Invited Trends Article

Genetic Engineering of Disease and Pest Resistance in Plants: Present State of the Art

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Rapid progress in gene technology has allowed, on the one hand, insight to be gained into the complex molecular mechanisms of plant/pathogen recognition and the natural defence strategies of host plants. On the other hand, this technology can also be used for the controlled and efficient generation of genetic variability and for the identification of desirable genotypes, far beyond the possibilities of classical breeding. The first successful attempts have been made to improve resistance against pathogenic viruses, bacteria, fungi and insects by engineering transgenic plants. The majority of these strategies were based on constitutively expressing single proteins that are either toxic to the pathogen/pest, or interfere with its reproductive cycle. More refined strategies, which are at the stage of testing, try to mimic and modify naturally-evolved defence reactions of plants and, thereby, will potentially confer a more durable resistance to a broad range of pathogens.

Introduction

Protection against attack by detrimental microorganisms and pests represents a major challenge for crop production in agriculture. A large percentage of the potential harvest yield is lost each year, due to infestation of crop plants with viroids, viruses, bacteria, fungi, nematodes and insects. In many areas of the world, such losses still cause famine and threaten the economic survival of farmers.

Presently, control of disease development is mainly based on three strategies: application of protective agrochemicals, breeding for resistant varieties, and various crop husbandry techniques, such as crop rotation. Despite the undoubted necessity of agrochemicals in assisting the fight against epidemic spread of diseases, the use of these compounds is limited by their high costs for the farmer and by their potentially harmful impact on the environment. In addition, extended application of agrochemicals reduces their efficiency due to coevolution of tolerant or resistant pathogens. Classical as well as more unconventional breeding, involving, for example, cell and tissue culture techniques, have created a large number of new varieties with desirable resistance traits [1]. However, these breeding programs are based on very time-consuming techniques (making crosses and backcrosses, selection) and, therefore, can barely keep pace with the rapid evolution of pathogenicity in microorganisms and pests.

The advent of molecular genetics in plant breeding offered the tools for improving the possibilities and efficiency of producing genetic variability in the plant population and selecting desirable types. This methodology allows the comparatively rapid identification, isolation and controlled recombination *in vitro* of genes and gene segments, and it renders possible the transfer of novel combinations far beyond the limits of sexual crosses and somatic hybridization [2].

In this article, we will summarize the different approaches currently being followed to create resistance* against various types of pathogens and pests by genetic engineering. To our knowledge, no successful attempts have yet been made to engineer the complex recognition processes between hosts and pathogens, or more than one of the coordinate steps of multienzyme pathways for the production of protective agents, such as phytoalexins or lignin. However, impressive improvements in the performance of plants have already been achieved by

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* The term "resistance" covers the range of protection from a delay to complete inhibition of disease development.

expressing single proteins that are mostly toxic to the pathogen/pest or interfere with its reproductive cycle, especially in the area of virus and insect resistance. Here, emphasis will be put on those novel transgenic lines which have been tested in the greenhouse or in the field. Strategies which have not yet reached this level but nevertheless have the potential to succeed, in our view, will be briefly discussed. For a more detailed description of non-conventional control measures against specific types of pathogens or pests (viruses, fungi or insects) we refer the reader to recently published reviews [3–7]. A speculative outlook on potential future strategies was lately presented by Lamb *et al.* [8].

Protection against Viral Infestation*

Due to the lack of "viricides", virus diseases are conventionally controlled by using certified virus-free planting material, by eradicating infected plants and by spraying chemicals against virus vectors. As these measures are not overly successful, the engineering of virus resistance into plants is expected to provide a direct and more efficient control of virus diseases. Engineering strategies are based on the introduction of a virus-derived or a virus-targeted sequence into the genome of a potential host plant. These sequences interfere with specific stages in the viral infection cycle, such as virus replication or spread, thus resulting in a virus-resistant plant. To develop strategies for the engineering of resistance, detailed knowledge of the complex viral genome organizations, and of the various modes of gene expression and replication are required.

The genome of viruses consists of single- or double-stranded RNA or DNA [9]. The vast majority of pathogenic plant viruses (about 80%) have genomes of plus strand (messenger sense) single-stranded RNA, but also viruses with minus sense or ambisense genomic RNA (TSWV), single-stranded DNA (geminiviruses) or double-stranded DNA (caulimo- and badnaviruses) cause significant losses to crop plants. All viruses encode proteins involved in the replication of their nucleic acid, the nature of which depends on their replication strategies. RNA viruses encode a (subunit of a) putative RNA-dependent RNA polymerase, while double-stranded DNA viruses, which replicate *via* RNA intermediates, code for a reverse transcriptase. Single-stranded DNA viruses contain genes for enzymes necessary for replication *via* complementary DNA. Besides the replication enzymes, the genome of most viruses also encodes a coat protein which protects the viral nucleic acid during the transfer of virus particles from one plant to another and can determine the specificity of the vector (mainly arthropods). Additionally, many viruses synthesize proteins that facilitate their movement from cell to cell in the host plant ("movement proteins"), and there are also several viral gene products with unknown function. The variability among the different virus groups is further demonstrated by their gene expression strategies. To enable monocistronic translation, some viruses have devided their genome into segments, each representing a mRNA for one protein, others synthesize subgenomic RNAs which are mRNAs for proteins encoded downstream of the 5′-terminal cistron. Finally, some viruses translate their genome into a polyprotein, which is processed into functional proteins by virus-encoded proteases.

To engineer resistance into host plants the following strategies are currently under investigation: coat protein-mediated protection, replicase-mediated protection, satellite RNA-mediated disease attenuation, defective interfering RNA or DNA protection, and antisense RNA/ribozyme-mediated protection. Transformation of plants with a coat protein gene was the first strategy that has been developed, and until recently has provided the strongest resistance. Its concept has been derived from the observation of cross protection: by pre-infection with a mild symptomless virus strain, plants are protected against a related but severely damaging strain [10]. Cross protection between viral strains has been used to reduce the devastating

* The following abbreviations are used to denominate viruses: ACMV, African cassava mosaic virus; AlMV, alfalfa mosaic virus; BMV, brome mosaic virus; CaMV, cauliflower mosaic virus; CMV, cucumber mosaic virus; CLRV, cherry leafroll virus; CyRSV, cymbidium ringspot virus; GCMV, grapevine chrome mosaic virus; PEBV, pea early browning virus; PLRV, potato leafroll virus; PVS, potato virus S; PVX, potato virus X; PVY, potato virus Y; RSV, rice stripe virus; SHMV, sunn hemp mosaic virus; TAV, tomato aspermy virus; TBSV, tomato bushy stunt virus; TCV, turnip crinkle virus; TEV, tobacco etch virus; TGMV, tomato golden mosaic virus; TMV, tobacco mosaic virus; TobRV, tobacco ringspot virus; ToMV, tomato mosaic virus; TRV, tobacco rattle virus; TSWV, tomato spotted wilt virus.

effects of several economically important viral diseases, such as infection of tomatoes with tomato mosaic virus, or citrus trees with citrus tristeza virus [11].

Coat protein-mediated protection (CPMP)

The first report on CPMP was published in 1986 by the groups of R. Beachy from Washington University, St. Louis (U.S.A.), and R. Fraley from MONSANTO, St. Louis (U.S.A.), who introduced the TMV coat protein gene into the genome of tobacco plants [12]. Transgenic plants expressing high levels of coat protein upon infection with TMV exhibited a delay in disease development. Subsequently, CPMP has been demonstrated for at least twenty RNA viruses (most of them with a plus sense single-stranded RNA genome [3]; for examples see Table I) whereas no effect on DNA viruses has yet been reported [13]. Mostly, tobacco has been used as model system to study CPMP, but resistance has also been engineered into important field crops, such as alfalfa, potato and tomato, and recently the CPMP approach has been extended to cereals [14]. The coat protein has been shown to interfere with early events of virus disassembly [15, 16], but some data indicate that other effects are involved in reduction of virus multiplication as well [17, 18].

Typically, CPMP was achieved by transforming plants with a functional sense coat protein gene under control of the CaMV 35 S promoter which mediates strong constitutive transcription in many cell types (as one of several examples see [19]). The degree of resistance was correlated with the amount of intact coat protein expressed in transgenic plants [20, 21], and CPMP could be overcome by high concentrations of virus inoculum [12, 19, 21] or infection with naked RNA [20, 22, 23], except from PVX and PVS [21, 24].

However, there are also results indicating that not only the coat protein itself is able to interfere with virus multiplication. In several cases the introduction of non-functional transgenes was reported to confer resistance. Plants containing antisense coat protein RNA of CMV [25] or PVX [21] were protected against viral infection, although only at low inoculum concentrations. High levels of resistance were obtained by introducing a coat protein cistron of PVY lacking a translational start signal [26]. An antiviral effect was also established by introducing untranslatable coat protein genes of TEV into tobacco plants [27]. The latter authors suggest that resistance resulted from hybridization of the coat protein RNA to the minus sense replication intermediates of the viral RNA, thus inhibiting the production of infectious virus particles. The formation of RNA : RNA hybrids was also postulated as a mechanism inhibiting multiplication of TSWV, a virus with an ambisense RNA genome. Resistance

Table I. Examples for protection against virus infection mediated by coat protein sequences.

Source of coat protein gene	Transformed plant species	Transgene expression protects against	References
TMV	tobacco	TMV	[12, 22]
TMV	tomato	TMV, ToMV	[30]
TMV	tobacco	PVX, CMV, AlMV, SHMV	[32]
AlMV	tobacco	AlMV	[19, 20, 23]
AlMV	tobacco	PVX, CVM	[32]
AlMV	tomato	AlMV	[19]
AlMV	alfalfa	AlMV	[33, 34]
TRV	tobacco	TRV, PEBV	[35]
CMV	tobacco	CMV	[25]
PVX	tobacco	PVX	[21]
PVX	potato	PVX	[36, 37]
PVX + PVY	potato	PVX + PVY	[31]
PVS	potato	PVS	[24]
PLRV	potato	PLRV	[38, 39]
TSWV	tobacco	TSWV	[28, 40]
TEV	tobacco	TEV	[27]
RSV	rice	RSV	[14]
GCMV	tobacco	GCMV	[41]

was mediated by the RNA of the nucleocapsid protein which is encoded in viral complementary sense on a genomic RNA fragment [28].

The effects of CPMP include a reduction in the number of "infectible sites", a reduced accumulation of virus particles, and a retardation or prevention of systemic disease development [3]. CPMP works against the virus strain from which the coat protein gene originates and against related strains or viruses, depending on the degree of relatedness [29]. In the meantime, the efficiency of CPMP has been tested in field trials with several plant species: for instance, tomato plants containing the coat proteins of TMV or ToMV, tobacco transformed with the coat protein gene of AlMV, and potato expressing the coat protein of PVX and/or PVY. The first field trial was conducted in 1987 with transgenic tomato plants [30]. Tomato lines expressing the TMV coat protein were grown in the field together with non-transgenic control plants. After mechanical inoculation with TMV, only 5% of the transgenic plants had developed disease symptoms compared to 99% of the control plants by fruit harvest, and the fruit yield was equal to that of non-inoculated control plants. Almost one third of the plants expressing the TMV coat protein gene were protected against ToMV, while all control plants exhibited disease symptoms. Russet Burbank potato plants transformed with the coat protein genes of PVX and PVY were tested to determine whether the simultaneous expression of the genes protected potato plants against the synergistic effects of PVX and PVY infection [31]. Plants from all four transgenic lines tested were significantly protected against PVX, but plants from three of these lines were not protected against infection with PVY. Plants from one line were resistant against both viruses. The yield of this line was unaffected by virus inoculation whereas the tuber yields from the other transgenic lines were markedly reduced. In summary, results from these and several other field trials demonstrate that CPMP might be very effective in preventing losses due to virus infection, but a large number of clones may need to be screened to identify the most resistant line. A better understanding of the mechanism(s) of CPMP should help to extend resistance beyond its current limitations.

Satellite RNA-mediated disease attenuation

Satellites are small extragenomic RNA species that depend on a helper virus for replication. Some satellite RNAs are known to attenuate the symptoms of their helper virus [42]. For example, in China, pepper plants are prophylactically inoculated with a CMV strain containing satellite RNA. These plants develop only mild disease symptoms and are protected against infection with a CMV strain which does not contain the satellite. Transgenic tobacco plants expressing the satellites of CMV [43] or TobRSV [44] were shown to be protected against severe effects of their respective helper virus. The expression of CMV satellite RNA also protected against the symptoms of infection with TAV, but not against a range of other viruses [45]. As a mechanism for satellite RNA-mediated disease attenuation, competition with the viral genomic RNA for a limiting amount of replicase enzyme has been proposed. However, inoculation with the satellite RNA of TobRSV also inhibited the accumulation of CLRV, a virus that is unable to replicate the satellite RNA of TobRSV [46]. This clearly suggests that other mechanisms than blocking replication might contribute to the disease attenuation as well.

The satellite RNA strategy is still associated with risks and needs further investigation. For example, strains attenuating disease symptoms on one plant species may induce severe symptoms on others, and an attenuating satellite could mutate into a molecule which promotes disease symptoms.

Defective interfering RNA/DNA protection

Defective interfering (DI) particles are deletion mutants of genomic viral sequences, which depend on their parent virus for replication. DI RNAs have frequently been described in animal virus systems but, thus far, only rarely in plant virus systems, such as TCV [47], TBSV [48], CyRSV [49], and TSWV [50, 51]. The occurrence of a DI viral DNA was reported for ACMV, a single-stranded DNA virus which produces subgenomic DNA particles [52].

Like satellite RNAs, DI particles can attenuate the disease symptoms of their helper virus by interfering with its replication, as found for TBSV [53] and TSWV [50]. Thus, DI RNAs or DNAs may also be used as tools for genetic engineering of plants. As an alternative to the introduction of naturally

occurring DI molecules, transformation with artificially constructed DI molecules has been described. For example, deletion mutants of BMV RNA 2 were shown to reduce the replication of BMV RNA 1 and 2 in barley protoplasts [54]. Similarly, the introduction of a defective subgenomic single-stranded DNA of the above mentioned ACMV (a tandem repeat of half DNA B) was demonstrated to interfere with the replication of components A and B, resulting in a reduction of disease development [55, 56]. The attenuation of disease symptoms correlated with the mobilization and replication of the defective B DNA, which seemed to compete with the intact DNA for replicase binding.

Antisense RNA/ribozyme-mediated protection

The expression of antisense genes has often been demonstrated to modulate the expression of plant nuclear genes [57]. However, only recently the expression of antisense RNA against viral RNA was reported to interfere with virus multiplication. As already mentioned, the expression of antisense coat protein RNA of CMV [25] or PVX [21] exhibited only limited protection, compared to plants expressing the corresponding sense genes. Very likely, the cytoplasmic localization of RNA virus replication, the high copy number of viral RNA, and the association of virus RNA with proteins impede the effect of antisense RNA. On the other hand, this implies that antisense RNA protects against viruses with different features, such as DNA viruses having a nuclear phase in their replication cycle. Protection of plants against the geminivirus TGMV was achieved by expressing antisense RNA of the *al* gene which encodes a protein required for replication [58]. Nevertheless, antisense constructs have generated protection against RNA viruses as well: antisense coat protein genes mediated a high level of resistance to PLRV, a virus which, in contrast to CMV, accumulates slowly and remains at a low titer [59]; Huntley and Hall reported that an antisense transcript of BMV RNA 3 intercistronic region interferes with viral RNA replication [60].

Ribozymes are RNA molecules catalyzing endonucleolytic cleavage reactions. Some viroids, virusoids and satellite RNAs perform self-cleavage reactions. Mostly, the cleavage site consists of a consensus structure, called the "hammerhead" motif. The nucleotide region directing the catalysis of the cleavage reaction could be separated from the region where the cleavage occurs and the recognition of the target could be modified by changing the nucleotide sequence of the regions flanking the cleavage site. Thus, ribozymes could be designed which cleave different sequences *in trans.* This activity of ribozymes has often been proven *in vitro* [61, 62], including the cleavage of viral RNA sequences [63]. However, only few reports have been published on the activity of ribozymes *in vivo,* mostly providing only circumstantial evidence [64, 65]. Recently, Steinecke *et al.* designed a ribozyme targeted against the mRNA of the *npt* gene, which was shown to reduce *npt* gene expression when transiently expressed in tobacco protoplasts [66]. A cleavage product of the NPT mRNA was detected, directly demonstrating the cleavage activity of ribozymes *in vivo,* but as in other systems a high molar excess of ribozyme over the target sequence was necessary to completely abolish gene expression. Whether ribozymes will also be active in stably transformed plants, and by which expression strategy a sufficient amount of ribozyme can be produced to achieve a reduction in target gene expression remains to be unravelled.

Replicase-mediated protection

As an alternative to the use of genes encoding structural proteins, such as coat protein, the introduction of sequences for non-structural proteins, for example replicase, has been exploited to create virus-resistant plants. The first example was reported by Golembowski *et al.,* who discovered that transgenic tobacco expressing part of the replicase gene (an open reading frame of 54 kDa) of a specific TMV strain was highly resistant to this and closely related strains [67]. The equivalent region of the PEBV replicase was found to confer resistance to PEBV and two relatives of this virus [68]. In both cases the 54 kDa protein itself rather than the respective RNA appeared to mediate the protection. In contrast, transgenic tobacco expressing functional AlMV replicase protein was not virus-resistant [69], and in barley protoplasts, intact BMV replicase did also not reduce virus replication [70]. Longstaff *et al.* changed the conserved NTP-binding or polymerase motifs of the PVX open reading frame 1 RNA (encoding the putative viral replicase), rendering the viral genome non-infectious

[71]. The mutant genes were transformed into tobacco and were then shown to confer extreme resistance against PVX infection. These results indicate the possibility of engineering virus resistance by expression of dominant negative mutant forms of viral genes.

Resistance to Bacterial Pathogens

In medicine, a large number of bactericidal compounds, such as antibiotics and lysozyme, are well known and applied for therapy. Functionally, antibiotics can be compared with phytoalexins, low molecular weight antimicrobial compounds which are synthesized in plants in response to pathogen attack [72–74]. The synthesis of phytoalexins is based on complex biosynthetic pathways which in most cases are not accessible for genetic engineering yet (an exception will be described below).

Lysozymes, which catalyze the hydrolytic cleavage of bacterial cell wall murein, have been detected in many plant species; most of these enzymes display only low lysozyme but high chitinase activity [75–77]. As the biochemical and molecular characterization of plant lysozymes and the corresponding genes is still in its infancy, lysozyme genes from other sources have been used for genetic plant engineering. The expression of lysozyme genes from hen egg white or bacteriophage T4 was achieved in tobacco and potato [78–80]. K. Düring and coworkers found an increased resistance of tuber slices from transgenic potato lines to maceration by the pathogenic soil bacterium *Erwinia carotovora* spp. *atroseptica* [80]. These transgenic plants constitutively expressed a fusion protein between the signal peptide from barley α-amylase and the bacteriophage T4 lysozyme, and the authors speculate that secretion of the antibacterial protein into the intercellular space is a prerequisite for its effect against pathogen invasion.

Endosperm and leaves of cereals contain small (5 kDa), cysteine-rich polypeptides, called thionins, which exert antimicrobial activity *in vitro* [81–85]. The group of Garcia-Olmedo obtained constitutive high level synthesis of functional thionin in transgenic tobacco leaves transformed with a barley α-thionin gene under control of the CaMV 35 S promoter [86]. After inoculation with *Pseudomonas syringae* pv. *tabaci* or *Pseudomonas syringae* pv. *syringae,* the number of necrotic lesions and the severity of disease symptoms were reduced in leaves of transgenic R_1 and R_2 progeny compared to leaves of control plants. The level of resistance coincided with the level of thionin expression, further proving that resistance depended on the presence of a functional α-thionin gene.

Another approach for engineering bacterial disease resistance was based on the transformation of plants with a gene encoding a toxin-detoxifying enzyme from the pathogen itself. Several lines of evidence suggest that the dipeptide tabtoxin is responsible for chlorosis during wildfire disease on tobacco caused by *Pseudomonas syringae* pv. *tabaci.* Very likely, *in planta* tabtoxin is converted to tabtoxinine-β-lactam which inhibits the target enzyme glutamine synthetase leading to an accumulation of cytotoxic ammonium [87, 88]. The pathogen protects itself against the toxin by expression of the tabtoxin resistance gene, *ttr,* which encodes an enzyme that acetylates tabtoxin. Transgenic tobacco plants constitutively expressing the *ttr* gene under control of the CaMV 35 S promoter did not produce the chlorotic halo seen for wildfire disease on non-transgenic plants [89]. Unfortunately, the authors did not address the question of whether only disease symptoms were reduced or also the multiplication of bacteria was inhibited in the transgenic plants. In a comparable strategy, protection against the damaging effects of a bacterial toxin was obtained by transformation of plants with a pathogen-derived toxin-resistant target enzyme. The gene-encoding ornithine carbamoyl transferase, an enzyme involved in citrulline biosynthesis, from *Pseudomonas syringae* pv. *phaseolicola* was introduced into the genome of tobacco plants. To target the enzyme to the plastids, the natural site of citrulline biosynthesis in plants, its coding sequence was fused to the transit peptide sequence of a *rbcS* gene [90]. Transgenic plants harboring this construct expressed an ornithine carbamoyl transferase which was insensitive to phaseolotoxin produced by *Pseudomonas syringae* pv. *phaseolicola,* and progeny of these plants displayed a reduction in disease symptoms compared to control plants.

Resistance to Fungal Pathogens

Antifungal proteins

Several characteristic features of fungal structure and cell metabolism represent potential targets for

inhibitory agents. The constitutive or pathogen-inducible expression of genes encoding proteins with fungitoxic or fungistatic capacity in transgenic plants may enhance resistance to fungal damage in these plants.

Chitin and β-1,3-glucans are major structural polysaccharides of the cell wall of many fungi [91]. The breakdown of these components by endochitinases and β-1,3-endoglucanases, both belonging to the group of naturally occurring PR ("pathogenesis-related") proteins of plants [92], is thought to inhibit fungal growth. Four classes (I–IV) of endochitinases and three major classes (I–III) of β-1,3-endoglucanases have been described [93–95]. Class I hydrolases are localized in plant vacuoles and have been proven to exert a strong inhibitory effect on fungal growth *in vitro* [6, 96–98]. Inhibition was far more pronounced when mixtures of the two types of hydrolases were applied, indicating that the proteins act synergistically. In contrast, class II hydrolases, which are very similar to class I proteins on the basis of their primary structure but are localized extracellularly, have no comparable effect on fungal growth *in vitro,* neither alone nor in combination with other proteins [6, 98].

Chitinase is generally found at low or basal levels in healthy plants, and its expression is increased during pathogen attack [92, 99]. As the outcome of the interaction between host and pathogen largely depends on the rapidity and intensity of defence response activation, Broglie and coworkers combined the bean endochitinase CH5B gene, encoding a class I protein, with the promoter region of the CaMV 35 S gene conferring high constitutive expression in a wide variety of plant cell types [100]. In the non-infected state, transgenic homozygous tobacco plants harboring this chimeric construct showed a 2- to 4-fold increase in the roots and a 23- to 44-fold increase over control plants in chitinase activity in the leaves. For phytopathological evaluation, these plants were grown in the presence of *Rhizoctonia solani,* an endemic, chitinous, soilborne fungus that infects numerous plant species. Seedling mortality or the loss of root fresh weight was clearly reduced in transgenic plants compared to control plants, depending on the amount of bean chitinase expressed. When 35 S-chitinase plants were infected with the non-chitinous fungal pathogen *Pythium aphanidermatum,* no difference in survival was detected compared to control plants. A similar effect on the level of resistance to *Rhizoctonia solani* as in tobacco, was exhibited by canola transformed with the 35 S-chitinase construct. In transgenic plants of both species the extent of disease resistance was dependent on the amount of fungal inoculum used, a property characteristic for quantitative resistance. The authors speculate that the inhibitory effect on fungal growth may be caused by enzyme-catalyzed hydrolysis of newly formed chitin and resultant disruption of growing hyphal tips. Protection against *Rhizoctonia solani* was also obtained in transgenic tobacco by transformation with a chimeric construct combining the CaMV 35 S promoter with a chitinase gene from the bacterium *Serratia marcescens* [101].

It remains questionable whether the engineering of chitinase expression alone can provide protection against a wide range of chitinous fungal pathogens. Transgenic tobacco plants bearing a class I chitinase from tobacco under control of the CaMV 35 S promoter did not reveal a substantial increase of resistance to the chitin-containing fungus *Cercospora nicotiana,* although most transformants expressed high levels of enzymatically active chitinase in the intracellular compartment of leaves [102]. Therefore, factors other than chitinase appear to be limiting in defence against this pathogen. On the other hand, in order to limit the spread of this pathogen the antifungal protein may have to be targeted to the extracellular space, the predominant site of growth of *Cercospora* and several other pathogenic fungi [6]. In an attempt to target class I hydrolases out of the cell, Cornelissen and co-workers have modified a chitinase and a β-1,3-glucanase from tobacco [103]. In transgenic plants, the two hydrolases were detected extracellularly, and both retained their antifungal activity *in vitro* which was enhanced when a combination of the proteins was applied.

The group of J. Ryals at CIBA-GEIGY, Research Triangle Park (U.S.A.), has reported on protection of transgenic tobacco plants against various fungal pathogens as a result of high-level expression of proteins associated with systemic acquired resistance (SAR) in this plant species [104]. The majority of these SAR proteins belong to the group of PR proteins. Typically, their mRNA accumulates not only in pathogen-infected (primary) leaves but also in non-infected upper (secondary) leaves [105]. The goal of this work is to achieve broad range protec-

tion against pathogens by over-producing combinations of SAR proteins in transgenic crops.

Ribosome-inactivating proteins (RIPs) from plants offer another possible approach for interfering specifically with fungal metabolism. These enzymes inhibit protein synthesis in target cells by N-glycosidic cleavage of 28 S rRNA [106, 107]. Two types of RIPs exist: single-chain type 1 proteins, and type 2 proteins consisting of two chains from which one bears a galactose-specific lectin domain that can bind to cell surfaces. The latter RIPs are among the most potent natural toxins, the best known of which is ricin. RIPs do not interfere with the function of "self" ribosomes but exert varying degrees of inhibitory activity towards ribosomes of distantly related species, including fungal ribosomes [108, 109]. Purified type I RIP from barley inhibits fungal growth *in vitro,* and its effect is synergistically enhanced by class I chitinases and β-1,3-glucanases. These characteristics of RIPs were used by Logemann *et al.* to improve resistance to *Rhizoctonia solani* in transgenic tobacco [110]. A barley type I RIP gene was put under control of the potato *wun 1* promoter which mediates wound- and pathogen-inducible transcription in the epidermis of leaves, stems and roots from tobacco [111]. Primary transformed regenerates and R_1 progeny grew more vigorously in soil inoculated with *Rhizoctonia solani* than non-transformed control plants. The authors could show that RIP mRNA and protein had accumulated in leaves of these transgenic plants in response to wounding. However, data proving a correlation between the expression level of the chimeric construct and the degree of resistance were not reported.

In the meantime, a large number of proteins with inhibitory effects on the growth of fungi *in vitro* have been identified from plants. Among these are osmotin, a vacuolar protein from tobacco produced in response to salt stress [112], and small, basic, cysteine-rich proteins of various origins [113, 114]. Also microorganisms, like the fungus *Aspergillus giganteus,* produce such antifungal peptides [115]. The function of these proteins in transgenic plants remains to be tested.

Phytoalexins

In several host/pathogen systems a correlation has been found between the concentration of phytoalexins and resistance to specific pathogens [116 and ref. therein]. Genetic evidence for a causal relationship comes from investigations on the pea/*Nectria haematococca* interaction. In this case, resistance of the host plant is overcome by the presence of the *pda* gene in the fungus, encoding an enzyme, pisatin demethylase, that detoxifies the phytoalexin pisatin from pea [117–119].

In *Vitis vinifera* and *Picea sitchensis,* stilbenes are involved in protection against fungal challenge [120, 121]. Different biotic and abiotic signals have been shown to stimulate their accumulation, and to activate genes for the key regulatory enzymes of their biosynthetic pathway, phenylalanine-ammonia lyase and stilbene synthase [122–124]. Most plants, for example tobacco, contain the precursors for the formation of stilbenes but lack the enzyme stilbene synthase. After transfer of a stilbene synthase gene from grapevine to tobacco, transgenic plants expressed the foreign gene in response to treatment with fungal elicitor or infection with the broad range fungal pathogen *Botrytis cinerea* and accumulated the stilbene resveratrol [125]. Compared to control plants, in two out of three transgenic tobacco lines bearing the grapevine stilbene synthase gene, the percentage of diseased leaf area was reduced after inoculation with *Botrytis cinerea.* The reduction of disease incidence on leaves directly correlated with the amount of accumulated resveratrol and with the rapidity of stilbene synthase gene activation. The authors also revealed evidence for stable inheritance of the increased resistance phenotype to F_1 progeny. This work supports the assumption that at least in some pathosystems the synthesis of phytoalexins plays a crucial role for the defence of the host plant. In order to decide whether this strategy is generally applicable for the improvement of disease resistance, the performance of the transgenic tobacco lines in response to a wide range of fungal pathogens remains to be analyzed.

Race-specific resistance genes and artificial generation of hypersensitive cell death

True pathosystems exhibit so-called cultivar/race-specific interactions in which certain cultivars of a plant species are resistant to particular physiological races of a pathogen species, depending on the presence and function of resistance genes in the host and avirulence genes in the pathogen. The genetic interdependence of host and pathogen

which determines the outcome of the interaction was first postulated in Flor's "gene-for-gene hypothesis" [126, 127]. Often, race-specific resistance is characterized by rapid and strictly localized cell death in the host (hypersensitive response) limiting the spread of the pathogen to non-infected tissue and preventing its propagation. Different approaches are being pursued for isolation and molecular characterization of resistance genes including map-based cloning and transposon tagging [128, 129]. Recently, transposon tagging led to the isolation of a race-specific resistance gene from maize, the genetically defined *HM1* locus, directed against race 1 of the fungus *Helminthosporium carbonum* [130]. The derived amino acid sequence of the *HM1* gene shows similarity with the amino acid sequence of plant NADPH- and NADH-dependent reductases, and very likely the *HM1* gene product is responsible for detoxification of the HC-toxin which mediates the specific virulence of *Helminthosporium carbonum* race 1 on maize.

In the past, crossing-in of race-specific resistance was frequently applied in breeding programs to protect novel varieties against fungal and other diseases. However, this procedure provides protection only against a limited number of pathogen races and large-scale growth of new varieties led to the rapid selection of new virulent races and epidemic spread in monocultures. Of course, the same problems could arise if transgenic plants carrying race-specific resistance genes from other cultivars or other species were grown in the field.

P. de Wit proposes a strategy for obtaining durable non-specific resistance by engineering plants in which the hypersensitive response is elicited by a wide range of pathogens [131]. The model proposes transfer of the *avr9* gene from the fungal tomato pathogen *Cladosporium fulvum* into a tomato cultivar which contains the corresponding *cf9* resistance gene. The author expects, that in transgenic plants harboring this two-component system, a hypersensitive response will establish in many host/pathogen interactions, if expression of the *avr9* gene is put under control of a promoter mediating rapid and local transcriptional activation in response to a broad spectrum of pathogens. Upon isolation of the *cf9* resistance gene, the *avr9-cf9* system could be introduced as a cassette also into plants that lack *cf9*. The strategy requires that the *avr9*-encoded elicitor is targeted to the correct cell compartment within the transgenic plants to give a functional interaction with the *cf9* gene product. Furthermore, the success of the concept largely depends on the availability of a suitable promoter to direct *avr9* expression. Besides being responsive to attack by a wide range of pathogens, it must also be completely inactive in all non-infected plant cells, because constitutive synthesis of elicitor and resistance gene product will result in cell death and, therefore, prevent regeneration of transgenic plants.

Our own laboratory is pursuing another strategy for artificial generation of programmed cell death in transgenic potato, potentially limiting the spread of a wide range of fungi in the host tissue. A promoter fragment of the potato *prp1-1* gene, which mediates rapid and local transcriptional activation selectively after fungal infection [132], has been combined with the barnase gene from *Bacillus amyloliquefaciens* encoding a highly cytotoxic RNase [133]. Rapid synthesis of this RNase in the vicinity of infection sites should initiate necrosis of host cells during early stages of compatible interactions and, therefore, restrict the growth and propagation of biotrophic pathogenic fungi also in this type of interaction, analogous to the naturally occurring hypersensitive cell death in incompatible interactions. In order to minimize the deleterious effects of a low potential background *prp1-1* promoter activity, transgenic plants were simultaneously transformed with a chimeric construct which constitutively produces barstar, the specific barnase inhibitor from *Bacillus amyloliquefaciens.* We expect that only in the close vicinity of infection sites the level of barstar expression will be exceeded by the level of barnase expression, resulting in a strictly localized rapid cell death. Two transgenic potato lines with increased quantitative resistance to *Phytophthora infestans,* the causal agent of late blight disease, were identified (Strittmatter, unpublished results). The molecular analysis of these transgenic lines is in progress.

Resistance to Plant-Feeding Insects

Bacillus thuringiensis δ-endotoxins

For more than 30 years crystalline δ-endotoxins, which accumulate during sporulation of the entomocidal bacterium *Bacillus thuringiensis (B. t.),*

have been available as an alternative to synthetic organic insecticides in controlling harvest losses by larval forms of agronomically important insects [134–136]. Although single proteins display only a very narrow range of target insects, *B. t.* toxin activity has been found against many species of insects within the orders of Lepidoptera, Diptera and Coleoptera, due to the enormous diversity of *Bacillus thuringiensis* strains with respect to δ-endotoxins. On the one hand, this diversity could be caused by the co-evolution of toxin-encoding genes in the bacteria and toxin target-encoding genes in the insects. On the other hand, natural populations of *Bacillus thuringiensis* could also be diverse because most toxin-encoding genes are located on self-transmissible plasmids allowing transfer between related cells and, consequently, creating bacterial strains with combinations of different toxins as well as novel recombined toxins.

Intensive investigations have led to detailed knowledge of the mechanism and specificity of *B. t.* toxin activity [134, 137]. Upon ingestion by susceptible insect larvae, the crystalline inclusions are solubilized in the midgut releasing one or more proteins of 27 to 140 kDa. Rapid proteolytic cleavage of the protoxins then produces active toxic polypeptides. These activated toxins bind to specific high affinity receptors on midgut cell membranes leading to the formation of pores that disturb the cellular osmotic balance; within minutes, midgut cells are paralyzed and disrupted. There is increasing evidence that the presence of specific binding sites on the midgut epithelium causes the difference between susceptible and resistant insects [138, 139]. Alternatively, the presence of specific proteases for the release of the active toxin from the protoxin could determine the target insects of *B. t.* toxins.

At present, the use of *B. t.*-based bioinsecticides, which usually are formulations of *B. t.* spores and crystalline toxin inclusions, is still very limited for several reasons: the low stability of the proteins in the field, the difficulty to reach internal and underground regions of the plant, the relatively high production costs and the limited insecticidal spectrum of single proteins. The first two restrictions can in principle be overcome by expressing *B. t.* toxins in transgenic crops, and indeed, transformation of plants with *B. t.* toxin genes was one of the first attempts made to genetically engineer disease or pest resistance.

In experiments carried out at "Plant Genetic Systems" (Gent, Belgium), insecticidal levels of *B. t.* toxin were produced in tobacco plants transformed with a truncated *bt2* gene from *Bacillus thuringiensis* strain berliner 1715 encoding the amino terminal half of the cryIA(b) protein. Expression of the transgene was under control of the *Agrobacterium tumefaciens* mannopine synthase gene promoter, which confers constitutive transcription [140]. Depending on the amount of *B. t.* toxin produced, the mortality of larvae from the lepidopteran insect *Manduca sexta* (tobacco hornworm) feeding on the transgenic plants dramatically increased and the damage to leaves was reduced when compared to control plants. The capacity to produce the toxin and concomitant protection against the pest were inherited to F_1 progeny. When kanamycin was used as a selecting agent during the regeneration of transformants, especially high levels of Bt 2 protein and, thereby, particularly high protection was observed with transgenic plants expressing the truncated toxin as a fusion protein with neomycin phosphotransferase, as a selectable marker. Transformation of plants with the full length *bt2* gene revealed only regenerates with low levels of the corresponding mRNA and protein, possibly due to cytotoxic effects of high concentrations of the full length protein; such plants did not exert significant insecticidal activity. Protection against feeding damage by *Manduca sexta* larvae was also reported for transgenic tobacco and tomato plants expressing the amino terminal half of the cryIA(a) and cryIA(c) δ-endotoxins, respectively, from *Bacillus thuringiensis* var. *kurstaki* strains [141, 142]. In these cases, transcription of the toxin gene was driven by the CaMV 35 S promoter; again, insecticidal activity of plants correlated with the amount of toxin produced, and the phenotype was inherited to progeny.

The level of *B. t.* toxin gene expression in the transgenic lines described above was low compared to other heterologous genes. Field trials with tomato plants indicated that higher levels of expression are required to control damage by agronomically important lepidopteran insects which are less sensitive than *Manduca sexta,* such as tomato fruit worm *(Heliothis zea)* [143]. In tomato, tobacco, cotton and potato significant increases in the concentrations of *B. t.* toxin (up to 1000-fold) were achieved by modifications of the truncated structural gene that had either no or only a minor effect

on the encoded amino acid sequence [144–147]. Alterations affected 5′- or 3′-terminal regulatory mRNA sequences, mRNA secondary structure, G+C content and/or the codon usage. To a certain extent, all of these modifications seem to contribute to an increased translational efficiency. The genes which expressed the *B.t.* insect control protein at the highest levels in cotton plants retained less than 80% DNA homology to the wild type sequence [145]. Field trials proved that expression of truncated and modified *B.t.* toxin genes leads to concentrations of insecticidal proteins which protect cotton, potato and maize against heavy infestation by various lepidopteran insects [147, 148]. In cotton, engineering of *B.t.* toxin synthesis conferred a similar level of protection against damage and yield loss as the application of chemical insecticides.

Plants transformed with *B.t.* toxin genes are considered as environmentally safe because of the high specificity of the encoded proteins as well as their short persistance in the field. A major concern arises from the potential selection of insects resistant to these toxins. Several major pest species have demonstrated the ability to adapt to *B.t.* toxins either in laboratory tests *(Heliothis virescens, Leptinotarsa decemlineata, Plodia interpunctella, Plutella xylostella)* or in the field *(Plutella xylostella)* [149]. Changes in the binding specificity of toxin receptors seem to represent the major cause of resistance development. Several strategies are discussed for the management of insect resistance, when deploying *B.t.* toxins through transgenic plants: developing and maintaining refuges for the survival of susceptible insects, growing mixtures of cultivars with different toxins, sequentially planting such different cultivars, expressing mixtures of toxins in single transgenic lines, and regulating the expression pattern and/or level of toxin genes by the use of promoter sequences which confer inducible and not constitutive transcription [149, 150]. None of these strategies alone has proven its general suitability, and extensive field trials are necessary to further evaluate their advantages and disadvantages.

Protease inhibitors

An alternative approach focuses on protease inhibitors, proteins which have evolved in plants for protection against herbivirous insects. Protease inhibitors are widely distributed within the plant kingdom and can accumulate to particularly high levels in seeds and storage organs, reaching concentrations of more than 10% of total protein in exceptional cases [151, 152]. Most plant protease inhibitors exert no effect on endogenous plant proteases but have specificities for animal or microbial enzymes [153], suggesting they may be involved in the protection of vulnerable plant tissues from pest and pathogen attack by an antinutritional interaction with digestive enzymes. Support for this idea originates from studies in which the detrimental effect of protease inhibitors was proven by feeding insects on artificial diets [154 and ref. therein]. Furthermore, rapid systemic accumulation of protease inhibitor mRNAs and proteins throughout the areal tissues of the plant was demonstrated in tomato and potato in response to insect attack or mechanical wounding [155–157]. Abscisic acid and a hormone-like proteinaceous factor PIIF (protease inhibitor-inducing factor), have been identified as two of possibly several mediators of this systemic response [158, 159].

In plants, at least eight non-homologous families of protease inhibitors have been recognized [160]. Together, these families cover inhibitors specific for each of the four mechanistic classes of proteolytic enzymes, *i.e.* serine, cysteine, aspartic and metalloproteases. The vast majority of known protease inhibitors, however, are effective against serine proteinases (trypsin, chymotrypsin, elastase, subtilisin, kallikrein), interfering with complex regulatory circuits that control the balance between food supply and digestive activities [161].

The first protease inhibitor protein expressed in transgenic plants was the cowpea trypsin inhibitor (CpTI) which belongs to the Bowman-Birk subfamily of serine proteinase inhibitors [162]. This protease inhibitor was selected for transformation of plants because of its demonstrated association with field resistance to the cowpea bruchid beetle (*Callosobruchus maculatus* F.) [163] and because of its activity against a broad spectrum of economically important pest species *in vitro* [154]. A full length CpTI cDNA sequence was brought under control of the CaMV 35 S promoter and transferred into *Nicotiana tabacum.* A wide range of CpTI expression levels was detected in independently transformed plants, reaching from below detection limit to about 0.9% of total soluble protein. Those primary regenerates expressing CpTI to the highest

levels were also rated as "potentially resistant" to tobacco budworm, *Heliothis virescens*. A further increase of the CpTI level was achieved by generating homozygous progeny of these plants. Bioassays considering, on the one hand, survival and insect biomass, and, on the other hand, the percentage of destroyed leaf area, have conclusively established that CpTI-expressing plants have significantly enhanced resistance to *Heliothis* and to a broad range of other lepidopteran pests feeding on tobacco [154, 162, 164].

The inhibitors PI-I and PI-II represent two distinct families of serine proteinase inhibitors which are unrelated to one another or to CpTI. They accumulate to high concentrations in unstressed potato tubers, but are also produced in leaves of potato and tomato in response to wounding [155]. Tobacco plants were transformed with fusions combining the coding sequences of PI-II genes from potato or tomato and a CaMV 35 S promoter fragment, and the resistance of these plants to insect infestation was then assayed with the tobacco hornworm, *Manduca sexta* [165]. Similarly to results obtained with transgenic CpTI plants, the severity of the effect on caterpillars and the reduction of leaf damage was related to PI-II expression in transgenic plants. In contrast, no inhibition of feeding by this particular insect was achieved with transgenic tobacco producing the PI-I inhibitor from tomato, indicating that not every protease inhibitor can be employed for control of a specific pest species.

Generally, the activity of protease inhibitors against a wide range of insects, their inactivation by cooking and the widespread occurrence of such inhibitors in the food of humans and animals are considered as advantages when employing the corresponding genes for genetic engineering [7]. However, these inhibitors may also become harmful to beneficial insects if they are constitutively expressed at the high levels necessary to accomplish protection.

Concluding Remarks

The first examples of genetic engineering of resistance to microorganisms and pests in plants clearly demonstrate the potential of this technology. Before gaining significant meaning for an integrated disease management in agriculture, however, the novel transgenic lines still have to prove that they are economic in production and safe for the environment. In the end, of course, the consumer will have to decide whether he/she accepts a menu or clothing including the products of transgenic plants. Hopefully, this decision will be made on a rational, rather than emotional, basis.

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