

Genetic engineering for fungal and bacterial diseases

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Significant new advances at the molecular level in the field of plant–pathogen interactions form the basis for novel transgenic approaches to crop protection. The cloning of disease resistance genes and the dissection of the signal transduction components of the hypersensitive response and systemic acquired resistance pathways have greatly increased the diversity of options available for transgenic disease resistance. These new approaches will supplement our rapidly increasing repertoire of antimicrobial peptides, defense-related proteins and antimicrobial compounds. The combinatorial deployment of these strategies will be exploited for engineering effective and durable resistance to pathogens in the field. The integration of transgenic approaches with classical resistance breeding offers a potentially chemical-free and environmentally friendly solution for controlling pathogens.

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Abbreviations

Avr	avirulence
BTH	benzo(1,2,3)thiadiazole-7-carbothioic acid <i>S</i> -methyl ester
HR	hypersensitive response
PR	pathogenesis-related
R	resistance
RIP	ribosome-inactivating protein
ROS	reactive oxygen species
SA	salicylic acid
SAR	systemic acquired resistance

Introduction

Since the early days of organized agriculture, bacterial and fungal pathogens have formed intimate, and often highly evolved, interactions with cultivated crops. These interactions often result in serious outbreaks of disease. Despite the use of sophisticated crop protection measures, many bacterial and fungal pathogens still remain formidable enemies posing a serious threat to crops. The intensive use of monoculture crops with little genetic diversity in modern agriculture has significantly enhanced their susceptibility to increasingly aggressive pathogens. With the exception of disease epidemics that lead to complete crop destruction, global loss because of pathogens is estimated to be 12% of potential crop production [1]. The highest losses, estimated at more than \$42 billion per year, occur in vegetables, fruits and rice. These losses occur despite the application of an increasing quantity of fungicides annually. In addition to causing yield losses, pathogens also reduce the quality of food and feed. Mycotoxins produced by *Fusarium* spp. and *Aspergillus* spp.

often contaminate grain and peanuts and affect human and animal health.

As the world population continues to increase, environmentally safe and economically viable means of disease control are needed. The classical *R* (resistance) genes will continue to be deployed in the development of disease-resistant crops; however, for certain less-specialized pathogens causing root and fruit diseases, classical resistance is not available. Such resistance is often limited by its lack of durability as pathogens quickly evolve to overcome it. Furthermore, classical resistance is often polygenic, making introgression into commercial cultivars via breeding time-consuming and difficult.

With the development of transformation technology for several important crops during the past decade, exciting opportunities for engineering crop protection have emerged. Several new advances in our understanding of the biology of plant–pathogen interactions [2•] form the basis for new and viable approaches for engineering resistance to pathogens in transgenic crops. The cloning and structure–function analysis of a number of *R* genes conditioning resistance to the bacterial and fungal pathogens in a race-specific manner, the molecular dissection of the downstream *R* gene interactions, and progress toward defining the various steps leading to the elicitation of hypersensitive response (HR) and systemic acquired resistance (SAR) have paved the way toward the production of transgenic crops with broad-spectrum resistance. Furthermore, several novel cysteine-rich antimicrobial peptides and other defense-related proteins with significant potential to impart *in planta* resistance have been isolated and cloned. In this review, I only discuss the implications of these very recent advances in developing engineered defense against pathogens in crops. The reader is referred to several earlier reviews on this topic [3–6].

Resistance through combinatorial expression of plant defense genes

Several defense-related genes encoding chitinases, glucanases, peroxidases and pathogenesis-related (PR) proteins are either constitutively expressed or induced upon pathogen infection. The proteins encoded by these genes display *in vitro* antimicrobial activity, suggesting a formal role in plant defense. Individually, some of these genes impart partial resistance to fungal pathogens in transgenic plants; however, the level of resistance appears to be insufficient for practical use [3–6]. The fungal cell wall degrading enzymes chitinases and glucanases have been examined extensively for their potential to afford resistance to fungal pathogens in transgenic plants. Synergistic *in vitro* antifungal activity between the basic isoforms of tobacco chitinase and glucanase has been previously

reported [7]. In tomato, the coexpression of tobacco genes encoding both enzymes leads to greatly enhanced resistance to *Fusarium* wilt disease [8••]. Transgenic carrot plants expressing this pair of tobacco enzymes displayed high levels of resistance to both *Alternaria* and *Cercospora* species in the field, consistent with their synergistic *in vitro* antifungal activity (LC Melchers, personal communication). Combinatorial expression of other chitinase and glucanase genes has also proven very effective in providing resistance to fungal pathogens in transgenic tobacco [9,10••]. The approach of using combinations of plant defense genes to achieve effective control of fungal pathogens has also been extended to ribosome-inactivating protein (RIP), which inhibits eukaryotic protein translation and is thought to play a role in plant defense. The antifungal activity of a barley RIP was synergistically enhanced in the presence of fungal cell wall hydrolases [10••]. Higher levels of fungal resistance were observed in transgenic plants that coexpressed the barley RIP and the chitinase.

There is a growing body of evidence to suggest that PR proteins are causally associated with disease resistance. Several members of the PR1, PR2 (glucanases), PR3 (chitinases), PR4 (chitin-binding) and PR5 (thaumatin-like) classes of proteins have displayed *in vitro* antimicrobial activity. In addition to PR2 and PR3, *in planta* efficacy of PR1a and PR5 from tobacco for fungal control has also been reported. Furthermore, when PR1a is coexpressed with another tobacco PR protein, SAR8.2, synergistic antifungal activity was observed (J Ryals, personal communication). Based on these studies, a combinatorial expression of plant defense genes where each single gene provides partial resistance appears to be a preferred avenue for engineering crop protection.

Resistance through expression of small antimicrobial peptides

The deployment of small antimicrobial peptides for defense against microbes represents a defense strategy that is conserved in evolution [11–13]. Recent evidence indicates that plants produce a number of antimicrobial peptides to ward off pathogenic attack [14]. Several distinct classes of peptides differing in their amino acid sequences have been reported. These include cysteine-rich antimicrobial peptides, plant defensins, thionins, lipid-transfer proteins and 2S albumins [14]. Of these, plant defensins share amino acid sequence homology with their insect and mammalian homologs and display strong, often broad-spectrum, *in vitro* antifungal activity [15•]. Two defensin-like peptides isolated from radish seed, Rs-AFP1 and Rs-AFP2, have been shown to inhibit the growth of several pathogenic fungi *in vitro* [16]. The expression of Rs-AFP2 in transgenic tobacco confers resistance to attack by *Alternaria longipes* [17••], although the spectrum of fungal resistance has not been fully investigated. Two homologous peptides, Rs-AFP3 and

4, are also induced in radish leaves upon infection by *A. longipes*, thus further substantiating the role of defensins in plant defense. Two sugar beet leaf defensins, AX1 and AX2, homologs of the radish AFP2, have been isolated after infection with the fungal pathogen *Cercospora beticola* [18]. The preliminary results indicate that the expression of these peptides in transgenic corn plants imparts significant resistance to Northern corn leaf blight caused by the fungal pathogen *Exserohilum turcicum*.

None of the other classes of antimicrobial peptides have yet been shown to confer resistance to fungal pathogens *in planta*. One potential problem with some of these peptides is that their *in vitro* antimicrobial activity is greatly reduced in the presence of physiological concentrations of inorganic cations. This may limit their *in planta* efficacy [19•]. In one report, the α -thionin gene from barley has been demonstrated to confer enhanced resistance to a bacterial pathogen *Pseudomonas syringae* in transgenic tobacco plants [20]. The isolation of antimicrobial peptides with potent *in vitro* activity is an active area of research in a number of laboratories and offers hope for providing enhanced resistance to pathogens in transgenic crops.

Resistance through manipulation of reactive oxygen species and phytoalexins

Reactive oxygen species (ROS; H_2O_2 , O_2^- , $OH\cdot$) play important roles in various defense responses of the plants [21,22•,23•]. A prolonged local oxidative burst is one of the earliest events correlated with plant resistance at the site of pathogen invasion. Besides being directly toxic to microbes, the ROS perhaps trigger the cell death pathway leading to the HR. The ROS are required for the covalent cross-linking of cell wall proteins and they activate expression of cellular protectant genes. There is also some evidence that ROS may have a signaling role in salicylic acid (SA) accumulation. The direct evidence that ROS are involved in conferring disease resistance was provided by the constitutive expression of an H_2O_2 -generating glucose oxidase gene from *Aspergillus niger* in transgenic potato [24••]. Transgenic tubers exhibited strong resistance to bacterial soft rot disease, caused by *Erwinia carotovora*, and this resistance was apparently mediated by elevated levels of H_2O_2 because it could be eliminated through the exogenous addition of catalase. Enhanced resistance to the potato fungal pathogens *Phytophthora infestans* and *Verticillium dahliae* was also demonstrated and it correlated with elevated levels of H_2O_2 . The preliminary results indicate that some PR genes, including those for acidic chitinase, basic glucanase and anionic peroxidase, are also activated in transgenic potato plants in the absence of pathogen infection (G Wu, personal communication). Thus, the expression of an H_2O_2 -generating enzyme in transgenic plants represents a novel strategy for engineering broad-spectrum resistance to bacterial and fungal pathogens.

The low-molecular weight antimicrobial compounds called phytoalexins are produced by plants upon pathogen infection and have long been implicated as playing an important role in disease resistance [25•]. Recently, the strategy of producing a foreign phytoalexin in transgenic plants has been shown to confer significantly enhanced resistance to fungal pathogens [26]. The expression of a grapevine stilbene synthase gene from its own promoter in tobacco leads to the pathogen-induced accumulation of resveratrol, promoting resistance to *Botrytis cinerea* [27]. The extent to which interspecific transfer of other phytoalexin biosynthesis genes to impart effective resistance will be possible remains to be seen. Genes encoding pinosylvin synthase from Scots pine and bibenzyl synthase from orchid have been cloned and introduced into tobacco, but data on their *in planta* efficacy are not available [26]. Given the complexity of phytoalexin biosynthesis pathways and the multitude of the enzymes involved, it is unlikely that the transfer of a single gene will impart effective resistance. Recently identified phytoalexin-deficient (*pad*) mutants of *Arabidopsis* will be particularly useful for the isolation of regulatory genes involved in the phytoalexin pathway [28]. Interestingly, these mutants are unaffected in their resistance to an avirulent isolate of the bacterial pathogen *P. syringae* but allow enhanced growth of a virulent isolate. One of these mutants, *pad4*, displays enhanced susceptibility to biotrophic fungal pathogens indicating that the *Pad4* gene is involved in conferring fungal resistance (F Ausubel, personal communication). The regulatory genes involved in a phytoalexin pathway may facilitate engineered resistance through manipulation of the endogenous phytoalexin levels.

Resistance through deployment of *R* genes

HR is triggered in response to an incompatible interaction between a plant and a nonpathogen or an avirulent pathogen and involves rapid, localized, programmed cell death. Although frequently associated with resistance, it is not clear if HR alone is sufficient to restrict a pathogen at the site of infection. HR is often accompanied by the activation of a multitude of local and systemic defense responses [29]. These responses may be critical for the resistance reaction of the host. A great deal of specificity has developed during the evolution of the host–pathogen interaction. The genetic analysis of this specificity has revealed that it is determined by specific *R* genes in the host and their corresponding *avr* (avirulence) genes in the pathogen, commonly in a one-to-one correspondence. The molecular basis for these gene–gene interactions for several host–pathogen systems is currently under intense investigation because of the recent breakthrough in the cloning of a number of *R* genes providing resistance against bacterial, fungal and viral pathogens. The reader is referred to several recent reviews that provide detailed descriptions of the cloning and partial characterization of the *R* genes, the structural domains and the signal transducing potential of their gene products and the partial characterization of their interaction with the downstream

signal transduction components [30,31•,32•,33,34,35••]. The relevance of these important discoveries to the genetic engineering of disease resistance in crops has been discussed at length in [31•,32•,35••]. The successful transfer of functional *R* genes between closely related species through genetic transformation clearly represents a significant step toward the goal of deploying *R* genes for engineered resistance in crops. For example, the *Pto* gene from tomato promotes HR-based resistance to *Pseudomonas syringae* pv. *tabaci* pathogens carrying *avrPto* in tobacco species [36•,37•]. The tobacco *N* gene provides resistance to tobacco mosaic virus (TMV) in tomato [38•]. The tomato *Cf9* gene functions in tobacco and potato (J Jones, personal communication). The intergeneric transfer of downy mildew *R* genes may also be feasible between *Arabidopsis* and *Brassica* species, as indicated by the observation that some of the downy mildew *R* genes from *Arabidopsis* recognize the downy mildew pathogens of *Brassica oleracea* [39•]; however, as noted by Bent [35••] and Crute and Pink [40•], it is difficult to predict the success of intergeneric transfer of *R* genes to distantly related species because some components of the signal transduction pathway will be specific to *R* genes and absent in distantly related species. Greater knowledge of the signal transduction pathway at the molecular level is needed to design rational strategies for the functional transfer of *R* genes in distantly related species.

Plant breeders have widely utilized the strategy of pyramiding *R* loci in traditional plant breeding to allow recognition by the host of multiple races of the evolving pathogen population in the field. Although this strategy for resistance breeding has yielded significant benefits to the farmer, generating an effective combination of *R* genes in many crop species is time-consuming, costly and often associated with yield drag. With molecular tools in hand to isolate large families of similar *R* genes recognizing multiple races of a pathogen, the transgenic approach of pyramiding *R* genes will be possible in the near future. Crute and Pink [40•] have discussed the rate-limiting factors impeding upon the success of such an approach. They also discuss the benefits of marker-aided selection for the directed transfer of *R* genes in crop species to provide durable disease control.

De Wit [41] proposed a two-component strategy for engineering broad-spectrum resistance using the cloned *R*–*avr* gene pair. It is based on eliciting an HR in plants containing an *R* gene with pathogen-inducible expression of the cognate *avr* gene. The advantage of this approach is that the resistance response is only triggered upon pathogen infection and is independent of race specificity. The challenge is to find a promoter that is only activated locally by pathogen invasion of the host. That such an approach might be feasible is indicated by a recent success in inhibiting the late blight disease caused by *P. infestans* in transgenic potato using pathogen-inducible *prp-1* promoter-driven expression of a

bacterial ribonuclease (barnase) that elicited localized HR [42••]. Further confidence in the proposed two-component system stems from the genetic experiments with the *Cf9-avr9* gene system in tomato. The tomato plants in which *Cf9* function is restored through somatic excision of a transposable element and in which *avr9* is constitutively expressed display defense-related somatic necrotic sectors and resistance to a number of pathogens such as late blight and powdery mildew [31•]. Whether or not this type of genetically imposed resistance, designated genetic acquired resistance, will be practically useful remains to be determined.

R genes recognizing multiple or all races of a pathogen are of special interest. The examples include *Bs2*, *Xa21* and *mlo*. The *Bs2* gene from pepper [43] recognizes a common virulence determinant of *Xanthomonas campestris* pv. *vesicatoria* and thus may provide durable resistance against Xanthomonads in general when introduced into transgenic plants. The *Xa21* gene of rice confers resistance to all known races of the bacterial vascular pathogen *Xanthomonas oryzae* pv. *oryzae* and has been recently cloned [44]. Furthermore, the cloned gene has been shown to confer resistance to 29 different isolates in transgenic rice plants [45•]. The recessive *mlo* gene from barley imparts non-race-specific resistance to powdery mildew. It has proven remarkably durable in the field against this pathogen and has been introduced into an estimated 700 000 ha of European barley. The *mlo* gene operates by a distinct mechanism: fungal penetration is arrested in the epidermal papillae that form before fungal attack and HR-like lesions seldom appear [46]. The *mlo* gene from barley has been cloned using map-based cloning (P Schulz-Leferet, personal communication); the availability of this gene from barley might allow the transgenic engineering of wheat for powdery mildew resistance through an antisense gene strategy. *R* genes capable of recognizing multiple isolates of a pathogen will be very useful for engineering broad-spectrum resistance in crops.

New insights from HR and SAR pathways

The strength of HR-based resistance is the induction of multi factorial defense pathways [47•]. A large number of lesion-mimic mutants that develop necrotic lesions spontaneously are providing important new insights into molecular mechanisms triggering cell death and the activation of defense mechanisms that are reminiscent of incompatible interactions [48,49•,50••]. As illustrated by Dangl *et al.* [50••], many of these mutants in maize and *Arabidopsis* behave as if constantly under pathogen attack and display many of the molecular markers associated with resistance responses, including increased SA levels, the activation of PR genes and heightened resistance to bacterial and fungal pathogens. Many of the lesion-mimic mutants of *Arabidopsis* have already been placed into an SA-dependent resistance pathway [50••]. Further detailed

genetic and biochemical characterization of these mutants, including the cloning of the genes involved, is in progress in a number of laboratories. Genes identified by these mutations might be useful for activating defense mechanisms in transgenic crops. Further support for this comes from several examples of plant and microbial transgenes that cause the lesion-mimic phenotype in transgenic plants and activate local and systemic disease resistance pathways. The recent noteworthy examples are transgenic tobacco plants expressing the bacterio-opsin gene from *Halobacterium halobium* [51], the yeast invertase gene [52] or the cholera toxin gene [53]. These genes will have little practical use because they compromise the growth of plants.

Tremendous progress has been made during the past few years in deciphering molecular aspects of SAR, an inducible plant defense response triggered by necrotizing infection that culminates in broad-spectrum, systemic resistance to bacterial, fungal and viral pathogens [54,55••]. Some of the important milestones of SAR research are outlined here. It is clear that SA accumulation is necessary for the establishment and maintenance of SAR. The question of whether SA is a systemic signal is still unresolved. Several SAR marker genes have been identified for tobacco, *Arabidopsis* and other plants. The chemical activators, such as 2,6-dichloro isonicotinic acid (INA) and benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester (BTH), of SAR have been identified. BTH will be useful agronomically as it provides effective control of powdery mildew in wheat without causing crop injury [56••]. This resistance to powdery mildew in BTH-treated wheat resembles that during the incompatible interaction and is correlated with the induction of a number of mRNAs. Several *Arabidopsis* mutants in the SAR signal transduction pathway have been identified. In addition to the lesion-mimic mutants mentioned above, mutants displaying the constitutive expression of PR genes and immunity have been described. These mutants, known as *cim* or *cpr*, are resistant to normally virulent pathogens and this resistance is not always associated with cell death. The third class of mutants, termed *nim* or *npr*, are deficient in the pathogen- or chemical-induced SAR. The molecular cloning of genes identified by these mutants is currently in progress. The *Npr1* gene of *Arabidopsis* has been recently isolated using a map-based cloning approach and encodes a novel protein containing ankyrin repeats [57••]. It will be interesting to determine if overexpression of the *Arabidopsis Npr1* gene or its homologs from other plants will lead to constitutive activation of SAR in crop plants. New evidence is emerging that there may be more than one pathway for triggering the SAR pathway in plants. One of these pathways induced by root-colonizing nonpathogenic biocontrol bacteria in *Arabidopsis* appears to be independent of SA and PR gene expression [58•]. Our greatly expanding knowledge base of this interesting and broadly effective resistance mechanism of plants will lead to engineered crops with enhanced resistance and to

the discovery of other novel crop protection chemicals with unique mode-of-action in near future.

Conclusions

The range of potential strategies for genetically engineered resistance in crops has expanded dramatically during the past few years. Our repertoire of novel transgenes encoding highly potent antimicrobial peptides, defense-related proteins and enzymes for the production of antimicrobial compounds (e.g. phytoalexin) has greatly increased. The combinatorial deployment of these transgenes in crops is likely to provide practically useful levels of disease control. This type of combinatorial resistance may, in fact, be desirable, as it may provide more durable resistance in the face of a constantly evolving pathogen population. The recent cloning of *R* genes and the characterization of signal transduction pathways for HR and SAR have greatly increased the diversity of transgenic approaches available for improved disease resistance. The careful integration of transgenic approaches with classical resistance breeding will form the basis for a new revolution in agriculture for enhanced productivity.

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