehn <i>et al.</i> , 1996 for a review

D-glucosamine residues that represent a main component of the cell wall of most phytopathogenic fungi, and may show a strong inhibitory activity in vitro on germination and hyphal growth (Lorito, 1997). Chitinase encoding genes from various plant origins have been transferred from one species to another, usually under consti-

tutive promoters, with a varying rate of success (Broglie *et al.*, 1991; Neuhaus *et al.*, 1991; Punja and Raharjo, 1996). For instance, transgenic carrots expressing a tobacco chitinase showed enhanced resistance to three out of five fungal pathogens tested, but no resistance was obtained when a petunia chitinase was used or if the

host plant for either the tobacco or the petunia gene was cucumber (Punja and Raharjo, 1996).

To date, although many important fungal pathogens have been found to be insensitive to some plant chitinases, several crops have been engineered for disease resistance by using plant chitinases alone, as well as in synergistic combinations with glucanases and other antifungal compounds (Zhu *et al.*, 1994; Jach *et al.*, 1995). The first attempts to use a transgenic chitinase to enhance plant defence were made by using the bacterial chitinase gene *chiA* from *Serratia marcescens*. Jones *et al.* (1988) demonstrated the feasibility of accumulating the *Serratia* chitinase in tobacco leaves under two different promoters, while Suslow *et al.* (1988) evaluated the role of transformed chitinase in plant defense by using this same gene. The *chiA*-expressing tobacco showed a significant reduction in necrosis and chlorosis development due to attack by *Alternaria longipes* (Suslow *et al.*, 1988) and exhibited tolerance to *Rhizoctonia solani* infection in the field (Howie *et al.*, 1994). More recently, El Quakfaoui *et al.* (1995) obtained expression in tobacco of a chitosanase gene from *Streptomyces* sp. strain N174, which was the first chitosanase in a transgenic plant. This gene appears to have an antimicrobial potential since its product inhibited in vitro *Fusarium oxysporum*, *Verticillium albo-atrum* and other fungi, but the effect on fungal disease resistance of the transgene has not been reported.

Terakawa *et al.* (1997) transferred to tobacco a gene

of fungal origin, the chitinase encoding gene (*chi1*) involved in cell autolysis of the filamentous fungus *Rhizopus oligosporus*. They obtained a three to four fold increase of the chitinase activity in transformed plants compared to controls and detected a significant reduction of the leaf symptoms caused by *Sclerotinia sclerotiorum* and *Botrytis cinerea*. Another approach was followed by Lorito *et al.* (1998) by expressing in tobacco and potato an endochitinase-encoding gene used by the biocontrol fungus *Trichoderma harzianum* to attack and parasitize a variety of phytopathogenic fungi (Schirmböck *et al.*, 1994; Lorito, 1998; Lorito and Woo, 1998; Woo *et al.*, 1999). The *T. harzianum chit42* gene encodes one of the most powerful chitinolytic enzymes characterized to date from any source, which is capable of degrading rapidly any chitin containing fungal structure (*i.e.* chlamydo-spores, mature hyphae and sclerotia) and inhibits a broad range of fungi in vitro (Lorito *et al.* 1993, 1994a, 1994b, 1994c, 1996a, 1996c). The fungal gene was expressed constitutively at high level in plant, and transgenic lines up to 400 fold more chitinolytic than controls were obtained with no apparent effects on plant development. The transgenic expression of the *Trichoderma* gene in tobacco and potato conferred almost complete resistance to the aerial pathogens *A. alternata*, *A. solani* e *B. cinerea* and to the soil-born pathogens *R. solani* e *S. sclerotiorum* (Lorito *et al.*, 1998 and unpublished) (Fig. 1). Interestingly, biochemical

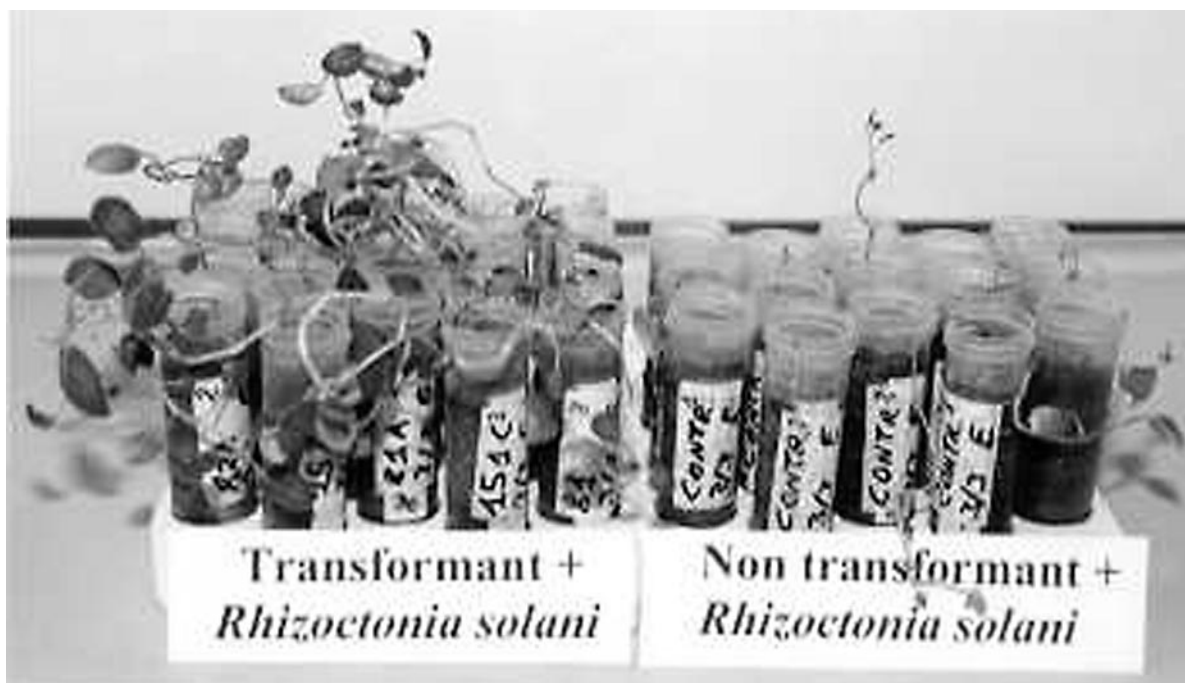


Fig. 1. Resistance to *R. solani* of transgenic potato plants cv. 'Desiree' expressing the *T. harzianum chit42* (endochitinase encoding) gene. Transgenic lines were obtained and tested against various pathogens as described by Lorito *et al.* (1998).

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 may be activated and help reaching a high level of disease resistance. This study conclusively demonstrates the usefulness of the fungal genome as a source of disease resistance genes, which apparently overcome the limitations of using plant chitinase genes. Following this successful approach, *T. harzianum* genes have been transferred into a variety of crops, including apple, petunia, pea, chickpea, brassica, wheat, grape, alfalfa, tomato etc., and positive results have been obtained with apple against *Venturia inaequalis*, grape against *Uncinula necator* and petunia against *R. solani* (Bolar *et al.*, 1997; Esposito *et al.*, 1999; B. Reisch *et al.*, unpublished).

Hydrogen peroxide and other reactive oxygen species seem to play multiple roles in plant defence, such as triggering of the hypersensitivity reaction (HR), exerting direct antimicrobial activity, diffusing the signal for activation of cellular defence genes and reinforcing the plant cell wall (Mourgues *et al.*, 1998). Therefore, a fungal glucose-oxidase encoding gene from *Aspergillus niger* was expressed in potato by Wu *et al.* (1995) to increase the level of H₂O₂ in transgenic tubers and leaves following bacterial infection. The progeny exhibited strong resistance to *Erwinia amilovora* subsp. *carotovora* under both aerobic and anaerobic conditions, while the addition of catalase counteracted the resistance. The transgenic lines also were less susceptible to late blight caused by *Phytophthora infestans*, indicating that transgenic expression of microbial enzymes that generate active oxygen species may confer resistance to a broad spectrum of fungal and bacterial diseases.

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 (*barstar*) to reduce detrimental effects of background activity of ribonuclease (RNase) on the non-infected tissues. When the progenies were challenged with *P. infestans* a strong *barnase* activity was induced only at the infection site, which significantly reduced

the sporulation of fungus on the leaves (Galiana *et al.*, 1997). Further, a similar method could also be successful for the control of bacteria diseases by using appropriate promoters. The work of Watanabe *et al.* (1995) has also been based on the use of microbial RNase activity. They transferred to tobacco the *pac1* gene of *Schizosaccharomyces pombe* encoding an RNase that degrades viral double stranded RNA (dsRNA). Although the level of resistance was modest, the transgene delayed symptoms of several unrelated single stranded plant viruses, probably by degrading double stranded replication intermediates.

Hohn and Ohlrogge (1991) and Zook *et al.* (1996a, 1996b) attempted to use a *F. sporotrichioides* gene coding for a trichodiene synthase (sesquiterpene cyclase) to alter plant sesquiterpenoid biosynthesis and produce in tobacco novel sesquiterpenoids toxic to insects and microbes. Although the transgenic plants were not evaluated for disease resistance, the accumulation of a novel trichodiene metabolite, 15-hydroxytrichodiene, was detected after elicitor treatments. This suggests that fungal synthase genes such as *Tri5* may be used both to increase pathogen resistance and study the role of phytoalexins in plant disease responses.

A limited number of toxins derived from fungi or bacteria have been transgenically expressed in plants to increase disease resistance. A gene encoding the A1 subunit of the cholera toxin, driven by a light inducible promoter from wheat (*Cab1*), was transferred to tobacco. The transgenic progenies accumulated salicylic acid and showed an enhanced resistance to *Pseudomonas syringae* pv. *tabaci* (Beffa *et al.*, 1995). The *Ustilago maydis* KP4 killer toxin has also been expressed in tobacco 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. Even though this strategy intends to provide a restricted resistance to specific *U. maydis* strains, it should be noted that only a small proportion of wild *U. maydis* strains are resistant to the toxin KP4 and that the overexpression of the transgene did not affect plant growth.

Finally, the best known and most successful case of microbial toxins expressed in plant concerns the family of insecticidal proteins produced by *B. thuringiensis* (*B.t.*). *B.t.* toxins from various *B. thuringiensis* subspecies have been used to obtain commercially available varieties of corn, potato and cotton resistant to European corn borer, Colorado potato beetle and bollworm. Although *B.t.* toxins are not effective against all insects and many concerns have been raised about the effect of *B.t.* crops on non-pathogenic species and the balance of

the ecosystem, this transgenic technology is regarded so far as the most successful applications of a microbial gene to reduce chemical pesticides. Interestingly, obtaining the expression of *B.t.* genes in plants to a desirable level was not an easy task and this provides a good case study on problems that can limit the expression of foreign genes in plant. The *B.t.* toxin case is too vast to be treated in this paper and the reader should refer to some of the excellent reviews recently published on this topic (see Diehn *et al.*, 1996).

MICROBIAL TRANSGENES THAT ACTIVATE PLANT DEFENSE RESPONSE OR MEDIATE RESISTANCE TO PATHOGEN TOXINS (TABLE 2)

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 have been transferred in plants to engineer resistance (Honée *et al.*, 1997), particularly, following De Wit's (1992) introduction of the two component sensor system method. 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. The avirulence gene *Avr9* of *Cladosporium fulvum* (causes leaf mould on tomato) 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.*, 1996), then transferred to tomato. Several 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 another work, the *P. infestans* gene *inf1*, responsible for HR on all cultivars of tobacco, and the *C. fulvum* *Avr9* gene, responsible for HR on *Cf9* transgenic tomato and tobacco, were functionally expressed in engineered potato virus X (PVX) (Kamoun *et al.*, 1999). Tobacco plants responsive to the protein elicitors AVR9 and INF1 produced localized HR and inhibited diffusion of the transformed virus. These results indi-

cated that HR may be a very effective mechanism to protect plants against different pathogens independently from the agent that causes the response.

In one case a virulence factor, the protein ECP2 secreted by *C. cladosporium* which is required for full virulence on tomato, proved to be useful for resistance engineering. Recombinant PVX expressing the gene encoding ECP2 was used to infect, and lines were identified among a collection of tomato genotypes that produced HR (Lauge *et al.*, 1998). Four tomato lines exhibited resistance dependent upon a single dominant gene, designated *Cf-ECP2*. These plants recognized specifically *C. fulvum* strains producing ECP2 and became resistant through induction of HR. In addition, a *C. cladosporium* strain lacking the *Ecp2* gene was pathogenic on *Cf-ECP2* plants. 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.

Avirulence genes from bacteria have also been utilized to confer resistance to plants expressing the corresponding resistance gene. HR and disease resistance occur when *P. syringae* pv. *tomato* with the avirulence gene *avrPto* infects tomato plants carrying the resistance gene *Pto*. *AvrPto* was transiently expressed in resistant and susceptible plants by using the recombinant PVX (Tobias *et al.*, 1999). In contrast to PVX which is virulent on tomato, the transformed virus only infected plants lacking *Pto*, therefore suggesting that the activated defence responses are effective against bacteria as well as viruses.

The possible use of a genotype specific HR elicited by the avirulence gene *avrB* from *P. syringae* in *Arabidopsis* plants carrying the matching resistance gene *RPM1* has been also investigated. An *Arabidopsis* *rps3* (*rpm1*) glabrous1 mutant was transformed with constructs expressing *avrB* and crossed with an *Arabidopsis* ecotype Columbia (*RPM1*) (Gopalan *et al.*, 1996). F1 progenies showed extensive necrosis on cotyledon leaves 10 days after germination indicating that expression of *avrB* caused a HR dependent on the presence of *RPM1*.

P. syringae pv. *tomato* carrying the *avrRpt2* avirulence gene induces specific cell death in *Arabidopsis* plants containing the complementary *RPS2* disease resistance gene. Since expression of *avrRpt2* in plants with *RPS2* leads to a generalized HR, transgenic lines were constructed by using the avirulence gene under the control of a glucocorticoid-inducible promoter (McNellis *et al.*, 1998). A specific hypersensitive cell

Table 2. Microbial genes expressed in plants, that activate plant defense responses or protect from pathogen toxins.

Gene encoding	Source	Activity / effect	Plant	Reference
Avirulence (<i>avr9, inf1</i>)	<i>Cladosporium fulvum</i> , <i>P. infestans</i>	Elicit HR. Resistance to potato virus X	Tomato, tobacco	Kamoun <i>et al.</i> , 1999
Avirulence (<i>avr9</i>)	<i>C. fulvum</i>	Elicit HR. Resistance to fungi	Tomato, tobacco	De Wit 1997; Honee <i>et al.</i> , 1997
Avirulence (<i>avrB</i>)	<i>P. syringae</i>	Elicit genotype specific HR.	<i>Arabidopsis thaliana</i>	Gopalan <i>et al.</i> , 1996
Avirulence (<i>avrPto</i>)	<i>P. syringae</i> pv. <i>tomato</i>	Elicit defense response. Resistance to bacteria and viruses	Tomato	Tobias <i>et al.</i> , 1999
Avirulence (<i>avrRpt2</i>)	<i>P. syringae</i> pv. <i>tomato</i>	Elicit defense response induced by dexamethasone. Resistance to bacteria	<i>A. thaliana</i>	McNellis <i>et al.</i> , 1998
Virulence (<i>Ecp2</i>)	<i>C. fulvum</i>	Elicit HR. resistance to <i>C. fulvum</i>	Tomato	Lauge <i>et al.</i> , 1998
β -cryptogein	<i>P. cryptogea</i>	Elicit defense response. Broad spectrum disease resistance	Potato, tobacco	Tepfer <i>et al.</i> , 1998; Keller <i>et al.</i> , 1999
Bacterio-opsin proton pump (<i>bO</i>)	<i>Halobacterium halobium</i>	Activate local and systemic response. Resistance to viruses, <i>P. syringae</i> pv. <i>tabaci</i> and <i>P. infestans</i>	Tobacco, potato	Abad <i>et al.</i> , 1977; Mourgues <i>et al.</i> , 1999
Pectate lyase (<i>PL3</i>)	<i>E. carotovora</i>	Activate disease response by producing elicitors from plant tissue. Resistance to <i>E. carotovora</i>	Potato	Wegener <i>et al.</i> , 1996
Oligogalacturonide lyase	<i>E. carotovora</i>	Degrade sugar oligomers into inactive products. Interfere with signalling and suppress pathogenicity in the potato/ <i>E. carotovora</i> interaction	Potato	Weber <i>et al.</i> , 1994
Tabtoxin resistance	<i>P. syringae</i> pv. <i>tabaci</i>	Inactivate tabtoxin. Resistance to <i>P. syringae</i> pv. <i>tabaci</i>	Tobacco	Anzai <i>et al.</i> , 1989
Ornithine carbamoyltransferase (<i>argK</i>)	<i>P. syringae</i> pv. <i>phaseolicola</i>	Resistance to bacteria phaseolotoxin. Repress symptom formation	Bean, tobacco	De la Fuente-Martinez <i>et al.</i> , 1992

death response similar to that caused by *P. syringae* was induced in transgenic lines by dexamethasone, suggesting that this system may provide a powerful new tool for analyzing avirulence gene effects and producing plant resistance to bacteria.

Disease resistance responses caused by elicitors other than the products of avirulence genes have been also used to increase plant resistance to pathogens. Tobacco were transformed with a *P. cryptogea* gene encoding the elicitor protein cryptogein under the control of the con-

stitutive 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 elicitors and is normally pathogenic to tobacco. Cryptogein accumulates within the cell and may be liberated upon fungal infection. Tobacco also have been transformed with cryptogein under the control of the pathogen inducible tobacco *hcr 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 eliciting a hypersensitive response, leading to resistance. Transgenic plants were also more resistant to other fungal pathogens such as *Thielaviopsis basicola*, *Erysiphe cichoracearum*, and *B. cinerea*. These results indicate that constitutive expression of a transgene is not necessary to generate plants resistant to many pathogens.

Unsaturated oligogalacturonates, the degradation products of pectic substances present in the plant cell wall, are thought to induce defence responses. Potato plants were transformed with constructs of the *E. carotovora* gene encoding the pectate lyase isoenzyme PL3 fused to the promoter of the potato patatin B33 and the 35S CaMV promoter (Wegener *et al.*, 1996). Plants transformed with the plasmid pB33-PL3 only produced the enzyme in tuber tissue, while plants containing the plasmid 35S-PL3 constitutively expressed the enzyme in various tissues. The transgenic plants were more resistant to tissue maceration by *E. carotovora*.

Unsaturated digalacturonides induce the production of pectic enzyme in *E. carotovora* which synthesizes an oligogalacturonide lyase to degrade these molecules to inactive products. Therefore, plants expressing this enzyme may be more resistant by interfering with the signal pathway in host/pathogen interactions. Weber *et al.* (1994) transformed potato plants with the bacterial gene encoding the lyase but pathogenicity tests have not been reported.

A completely different approach to engineer resistance in 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 this 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 several viruses and completely resistant to *P. syringae* pv. *tabaci* (Mourgues *et al.*, 1998). Transgenic potatoes also exhibited high levels of salicylic acid, all other characteristics of systemic ac-

quired resistance (SAR) (Abad *et al.*, 1997), and were more resistant to the US1 isolate (A1 mating type) but not to the US8 isolate (A2 mating type) of *P. infestans*. In addition, most of the lesion mimic potato plants were more susceptible to PVX whereas the tubers were not resistant to *E. carotovora*.

In another strategy plants were transformed with genes whose products can inhibit pathogen virulence factors such as toxins. Generally, pathogens that produce toxins to attack host plants are also able to detoxify these compounds for self-protection. *P. syringae* pv. *tabaci* (wildfire disease) produces a toxin, tabtoxin, that causes chlorotic symptoms on tobacco while a gene encoding a tabtoxin acetyltransferase inactivates the toxin. The gene has been isolated and transferred to tobacco under the control of a constitutive promoter (Anzai *et al.*, 1989). Transgenic plants challenged with the pathogen did not show any chlorotic symptoms.

A similar approach was followed with *P. syringae* pv. *phaseolicola* that produces phaseolotoxin and an ornithyl transcarbamylase (involved in arginine biosynthesis) resistant to the toxin. The gene encoding the enzyme, *argK*, was cloned and transferred to tobacco and bean (de la Fuente *et al.*, 1992). Transgenic tobacco plants were insensitive to the toxin and less susceptible to infection by *P. syringae* pv. *phaseolicola*, whereas transgenic beans were completely resistant to the bacterium. These results indicate that toxin detoxification may be used to engineer resistance even if it is useful against a limited number of pathogens.

MICROBIAL GENES EXPRESSED IN PLANTS FOR VARIOUS PURPOSES OTHER THAN INCREASE DISEASE RESISTANCE (TABLE 3)

The fast development, mainly in the last ten years, of plant biotechnology and the cloning of hundreds of potentially useful genes from fungi and bacteria, has opened countless opportunities for using plants as bioreactors to produce recombinant proteins in large quantities, at a relatively low cost. In addition, model plants can be used today as living laboratories to study the effect of foreign genes with the purpose of understanding basic physiological, genetic and pathogenic processes or screening for useful genes. Further, microbial genes may be useful to improve plant tolerance to environmental stresses. Table 3 provides a representative, but by no means complete, list of microbial genes already expressed in crop and model plants with a variety of purposes. The interest in the industrial production of microbial enzymes in plants is a growing field in biotechnology (see Herbers and Sonnewald, 1999 for a review). Fungal and bacterial genes have been used successfully

Table 3. Microbial genes expressed in plants for purposes other than to increase disease resistance.

Gene encoding	Source	Activity / purpose	Plant	Reference
Xylanase (<i>xynD</i>)	<i>Clostridium thermocelum</i> , <i>Ruminococcus flavefaciens</i>	Xylanase. Production of industrial enzymes	Tobacco, <i>Pisum sativum</i>	Herbers <i>et al.</i> , 1995, 1996; Herbers and Sonnewald, 1999
α -amylase	<i>Bacillus licheniformis</i>	Starch degradation. Production of industrial enzymes	Tobacco, <i>Vicia narbonensis</i> , <i>P. sativum</i>	Pen <i>et al.</i> , 1992; Czihal <i>et al.</i> , 1999
Biodegradable thermoplastic	<i>Alcoligenes eutrophus</i>	Biosynthesis of biodegradable compounds. Production of industrial polymers	<i>A. thaliana</i>	Poirier <i>et al.</i> , 1995
Acyltransferase (<i>SLC1-1</i>)	<i>Saccharomyces cerevisiae</i>	Increase seed oil content	<i>A. thaliana</i> , <i>Brassica napus</i>	Zou <i>et al.</i> , 1997
Phytase	<i>A. niger</i>	Hydrolyze phytate. Improve feed quality	Tobacco, soybean	Pen <i>et al.</i> , 1993; Verwoerd <i>et al.</i> , 1995; Li <i>et al.</i> , 1997; Denbow <i>et al.</i> , 1998
β -(1,3-1,4)-glucanase	<i>R. flavefaciens</i> , <i>B. amyloliquefaciens</i> , <i>B. macerans</i>	Hydrolyze plant glucans. Improve food quality	Tobacco, barley	Jensen <i>et al.</i> , 1996; Herbers <i>et al.</i> , 1996
Tryptophane monooxygenase (<i>iaaM</i>)	<i>P. syringae</i> pv. <i>savastanoi</i>	Parthenocarpic fruit development. Production of seedless fruit	Tobacco, eggplant	Rotino <i>et al.</i> , 1997
Cholera toxin subunit (CT-B)	<i>V. cholerae</i>	Antigen production	Potato	Arakawa <i>et al.</i> , 1997
Enterotoxin subunit (LT-B)	<i>E. coli</i>	Antigen production	Tobacco, potato	Haq <i>et al.</i> , 1995
HAL1	<i>S. cerevisiae</i>	Improve salt tolerance	Melon	Bordas <i>et al.</i> , 1997
Fe(III) reductase (<i>FRE1</i> , <i>FRE2</i>)	<i>S. cerevisiae</i>	Improve Fe nutrition. Tolerance to Fe deficiency	Tobacco	Samuelsen <i>et al.</i> , 1998
Aminolevulinatase synthase (ALA-S)	<i>S. cerevisiae</i>	Synthesis of ALA. Tolerance to inhibitors of the C5 pathway	Tobacco	Zavgorodnyaya <i>et al.</i> , 1997
Herbicide resistance	Various bacteria species	Resistance to glufosinate, glyphosate, bromoxynil, sulphonylurea	Corn, cotton, soybean	
Lignin degrading enzymes (<i>LiP</i> , <i>MnP</i>)	<i>Phanerochaete chrysosporium</i>	Alter growth and development. Study effect of pathogen enzymes on plant	Tobacco	Austin <i>et al.</i> , 1994

continued

Table 3 (continued)

Gene encoding	Source	Activity / purpose	Plant	Reference
<i>rolA</i>	<i>Agrobacterium rhizogenes</i>	Alter development. Study function of <i>rolA</i> protein	Tobacco, <i>A. thaliana</i>	Spena and Langenkemper 1997; Vilaine <i>et al.</i> , 1998
TL-DNA (various ORFs)	<i>A. rhizogenes</i>	Alter morphogenesis and response to hormones. Study role in pathogenesis of TL-DNA genes	Tobacco	Lemke and Schmulling 1998
Inorganic pyrophosphatase (<i>ppa</i>), invertase (<i>suc2</i>)	<i>E. coli</i> , <i>S. cerevisiae</i>	Alter photoassimilate partitioning. Study role of PPI	Tobacco	Lerchl <i>et al.</i> , 1995
Invertase	<i>S. cerevisiae</i>	Study effect on development and photosynthesis	Potato	Bussis <i>et al.</i> , 1997
FLP recombinase (<i>FLP/FRT</i>)	<i>S. cerevisiae</i>	Site specific recombination. Study effect on development	Tobacco	Lloyd and Davis, 1994; Sonti <i>et al.</i> , 1995
Mitotic inducer (<i>cdc25</i>)	<i>S. pombe</i>	Alter mitosis. Study effect on development	Tobacco	Bell <i>et al.</i> , 1993
Xylanase	<i>Cryptococcus albidus</i>	Test intron splicing	Tobacco	Laliberte <i>et al.</i> , 1992
Presequence of mitochondrial ATPse (β -subunit)	<i>S. cerevisiae</i>	Target proteins to mitochondria	Tobacco	Schmitz and Lonsdale, 1989
Antibiotic resistance and reporters (<i>NptII</i> , <i>Hpt</i> , <i>bar</i> , <i>uidA</i>)	<i>E. coli</i>	Selection markers for transformation and reporter genes	Various plants	-

to increase desirable traits in crops (Rotino *et al.*, 1997; Zou *et al.*, 1997) and improve plant food (Verwoerd *et al.*, 1995; Jensen *et al.*, 1996). Microbial antigen production in transgenic plants, as obtained with a cholera toxin and a *E. coli* toxin subunits in tobacco and potato, also appears today as an attractive strategy for oral immunization of animals and humans (Haq *et al.*, 1995; Arakawa *et al.*, 1997). *S. cerevisiae* genes have been transferred to melon and tobacco to improve salt tolerance, iron deficiency or inhibitors of the C5 pathway (Bordas *et al.*, 1997; Samuelsen *et al.*, 1998), whereas bacterial genes have been used to provide resistance to herbicides (see Botterman and Leemans, 1988 for a review) in commercially available varieties. Finally, a number of bacterial and fungal genes have been expressed in tobacco, *Arabidopsis*, alfalfa or potato to study the role

of pathogen enzymes (Austin *et al.*, 1994), proteins and genes. In particular plant-*A. rhizogenes* interaction (Spena and Langenkemper, 1997; Lemke and Schmulling, 1998), pyrophosphate metabolism (Lerchl *et al.*, 1995), effect of invertase (Bussis *et al.*, 1997), site specific recombination (Lloyd and Davis, 1994), mitotic inducers (Bell *et al.*, 1993), intron splicing (Laliberte *et al.*, 1992), targeting proteins to mitochondria (Schmitz and Lonsdale, 1989) have been considered. It should also be noted that the most common antibiotic resistance genes generally used as markers for plant transformation, such as neomycin phosphotransferase (*NptIII*), hygromycin phosphotransferase (*Htp*) and phosphinothricin acetyltransferase (*bar*), are of bacterial origin, as well as some of the most frequently used reporter genes (*i.e.* *uidA* which encodes the β -glucuronidase GUS).

CONCLUSIONS AND OUTLOOK

Unlike conventional plant breeding, the transgenic approach has no limits in respect to the gene source providing disease resistance. Today many genes have been identified which encode for antimicrobial or pesticidal compounds, and pathogen-derived components have been found that block the infection process or symptom formation. In this scenario, the genomes of bacteria and fungi appear as a very rich source of genes for conferring disease resistance. As presented in this review (Table 1 and Table 2), the contribution to date of microbial genes used for engineering resistant plant varieties is already significant, although limited to the laboratory level. The potential of this approach is tremendous and merits further investigation. Often, microbes can provide useful genes that are either absent in plants or more potent in biological activity than their plant equivalent. A typical example, which involves compounds with direct antimicrobial activity, considers the use of antifungal chitinolytic enzymes. The chitinase encoding genes from plants have been used for a decade to transform a number of crops, but inadequate levels of disease resistance have been obtained and to date no varieties have been produced that are close to reaching the market. Differences in levels and specificity of the antifungal chitinase activity indicate that the type and the source of the transgenic enzyme is a critical factor. 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 pathogens (Terakawa *et al.*, 1997; Lorito *et al.*, 1998). This result should not be surprising since microbial chitinases, especially those from mycoparasitic fungi, are optimized to degrade fungal cell walls. Therefore, a strategy based on transferring 'antimicrobial' genes in plant should start by selecting from an appropriate source well characterized, powerful gene products with desirable traits, such as stability, high specific activity and ability to synergize with other compounds.

The work performed thus far with microbial genes indicates that a significant improvement of plant resistance may be obtained in a variety of ways, but attained mainly by producing enzymes that degrade pathogen structures, enzymes that synthesize antimicrobial compounds or selective toxins. Clearly, each of these approaches will need a tailor made strategy to minimize any detrimental effects on the acceptor plant and the ecosystem. Many concerns have been raised on the effect of such transgenes on beneficial microorganisms such as *Rhizobium*, endophytic microbes and mycorrhiza forming fungi, but these aspects will not be dis-

cussed here. Unfortunately, only a limited number of studies have been published on non-target effects of antimicrobial and insecticidal compounds in transgenic crops, and these have been mainly focused on mycorrhizal symbiosis (Lorito *et al.*, 1997; Mourgues *et al.*, 1998) and the effect of *B.t.* toxin on beneficial insects (Losey *et al.*, 1999). Adequate answers to these questions are even more important when genes active on a wide spectrum of different pathogens are utilized.

Obviously the use of inducible instead of constitutive promoters may be very useful in reducing undesired effects. Today, the range of available sequences mainly includes promoters that respond to diverse biotic and abiotic stimuli, but new promoters which respond to specific fungal or bacterial infections or chemicals (*i.e.* tetracycline or glucocorticoids) are being identified (Martini *et al.*, 1993; Pontier *et al.*, 1994; Gatz, 1996; Aoyama and Chua, 1997). Further, a few promoter sequences recently cloned from biocontrol microbes and inducible during the antagonistic interaction with pathogenic fungi (Lorito *et al.*, 1996b; Mach *et al.*, 1999; Zeilinger *et al.*, 1999), may be tested in plant and eventually provide novel pathogen or stress inducible promoters for transgenic expression of useful genes.

The cloning of avirulence and R-genes has opened a new range of possibilities for studying the molecular basis of plant/pathogen interactions and improvement of crops by genetic engineering (Staskawicz *et al.*, 1995). The identification of the key factors that regulate the mechanisms of pathogen recognition has suggested the use of pathogen derived components transgenically expressed in plant to disrupt the infection process. This approach does not enhance the antimicrobial weaponry or the defence potential of the plant, but alters the plant perception of the pathogen and modifies the process of endogenous defences activation. Following this strategy, a variety of microbial genes, mainly coding for avirulence factors, compounds that activate local and systemic defence responses, or substances that confer resistance to pathogen toxins, have been successfully applied to improve disease resistance (Table 2). The theoretical advantage of this approach is that the transgene should be effective only in response to pathogen attack, and this should minimize the alteration of functions important for maintaining the agronomic qualities of the engineered crop. In addition, the resistance obtained could be targeted against one or few specific pathogens, therefore reducing the risk of affecting the beneficial microflora that interact with the transgenic plant. On the other hand, the behaviour in a natural environment of highly-reactive plants is not readily predictable. Moreover, if programmed cell death is involved, the spread of a necrogenic defence responses from the infection site to

the rest of the plant must be avoided.

The existing approaches can be refined and new strategies developed to achieve desirable traits of disease resistance by (i) unravelling new details of the signalling and recognition events which determine the compatible/incompatible plant/pathogen reaction; (ii) identifying additional avirulence and defence eliciting genes; (iii) screening transgenically in plants the available sequences under different promoters.

The extensive use of transgenic crops has raised obvious concerns about the fate of the transgene and the environmental impact, for instance, of horizontal gene transfer (no parent-to-offspring transfer of genes) from the plant to phytosphere bacteria. The latter point is a key issue in any serious risk assessment program considering the biosafety of a genetically modified crop. In this respect, transgenes from microbial origin, such as the bacterial kanamycin resistance gene, have received a lot of attention for two main reasons. First, they are the most frequently used selectable marker genes for plant transformation and, therefore, are becoming ever present in the environment. This increases the possibility of transfer to other organisms, which may produce genetically modified microbial strains that have a deleterious impact on the ecosystem, or represent a direct threat for animals and humans. Second, the limited data available so far on horizontal gene transfer from plants to microbes indicates that this phenomenon is influenced by the level of DNA homology between the transgenic sequences and the genome of the acceptor strain. This seems to imply that there may be a higher risk of transfer or recombination by using crops expressing microbial transgenes. However, studies performed mainly on the kanamycin resistance gene have indicated that the frequency of horizontal transfer is extremely low, and the likelihood of environmental impact is remote because the transferred gene will be readily lost by effect of natural selection (Nap *et al.*, 1992; Drogue *et al.*, 1998; Nielsen *et al.*, 1998). Regardless, the choice of a microbial gene for transgenic expression in plant must seriously consider the consequences of such a transgene on native microbe populations. A responsible use of fungal and bacterial genes for genetic plant improvement should produce a minimal effect on the ecosystem and contribute safely to a more productive and less chemically dependent agriculture.

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