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Engineering Disease Resistance in Plants: An Overview

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I. INTRODUCTION AND SCOPE

The goal of genetically manipulating disease resistance in plants has become a reality in the last 3 or 4 years. This is primarily due to advances in two areas: the technology of plant transformation and our better understanding of the molecular basis of plant-pathogen interactions. Transgenic plants expressing either novel proteins from foreign organisms or overexpressing a part of their own defensive arsenal have been engineered, tested in both laboratory and field situations, and evaluated for disease resistance. Engineered viral resistance through expression of viral coat protein genes,¹ replicase components,² or antisense RNA³ has proved effective in several instances. To date, there are fewer reports on engineered resistance against fungi and bacteria. However, we believe that the next 5 years will witness a number of successes in this area, utilizing a range of different, and often ingenious, strategies. The purpose of this chapter is to outline the principles upon which strategies for fungal and bacterial resistance may be based, to evaluate the types of genetic manipulations which may lead to increased resistance, and to review those examples in which success has already been reported.

II. MOLECULAR FEATURES OF PLANT-PATHOGEN INTERACTIONS

The processes of plant-pathogen coevolution have led in many cases to interactions in which the outcome (compatibility or incompatibility) is determined by a single dominant gene for resistance in the host, the functional realization of which is determined by the presence of a corresponding, dominant avirulence gene in the fungal or bacterial pathogen.⁴ In such gene-for-gene interactions, incompatibility is associated with the rapid activation of a battery of defense-response genes, whose products may include biosynthetic enzymes for the production of antimicrobial phytoalexins and wall-bound phenolics, hydrolytic enzymes and other so-called pathogenesis-related (PR) proteins, and hydroxyproline-rich glycoproteins.⁵ These products usually accumulate locally around the site of attempted microbial ingress. In compatible

interactions, such defense-response genes are either not activated or are induced too late in the interaction to prevent disease symptoms.^{6,7} These observations suggest that resistance could be engineered in plants by (1) altering the timing and extent of induced defenses by constitutive expression of a natural induced defense-response gene or by putting naturally occurring defense-response genes under the control of stronger inducible promoters, or (2) by genetic manipulation of the dominant resistance genes per se. The strategy of altering expression of defense-response genes could also include targeting expression of novel antimicrobial proteins from foreign organisms, either constitutively or to the plant-pathogen interface.

Some fungal and bacterial pathogens produce toxins which are responsible for the disease symptoms.⁸ In such cases, virulence is dominant and resistance is expressed through the ability of the host either to not recognize the toxin (i.e., by lacking a toxin binding site) or to detoxify it. In such cases, incorporation of toxin-insensitive binding sites or enzymes for detoxification may provide means of engineering resistance.

Some fungal pathogens have acquired virulence by being able to detoxify the phytoalexins the host produces as a part of its defensive arsenal.⁹ A basis of information now exists for engineering modified phytoalexin structures which may be resistant to detoxification, or for transferring a phytoalexin biosynthetic pathway from one plant to another which lacks that particular pathway. Such strategies will generally necessitate the transfer of several genes; although this may pose complications, attempts in this area should lead to further insights into the control of plant gene expression and the roles of secondary metabolites in plants.

The following sections review the prospects for engineering fungal and bacterial resistance in plants based on the above features of plant-pathogen interactions. For a more detailed background on the molecular basis of resistance in plant-microbe interactions, the reader is referred to the reviews by Lamb et al.,¹⁰ Dixon and Lamb,¹¹ Dixon and Harrison,⁵ and Keen.¹² More details of engineered resistance strategies can be found in the recent review by Lamb et al.¹³

III. CHOICE OF PROMOTERS

A large number of plant defense-response genes have now been cloned.¹⁴ Most of these are transcriptionally activated in response to infection or exposure to microbial elicitor macromolecules.⁵ The promoters of such genes could therefore be used to target expression of engineered transgenes encoding proteins to enhance resistance. Before selecting a defense-response gene promoter for such studies, several features of the promoter must be assessed. These include whether or not its expression is tissue or cell type specific, whether it is affected by developmental or environmental cues other than infection, its kinetics of activation in response to infection, and its extent of expression (i.e., promoter strength). If the protective factor being introduced is not toxic to the plant, it may be best to use a promoter which will deliver high-level constitutive expression; the cauliflower mosaic virus 35S promoter¹⁵ or higher expression derivatives with double enhancer elements¹⁶ have been used successfully in a number of cases. Indeed, the importance of the timing of defense gene activation in determining the outcome of many plant-pathogen interactions suggests that having the newly engineered defensive barrier in place prior to pathogen ingress should be beneficial. On the other hand, inducible promoters would be a necessity if constitutive expression of the transgene or its ultimate product (e.g., phytoalexins) were toxic to the plant or in any way compromised the ability of the plant to defend itself (e.g., by affecting amino acid or energy metabolism in the case of very highly expressed proteins).

The properties of several plant defense-response gene promoters are outlined in Table 1. Many of these show highly specific patterns of tissue and cell type expression. In some cases, it has proved possible to separate *cis*-elements conditioning infection or elicitor inducibility from those determining tissue-specific expression;²² it may thus be possible to engineer a promoter which is only expressed in response to pathogen attack.

To be of general use, a promoter must retain its potential for correct activation in species other than that from which the gene was isolated. The examples in Table 1 indicate that most defense-response gene promoters studied to date are active in heterologous species. Whether or not this is likely to be universally true is not yet known, although it is interesting to note that the bean *chs8* promoter, the activation of which is a component of the induction of isoflavonoid phytoalexins in the host species, is also induced by infection in tobacco,²⁰ which does not use the flavonoid pathway for defense and does not make isoflavonoids at all. Some monocot defense gene promoters are correctly expressed in

Table 1 Properties of plant defense-response gene promoters

Gene	Source	Protein encoded	Promoter expressed in:	Tissue specificity ^a	Induction ^b				Ref.
					L	W	I	E	
<i>pal1</i>	<i>Arabidopsis thaliana</i>	L-Phenylalanine ammonia-lyase	<i>Arabidopsis</i>	V, Se, A, C	+	+	ND	+	17
<i>pal2</i>	<i>Phaseolus vulgaris</i>	L-Phenylalanine ammonia-lyase	Tobacco	P, A, St, R, St	+	+	ND	ND	18
<i>chs8</i>	<i>P. vulgaris</i>	Chalcone synthase	Tobacco	RT, V, St	ND	-	ND	+	19, 20 20a
<i>ifr</i>	<i>Medicago sativa</i>	Isoflavone reductase	Tobacco <i>Medicago sativa</i>						
<i>5B</i>	<i>P. vulgaris</i>	Basic chitinase	Tobacco	ND	ND	ND	+	+	21
<i>RCH10</i>	<i>Oryza sativa</i>	Basic chitinase	Tobacco	RT, R, V, St, O, A	ND	+	ND	+	22
—	<i>A. thaliana</i>	Acidic chitinase	<i>Arabidopsis</i> , tomato	R, V _L , H, G, A	ND	ND	+	ND	23
<i>hrgp4.1</i>	<i>P. vulgaris</i>	Hydroxyproline-rich glycoprotein	Tobacco	SN, RT, Sty, St	ND	+	+	ND	24

^a A = anthers; C = carpels; G = guard cells; H = hydathodes; ND = not determined; O = ovaries; P = petals; R = roots; RT = root tips; Se = sepals; SN = stem nodes; St = stigmas; Sty = styles; V = vascular tissue; V_L = leaf vascular tissue.

^b L = light; W = wounding; I = infection; E = elicitor.

Table 2 Pathogenesis-related (PR) Proteins Induced During Plant Defense

Class	Biological activity <i>in vitro</i>	Typical sources
Class I chitinase, basic	Antifungal	Bean, ²⁷ tobacco, ²⁸ maize, ²⁹ rice, ³⁰ <i>Brassica</i> ³¹
Class I chitinase, acidic	Antifungal	Bean ³²
Class II chitinase, acidic	Antifungal	Tobacco ³³
Class III chitinase	Bifunctional lysozyme/chitinase	Cucumber, ³⁴ tobacco ³⁵
Acidic extracellular glucanase	(Antifungal)	Bean, ³⁶ tobacco ³⁷
Basic vacuolar glucanase	Synergist for chitinase, antifungal	Bean, ^{38,39} pea ⁴⁰
PR-1a	Antifungal	Tobacco ⁴¹
PR-1b	ND ^a	Tobacco ⁴¹
PR-1c	ND	Tobacco ⁴¹
Pv PR1, Pv PR2 (birch pollen allergen-like)	ND	Bean ⁴² (similar in parsley, pea, potato)
Pv PR3	ND	Bean ⁴³
Ao PR1	ND	Asparagus ⁴⁴
PR-4 (hevein-like, no lectin domain)	ND	Tobacco ⁴⁵
PR-5 (thaumatin-like)	Antifungal, synergist for PR-4	Tobacco, ⁴⁶ barley ⁴⁷
PR-5 (osmotin-like)	Antifungal	Tobacco ⁴⁸

^aNot detected or not determined.

dicot tissues²² and the constitutive 35S promoter has been used successfully in some monocots.²⁵ Overall, signals for defense gene activation in plants would appear to be conserved even if the sets of genes upon which they act are different in different species.

To date, very few studies have attempted to engineer inducible defense responses using promoters such as are outlined in Table 1. Even in cases where potentially toxic secondary metabolites are being engineered, constitutive expression of a gene encoding a modifying enzyme may be acceptable if the earlier stages of the pathway which provide the substrate for that enzyme are only expressed locally in response to infection.

IV. MANIPULATION OF SINGLE GENE TRAITS TO DIRECTLY CONFER ANTIMICROBIAL ACTIVITY

A. PATHOGENESIS-RELATED PROTEINS

PR proteins are low-molecular weight proteins which accumulate to significant levels in infected plant tissues. They were initially defined and classified on the basis of their physical properties and induction characteristics in virus-infected tobacco.²⁶ The major classes of PR proteins are outlined in Table 2. Antimicrobial activity *in vitro* has not been demonstrated for all these proteins; it is possible that some are only active in combination with others.

On the basis of our present knowledge, the most attractive PR proteins for engineering resistance based on constitutive expression are the chitinases and 1,3,- β -D-glucanases, at least against those fungal pathogens which contain chitin in their cell walls. A basic, vacuolar chitinase of bean (*Phaseolus vulgaris*) has been expressed constitutively to high levels in transgenic plants of tobacco and *Brassica napus*. This expression resulted in significant protection of the plants from post-emergent damping off caused by the pathogen *Rhizoctonia solani*.²⁷ In the case of *B. napus*, although the protection was a delay rather than a complete inhibition of symptoms, it was concluded that the level of protection was sufficient to be of economic significance in field situations.²⁷ It would, however, be dangerous to assume that expression of a single chitinase gene will be of general efficacy in conferring resistance; indeed, constitutive expression of a tobacco basic chitinase gene in *Nicotiana sylvestris* proved ineffective against *Cercospora nicotianae*.⁴⁹ Such conflicting results should not be surprising, as we do not yet understand the basis of the protection conferred by hydrolytic enzymes such as chitinase. This may involve a direct lytic effect in which invading hyphae are killed, a perturbation of growth allowing

other induced defenses to become effective, a release of fungal wall components which can elicit other defenses, or a combination of all three.

To date, there have been no reports of increased resistance from expression of a 1,3,- β -D-glucanase gene in transgenic plants. Likewise, down-regulation of glucanase expression by antisense RNA did not increase the susceptibility of transgenic *N. sylvestris* plants to infection by *C. nicotianae*.⁵⁰ *In vitro*, chitinases generally show greater antimicrobial activity than glucanases. However, glucanase has been shown to act as a powerful synergist for chitinase.⁵¹ This is presumably because, by digesting the β -glucan portion of the fungal cell wall, this enzyme renders the chitin more digestible. These observations suggest the strategy of coexpression of chitinase and glucanase in transgenic plants. Transgenic tobacco plants expressing a basic rice chitinase exhibit slightly delayed symptoms on infection with *C. nicotianae*. If these plants are crossed with tobacco expressing an alfalfa acidic glucanase (which itself does not appear to confer significant protection), the delay of symptoms in progeny expressing both genes is greater than in plants expressing chitinase alone.^{51a} There is considerable scope for optimizing and fine tuning such a protection mechanism, utilizing different combinations of chitinases and glucanases. These enzymes often exist in multiple forms, the basic forms generally being vacuolar and the acidic forms extracellular. As well as mixing and matching naturally occurring forms, it should be possible to modify targeting, for example, by removing the vacuolar targeting signals⁵² from the carboxy termini of the basic forms in order to direct them to the extracellular space.

A number of groups have attempted overexpressing other PR proteins in plants with a view to understanding their function. The possibility that some of these proteins are only active in combination with others makes negative results difficult to interpret and necessitates the laborious testing of a matrix of different PR protein combinations.⁵³ It is also possible that individual PR proteins exhibit some degree of pathogen specificity. Thus, overexpression of the tobacco PR-1 gene does not protect tobacco against tobacco mosaic virus,⁵⁴ although it can delay the onset of infection by the blue mold pathogen *Peronospora tabacina*.⁵⁵ Some PR proteins other than chitinase and glucanase exhibit antimicrobial activity *in vitro* (e.g., the osmotin-like PR5 protein),⁴⁸ and are therefore candidates for further evaluation.

B. NOVEL ANTIMICROBIAL PROTEINS

Plants, and indeed other organisms, may contain antimicrobial proteins not necessarily associated with induced defense responses, which are potential subjects for engineered protection strategies. Floral organs and seeds often contain high levels of antimicrobial proteins, presumably to protect the vulnerable tissues of the reproductive phase of the plant. Table 3 lists a selection of antimicrobial proteins which could find uses in plant protection in the next several years.

The ribosome-inactivating proteins (RIPs) have *N*-glycosidase activity which cleaves a specific adenine residue from the large subunit ribosomal RNA. They exist as single-chain proteins (Type I, e.g., the pokeweed antiviral protein) or double chains possessing a galactose-specific lectin which targets them to cell surfaces (Type II, e.g., ricin). A recent review lists nearly 40 RIPs from a range of plant families.⁶⁶ RIPs do not inhibit ribosomes from the plant of origin; some are active against fungal ribosomes and the barley RIP exhibits antifungal activity *in vitro*,⁶⁸ an activity which is enhanced in the presence of enzymes which can degrade fungal cell wall polysaccharides. A major potential complication in using RIPs for plant protection concerns their potential cytotoxicity, and, if this is likely to be a problem, the need to express them from nonleaky, inducible promoters and/or to target them to the extracellular space. The barley RIP has been expressed under a wound-inducible promoter in transgenic tobacco and shown to afford protection (measured by overall plant growth parameters) against the soilborne pathogen *R. solani*.⁶⁹ The fact that the expression of this transgene, which occurs in floral tissues and pollen, did not effect the fertility of the primary transformants suggests that cytotoxicity may not be a serious problem, at least in this case.

C. ENGINEERED TOXIN INSENSITIVITY

The molecular targets of several fungal or bacterial toxins from plant pathogens are now known. One example will illustrate the strategy for engineering resistance by transfer of toxin-insensitive targets. The bacterial halo-blight pathogen of bean, *Pseudomonas phaseolicola*, produces a tripeptide toxin, phaseolotoxin, which causes the chlorotic halos symptomatic of the disease.⁷⁰ Phaseolotoxin inhibits the enzyme ornithine transcarbamylase, a key step in the biosynthesis of the amino acid arginine. Bacteria have been selected which contain a phaseolotoxin-insensitive ornithine transcarbamylase, and the gene encoding this enzyme has been cloned and transferred to tobacco, where its expression has

been shown to prevent the symptoms caused by application of the toxin.⁷¹ The lack of a transformation system for bean has so far precluded analysis of this manipulation with respect to resistance of the true host species.

D. ENGINEERED TOXIN DETOXIFICATION

An alternative to engineering plants with modified sites of action for microbial toxins would be to introduce genes encoding enzymes which can inactivate the toxin. In maize, resistance to the cyclic tetrapeptide toxin produced by *Cochliobolus carbonum* is associated with the presence of a pyridine nucleotide-dependent reductase which acts on an essential carbonyl group of the toxin. This enzyme activity is present only in resistant germplasm containing the dominant allele of the *hm1* locus.⁷² This toxin-resistance gene has recently been cloned by transposon tagging.⁷³ This opens up the possibility of engineering resistance to *C. carbonum* in previously susceptible maize genotypes.

E. EXPRESSION OF ANTIBODIES IN PLANTS

With the development of the technology for cloning individual, specific antibody genes,⁷⁴ it has become possible to express monoclonal antibodies in plants. Although expression levels are very variable and the system is still far from optimized, especially with respect to targeting, it has been possible to produce functional antibodies in plants by either transforming separate plants with constructs containing the heavy or the light chain, followed by crossing to yield progeny in which both chains are expressed and the antibody assembles,⁷⁵ or by expressing a chimeric construct harboring both heavy- and light-chain genes.⁷⁶ Expression of an antibody targeted against an antigen essential for the *in planta* growth of a pathogen, or its ability to cause symptoms, could decrease the titer of the antigen to a level which could prevent disease and/or ameliorate the symptoms. Obvious targets for such antibodies would be viral replicase or systemic movement functions, or perhaps viral coat proteins. This strategy is currently being assessed for several antigens of tomato spotted wilt virus.^{76a} Monoclonal antibodies have been produced against cell surface and extracellular components of fungal⁷⁷ and bacterial⁷⁸ plant pathogens, but it is not known if these would be effective in inhibiting pathogen growth *in planta*. Future development of this technology requires effective assembly of immunoglobulin chains in the cytoplasm of plant cells, a process which has not yet been achieved.⁷⁹ Alternatively, the design of single-chain antigen-binding constructs⁸⁰ could alleviate the need for assembly of the antibody complex.

Table 3 Antimicrobial proteins with potential for engineering disease resistance in plants

Class	Source	Activity	Ref.
Fungal, highly basic	<i>Aspergillus giganteus</i>	Antifungal	56
Polygalacturonase inhibitors	Bean (<i>Phaseolus vulgaris</i>), alfalfa	Inhibit fungal cell wall-degrading enzymes of pathogens	57,58
Chitin-binding proteins	Rubber tree (hevein)	Antifungal	59
	Stinging nettle (lectin)	Antifungal	60
	Barley	Antifungal, synergist with barley chitinase or PR-5	47
	<i>Amaranthus caudatus</i>	Antifungal (very potent); bactericidal	61
Thionins (cysteine-rich cell wall and vacuolar proteins)	Barley	Antifungal	62,63
Basic oligomeric proteins	Radish	Antifungal	64
2S storage albumins	Radish	Antifungal	64
Elicitins	<i>Phytophthora</i> spp.	Induce host defense responses	65
Ribosome-inactivating proteins	Range of plant species	Cytotoxic, antiviral, antifungal	66
Zeamatin	Maize	Antifungal, membrane permeabilizing	67

V. MANIPULATION OF PHYTOALEXINS TO INCREASE DISEASE RESISTANCE

Phytoalexins (antimicrobial secondary metabolites) are thought to contribute to the resistance of plants to disease. Phytoalexins have been identified in many different plant species and are structurally diverse, being synthesized from a wide range of precursors.⁸¹ In many cases it has been shown that they quickly accumulate to very high levels around the site of pathogen attack, but not to a high degree in the surrounding uninfected tissue.⁸²⁻⁸⁴ In some cases, before infection, significant amounts of the phytoalexin may be constitutively accumulated, but usually in special cells or organelles, or in a conjugated, inactive form.⁸⁵⁻⁸⁷ During infection, the "stored" phytoalexins are mobilized, while genes for biosynthetic pathways are induced and the synthesis of more phytoalexin begins. Little is known about the turnover or degradation of phytoalexins by the whole plant following accumulation; studies with elicited cell cultures indicate that plant peroxidases may cause degradation of phytoalexins.^{88,89} Much more significant can be the degradation of phytoalexins by plant pathogens.⁹ Successful pathogens either have very effective detoxification machinery, are not sufficiently sensitive to the phytoalexins of the host plant, or infect without inducing phytoalexin synthesis.

Given an understanding of the interaction between a host plant and a particular pathogen, several strategies can be outlined for improving plant disease resistance by modifying phytoalexin production. These strategies fall into the three general categories of (1) introducing an entirely new class of phytoalexins, (2) modifying the structure(s) of the phytoalexin of the host, and (3) altering the level and/or timing of phytoalexin synthesis. Specific examples of published, on-going, or proposed/possible manipulations are described below, followed by a potential "checklist" of concerns that should be addressed before undertaking such projects.

A. INTRODUCING NEW PHYTOALEXINS

A successful pathogen may have evolved to detoxify or avoid the natural phytoalexins of its host plant, but might be sensitive to phytoalexins from other plants. Two groups have succeeded in transferring single enzyme genes into tobacco, resulting in the production of novel secondary metabolites. First, introduction of a stilbene synthase gene from peanut into tobacco resulted in the measurable production of the peanut stilbene resveratrol.⁹⁰ Various types of stilbenes are important phytoalexins in peanut (*Arachis hypogaea*), grape (*Vitis* sp.), and conifers such as pine (*Pinus* sp.) and spruce (*Picea sitchensis*),⁹¹ but stilbenes are not normally made in tobacco. Stilbene synthase converts *p*-coumaroyl-CoA and malonyl-CoA (1:3 ratio) to a C₁₄ molecule in much the same way that chalcone synthase converts the same precursors to flavonoids (Figure 1a).⁹² Results of any pathogen challenges on these transgenic tobacco have not yet been reported.

Second, introduction of a fungal gene for a sesquiterpene cyclase, trichodiene synthase, resulted in the accumulation of low levels of trichodiene, the precursor of many fungal mycotoxins.^{93,94} Solanaceous plants do accumulate sesquiterpenoid phytoalexins, but these contain carbon skeletons unlike trichodiene.⁸¹ Sesquiterpene cyclases are found in many plants and fungi; all use farnesyl pyrophosphate (FPP) as their substrate, but fold and cyclize the molecule in a number of different ways. Further modification results in the hundreds of known sesquiterpenoids, including the phytoalexins of cotton^{81,95} and sweet potato (Figure 1b).⁹⁶ Successful production of trichodiene demonstrates that a wide variety of sesquiterpenoid skeletons may be introduced into plants, but the initial cyclization products are not as antimicrobial as the final modified phytoalexins. Geranylgeranylpyrophosphate, produced by the addition of a five carbon unit to FPP, is an intermediate in diterpene biosynthesis found in many plants. Expression of the casbene synthase gene recently cloned from castor bean⁹⁷ may likely lead to the accumulation of the diterpene phytoalexin casbene, which is directly antifungal.

Initial metabolite and enzyme accumulation was very low in both of the above examples, but this was possibly due to lack of optimization of the expression vectors used. These two cases represent rare examples where introduction of one gene can produce a relatively new molecule. To introduce other new phytoalexins could require the cloning and introduction of several genes. For example, to generate tobacco plants that could make pisatin, the first characterized phytoalexin, would require the introduction of at least nine enzymatic steps, and most of these enzymes/genes have not yet been cloned.

B. MODIFYING EXISTING PHYTOALEXINS

There is much evidence that small modifications in the structure of an existing phytoalexin might greatly alter its toxicity to pathogens and/or its rate of degradation by detoxifying enzymes. Certain pathogens

Figure 1a Conversion of coumaroyl-CoA and malonyl-CoA to a chalcone and a stilbene.

Figure 1b Conversion of farnesyl pyrophosphate to trichodiene and three sesquiterpenoid phytoalexins.

Figure 2 Differential metabolism of pterocarpan phytoalexin stereoisomers by *Nectria haematococca*.

have evolved extremely specific detoxification enzymes, and the ability to detoxify phytoalexins appears to play an important role in determining the virulence of pathogens.⁹ In one example, isolates of the red clover pathogen *Nectria haematococca* could readily degrade (-) maackiain, the pterocarpan phytoalexin isomer found in red clover, but could not degrade (+) maackiain at all (Figure 2),⁹⁸ and were much more sensitive to (+) than (-) maackiain in bioassays.⁹⁹ Many legumes contain pterocarpan phytoalexins, either (-) (alfalfa, red clover, chickpea), (+) (certain cultivars of peanut), or both isomers (*Sophora japonica*).¹⁰⁰ Van Etten and others⁹ have proposed that by moving the appropriate genes from a (+) pterocarpan-producing legume to a (-) pterocarpan-producing legume, the recipient plant could be engineered to make phytoalexins of the opposite stereochemistry.⁹ In general, pathogens with stereo-specific detoxification (such as the above mentioned *Nectria*) would be unable to degrade the "unnatural" isomer and may therefore be unable to infect. It is thought that only two enzymes control which isomer forms in the legume, isoflavone reductase and pterocarpan synthase; isoflavone reductase has been cloned from alfalfa¹⁰¹ and chickpea¹⁰² and pterocarpan synthase genes should be available in the near future.^{103,104}

Several authors have proposed structure-activity relationships based on bioassays with various plant pathogens.^{100,105} Striking increases in bioactivity were correlated with prenylation of isoflavonoids, presumably due to the increase in lipophilicity. For example, wighteone, kievitone, and phaseollidin are all much more antifungal than their unprenylated precursors (Figure 3).¹⁰⁶⁻¹⁰⁸ Moving prenyltransferases into legumes which currently accumulate unprenylated isoflavonoids may result in the production of novel antimicrobial compounds. For example, a prenyltransferase (dimethylallyl pyrophosphate: 3,9-dihydroxypterocarpan 10-dimethylallyl transferase) involved in the biosynthesis of the bean phytoalexin phaseollin has been purified from bean cell cultures and found to act also on the alfalfa phytoalexin medicarpin.¹⁰⁹ The product has not yet been identified, but expression of this enzyme in alfalfa may greatly increase the antifungal activity of the resulting phytoalexins.

Methylation of free hydroxyls has also been shown to increase the antifungal activity of isoflavonoids, again presumably by increasing lipophilicity, and may also help protect hydroxyl groups from oxidative detoxification reactions. Methyltransferases for isoflavones and pterocarpan have been partially characterized from alfalfa¹¹⁰ and pea¹¹¹ and, once cloned, may prove useful in modifying phytoalexins. The substrate specificity of such biosynthetic enzymes can be very high; the *O*-methyltransferase which carries out the final methylation to produce pisatin (pea pterocarpan phytoalexin) is totally inactive on the pterocarpan (-) medicarpin and therefore could not be used to directly methylate this alfalfa phytoalexin. In contrast, the purified alfalfa *O*-methyltransferase was active on a number of isoflavonoid substrates.

Another way in which phytoalexin modification may increase resistance is by the production of phytoalexin analogs which act as inhibitors of detoxification enzymes, even if they have no antimicrobial activity of their own. A well-characterized example of this comes from studies of β -lactam antibiotics. Clavulanic acid alone is not toxic to *Escherichia coli*, but is a powerful inhibitor of β -lactamases; the addition of a small amount of clavulanic acid to penicillin or other β -lactam antibiotics can prevent

Figure 3 Prenylation to form the phytoalexins wightone, kievitone, and phaseollidin.

resistant *E. coli* strains from degrading the antibiotic.¹¹² Initial screening for such inhibitors could be done by testing for synergistic effects between compounds in simple bioassays.

C. ALTERING THE AMOUNT OR TIMING OF PHYTOALEXIN PRODUCTION

There are correlations between disease resistance and production of phytoalexins. For example, cultivars of *Medicago sativa* (alfalfa) which produce higher levels of medicarpin were more resistant to the fungus *Verticillium*.^{113,114} Similarly, susceptible cultivars of chickpea make less medicarpin and maackiain during infection by *Ascochyta rabei* and the differences are retained in elicited cell culture systems.¹¹⁵ The lower production in the susceptible cultivar is apparently due to lower levels of isoflavone 2'-hydroxylase activity, an enzyme late in the biosynthetic pathway. Increased expression of this rate-limiting enzyme could increase the amount of phytoalexins produced.

In the soybean/*Phytophthora megasperma* f. sp. *glycinea* race-specific interaction (Section VI.A), the resistant cultivars quickly make high levels of glyceollins, while susceptible cultivars produce lower amounts much more slowly. However, when challenged with abiotic elicitors the two cultivars can produce comparable levels of phytoalexins.¹¹⁶ Thus, low phytoalexin production is due to lack of early recognition of the pathogen. Increased and earlier phytoalexin production could be obtained by introducing cloned "resistance" genes (Section VI.A) or by linking the phytoalexin genes to different promoters. Candidate promoters would be ones which are activated early in both compatible and incompatible interactions, as well as ones which could be activated by spraying inducing chemicals. Many genes thought to be involved in systemic acquired resistance (SAR) are highly induced when plants are sprayed with salicylic acid or methyl-2,6-dichloroisonicotinic acid.¹¹⁷ If the phytoalexin biosynthetic genes were put under the control of inducible SAR gene promoters, relatively nontoxic and cheap inducers could be sprayed to cause the plant to synthesize internally its own fungicides; the expression of endogenous SAR genes (mostly PR proteins; Section IV.A) would add to the defense. Alternatively, constitutive

expression of phytoalexins could theoretically be achieved by altering the expression of promoter-binding factors or other proteins involved in the defense response signal transduction pathway.¹³

D. CONCERNS REGARDING PHYTOALEXIN MANIPULATIONS

The above examples are meant to illustrate ways in which simple phytoalexin manipulations may result in increased pathogen resistance. There is a potential for high economic payoff due to reduced use of pesticides and energy and/or increased crop production. However, there are several concerns that should be addressed before such an undertaking is proposed.

1. In modifying an existing phytoalexin pathway, does the new enzyme get targeted to the correct cell compartment? Can it interact with other enzymes in the pathway? The prenyltransferases involved in phytoalexin biosynthesis in bean and soybean are associated with plastid membranes,¹¹⁸ and many of the hydroxylating enzymes have been shown to be P450s which require association with a membrane for activity.¹¹⁹ It has been proposed that several of the enzymes involved in isoflavonoid biosynthesis are loosely associated in series on a membrane surface, and that intermediates are "channeled" from enzyme to enzyme.^{120,121} Late stages in alkaloid and sorghum deoxyanthocyanidin phytoalexin biosynthesis have been shown to occur inside vesicles.^{83,122} Chalcone reductase, a key enzyme early in pterocarpin biosynthesis, requires close protein-protein interaction with chalcone synthase in order to mediate a change in the product chalcone.¹²³ Any attempts at manipulating such pathways may require the inclusion of appropriate protein leader sequences, membrane-anchoring domains, or other protein sequences required for effective integration into the pathway.

2. Are the necessary precursors present in the plant cell, at the correct time? Is the new enzyme expressed at the correct time and in the correct cell type? Since tobacco already produces sesquiterpene phytoalexins, addition of a new sesquiterpene cyclase is likely to succeed because the FPP substrate must be available. In contrast, legumes in general produce phenylpropanoid phytoalexins; there is no evidence that FPP would be present during fungal attack in sufficient quantities to produce novel phytoalexins. Similarly, use of a "constitutive" promoter such as CaMV 35S may result in poor expression of a phytoalexin-modifying enzyme. The 35S promoter is much less active in older leaves than in younger leaves and roots; the native phytoalexin gene promoters can be highly activated in leaves or roots of any age.¹²⁴ The level of phytoalexin modification may be greatly increased by the use of promoters which normally drive the phytoalexin enzyme genes of the host plant by ensuring the proper tissue specificity and correct timing/inducibility of expression.

3. Is the new phytoalexin toxic to the host plant? The native phytoalexins are often toxic to the host as well as the pathogen¹²⁵ and are therefore "contained" or expressed only in the lesion tissue, which will die anyway. The concentration of phytoalexins in lesion tissue can be very high, over 100 times that of the surrounding uninfected tissue (pea,⁸⁴ potato,⁸² sorghum⁸³). In some members of the Asteraceae, thiarubrines are accumulated constitutively in specific cells or veins;¹²⁶ these thiophenes would be very toxic to the plant if applied externally, and it is not understood how they are contained in these cells without damage to the host. Medicarpin and maackiain are accumulated as malonylated glucosides in roots;^{85,86} it is thought that the charged side chain facilitates movement to and storage in the vacuole, away from most of the metabolic activity of the cell. Sorghum produces high concentrations of deoxyanthocyanidin phytoalexins in vesicles which move toward the site of infection and later release their contents, coating the pathogen.⁸³ Accumulation of high levels of a toxic metabolite in cells of a plant species which does not have the ability to protect itself could cause more damage than the pathogen.

4. Is the new phytoalexin toxic to the symbionts of the host? Many plants have internal symbionts, including *Rhizobia*, endophytes, and mycorrhizal fungi, and there is evidence that rhizosphere bacteria such as Streptomycetes and Pseudomonads¹²⁷ may have beneficial effects on plant nutrition and defense. A new phytoalexin may decrease nitrogen fixation or otherwise be detrimental to symbionts as well as pathogens, and the effects may not be easy to assess with *in vitro* assays.

5. Does the change in phytoalexin content have an effect on food or forage quality? Many foods contain phytoalexins and other secondary metabolites which are not considered harmful. However, there are examples of plants which were bred for increased disease resistance, which was later correlated with an increase in a particular metabolite, and subsequently found to have detrimental effects on food quality. In celery, increases in the antifungal psoralens improved the harvest quality, but caused photoactivated blistering in field workers and grocery store personnel who handled the produce.¹²⁸ Subterranean clover and red clover with high coumestrol and isoflavone contents were found to perform

well as forage crops in Australia, but these compounds were later found to have estrogenic effects on sheep and cattle, causing infertility.¹²⁸

6. Does the modification have an effect on allelopathy? Medicarpin has been shown to inhibit the germination of alfalfa seeds,¹²⁹ a plant which produces medicarpin, and may play a role in the gradual thinning of alfalfa stands; increased production of medicarpin may worsen the problem. Scopoletin (a methoxycoumarin derivative) is known as a phytoalexin in several species and has been implicated in the allelopathic suppression of weeds in oats (*Avena* sp.);¹³⁰ increased production may improve both disease resistance and weed suppression.

7. Is the pathogen really more sensitive to the new phytoalexin? Does the pathogen use an “avoidance” mechanism of resistance, rather than detoxification or insensitivity?⁹ If attack by the pathogen does not trigger production of the phytoalexin by the plant, phytoalexin modification will be useless; constitutive production of the phytoalexins might be useful if it is not detrimental in other ways. Ideally, bioassays against virulent pathogens of the host plant with the proposed new phytoalexins should be carried out long before any manipulation is begun.

Currently, the major limitation to the manipulation of phytoalexins is the lack of cloned genes for secondary metabolite biosynthesis, a problem which is, however, quickly being overcome.

VI. POTENTIAL FOR MANIPULATION OF DISEASE-RESISTANCE GENES

Breeding of resistant cultivars has long been considered to be one of the most important aspects of crop improvement.^{131–134} Resistance of plant species can be classified broadly into (i) horizontal resistance, which is governed by polygenes or minor genes and (ii) vertical resistance, governed by major genes. We here only address the application of vertical resistance in engineering disease resistance of crop plants.

A. GENETICS OF HOST-PATHOGEN INTERACTIONS

In a host species, some of the cultivars are resistant while others are susceptible to a pathogen that causes a disease on that host. This resistance of the host is termed host resistance, also commonly known as cultivar or race-specific resistance. Resistance conferred by a nonhost towards a nonpathogen is known as nonhost resistance. There are obvious cytological differences between host and nonhost resistant responses of plants to fungi.¹³⁵ This distinction is less obvious in plant-bacterial interactions.¹³⁶ The genetics of host-pathogen interactions were first studied in detail by Flor¹³⁷ using flax (*Linum usitatissimum*) and flax rust (*Melampsora lini*) as a model system. From his study he concluded that “for each gene conditioning resistance in the host there is a specific gene for pathogenicity in the parasite”. This hypothesis is known as the “gene-for-gene” hypothesis,¹³⁸ and was subsequently redefined as “for each gene for resistance in the host, there is a corresponding gene for avirulence in the parasite”.¹³⁹ As the definition implies, the hypothesis deals with the cultivar or race-specific resistance of host plants. Mutational analysis indicated that the specificity of host-pathogen interactions, as dictated by this hypothesis, resides in the interaction between gene products of dominant resistance genes and avirulence genes.^{140–142} Flor¹⁴¹ reported from his mutational analysis of *M. lini* that deletion of an avirulence gene resulted in a compatible interaction with the host. In a gene-for-gene system a compatible interaction between the host and the pathogen occurs due to the absence of a correspondence between resistance gene in the host and avirulence gene in the pathogen. The compatible interaction, therefore, can occur due to either the absence of a resistance gene or the presence of a virulence gene in the absence of a correspondence between resistance gene in the host and avirulence genes in the pathogen. In recent years, a number of avirulence genes from bacterial^{143–148} as well as fungal¹⁴⁹ pathogens have been cloned.

B. GENETICS OF NONHOST RESISTANCE AND EVOLUTION OF HOST-PATHOGEN SPECIFICITY

Cloning of avirulence (*avr*) genes from bacterial pathogens has led to a greater understanding of nonhost resistance and the probable mechanisms of coevolution of plant and pathogen, in addition to confirming the gene-for-gene hypothesis. Kobayashi et al.¹⁵⁰ cloned three *avr* genes from a nonpathogenic bacterium of soybean for which there are three corresponding resistance genes in the nonhost soybean plants.^{150,151} Similarly, Whalen et al.¹⁵² also found an *avr* gene from a tomato pathogen for which there is an incompletely dominant corresponding resistance gene in beans. The *avrRpt2* locus of *Pseudomonas syringae* pv. *tomato* encodes an avirulence gene that acts in a gene-for-gene manner with both *Arabidopsis* and the nonhost soybean.¹⁵³ This suggests that soybean may have a resistance gene functionally equivalent

to that in *Arabidopsis*. Likewise, it has recently been shown that a bean pathogen carries an avirulence gene that has avirulence function not only on bean, but also on pea, and a pea pathogen carries an avirulence gene that confer specificity to interactions on both pea and beans.¹⁵⁴ Interestingly, it has recently been reported that a pathogenicity gene from *Xanthomonas citri*, when introduced into *X. phaseoli* and *X. campestris* pv. *malvacearum*, can function like an avirulence gene on both bean and cotton which are otherwise nonhost species of *X. citri*.¹⁵⁵ Dangl et al.¹⁵⁶ went one step further and showed that the avirulence gene *avrPpiA1* of the pea pathogen *P. syringae* pv. *pisi* confers avirulence against some genotypes of the nonhost *Arabidopsis* and has a very high level of identity to the avirulence gene *avrRpm1* of the *Arabidopsis* pathogen *P. syringae* pv. *maculicola*. The corresponding resistance genes in *Arabidopsis* for these two *avr* genes may be the same gene, *RPM1*, or, if not, they are very tightly linked. From these studies it appears that nonhost species carry disease-resistance genes corresponding to avirulence genes of pathogens. This opens up the possibility of utilizing nonhost disease-resistance genes in crop improvement for disease resistance.

Conventional breeding also suggests that genes available in nonhost species, or even in different genera, can be utilized for improving disease resistance in cultivated species.^{133,134,157–159} For example, there are two major genes conditioning avirulence of the nonpathogen *Erysiphe graminis* f. sp. *agropyri*, Ak-1, to wheat (*Triticum aestivum*, cultivar 'Norin 4'). Through genetic analysis, it was shown that the two corresponding resistance genes are located on chromosomes 1D and 6B of wheat. Thus it was suggested that the gene-for-gene relationship should also fit the forma specialis-genus specificity.¹⁵⁸

The rice nonpathogen *Eragrostis curvula* carries three independently segregating avirulence genes showing specificity towards different rice cultivars, presumably because of the gene-for-gene correspondence between the nonpathogen avirulence genes of *E. curvula* and nonhost disease-resistance genes in rice. In addition to major avirulence genes from the nonpathogen there are also minor genes that are responsible for determining disease symptoms on rice. Because of these minor genes, it was not possible to detect the segregation of avirulence genes from *E. curvula* until the fourth and fifth generations of backcrossing to the recurrent parent, the rice pathogen *Magnaporthe grisea*.¹⁶⁰ This is a good example of the role of minor genes from a nonpathogen in plant-pathogen interactions. A similar situation can be seen for the nonhost resistance genes of the plant. Thus, in addition to the existence of nonpathogen-specific resistance genes as suggested by Tosa,¹⁵⁸ there may also be a series of minor resistance genes in the nonhost conferring resistance to nonpathogens. This, however, requires experimental confirmation.

It has been suggested that there may be a gene-for-gene basis for the polygenes of the host and pathogen involved in horizontal resistance.¹⁶¹ This assumes a much greater stability of horizontal resistance than that proposed in the addition model of Van der Plank.¹⁶² Polygenes or minor genes encoding horizontal resistance could be a potentially powerful defense against nonpathogens, since in plant-pathogen coevolution the nonpathogens may not have lost most of the minor genes that encode avirulence functions on the nonhost. Therefore, major genes and minor genes could together create a potentially stable resistance to nonpathogens. Transfer of major disease-resistance genes from putative nonhost species by conventional breeding, and the presence of major disease-resistance genes in the nonhost for a nonpathogen, suggest that disease-resistance genes may be spread horizontally to related genera or species during the course of evolution. Similarly, avirulence genes may have been distributed to related forma speciales or pathovars of a pathogen species during their evolutionary process. Elimination or loss of the corresponding disease-resistance and avirulence genes of both major and minor gene categories through mutation during evolutionary processes may have resulted in different host and pathogen combinations.

In the coevolution of host and pathogen the selection pressure in the host is for gaining resistance genes, while in the pathogen for losing avirulence genes. There is evidence that a host carries few resistance genes while a pathogen carries many virulence genes.¹⁶³ Therefore, in engineering high-yielding cultivars with a durable resistance, one has to consider the frequent occurrences of new virulent races which presumably occur due to mutation of the existing avirulence genes. Use of multiline cultivars carrying different resistance genes is a possibility for obtaining durable resistance in such a situation.^{164,165}

C. ENGINEERING DISEASE-RESISTANCE GENES FOR CROP IMPROVEMENT

Attempts to isolate disease-resistance genes have gained momentum in the past several years, primarily because of the development of map-based cloning and gene tagging strategies (see Chapter 16) and rapid progress in cloning these genes for different host-pathogen interactions has been observed during

the last year. Isolation of these genes will enable us to learn more about the mechanisms of host-pathogen interactions and also to design strategies for improving crop resistance.

The *HMI* gene from maize which confers resistance to *C. carbonum* Nelson race 1 has recently been cloned.⁷³ In this plant-pathogen interaction, however, virulence is dominant over avirulence, a major difference from those host-pathogen interactions that follow Flor's gene-for-gene hypothesis. As described earlier, the *HMI* locus encodes an NADPH-dependent HC-toxin reductase that inactivates the HC-toxin of *C. carbonum* race 1. Apart from its role in improving resistance of corn to *C. carbonum*, the potential of this resistance gene could be explored for improving resistance of other crop species whose pathogens produce a structurally similar toxin. Cloning of the *HMI* locus also opens up the possibility of searching for putative toxin-detoxifying enzymes from other host species.

Dangl and co-workers¹⁵⁶ have isolated a yeast artificial chromosome (YAC) clone of *Arabidopsis* apparently carrying the *RPM1* gene that confers resistance to *P. syringae* pv. *maculicola* isolates.¹⁶⁶ Similarly, a YAC clone of tomato apparently carrying the *Pto* gene that confers resistance to *P. syringae* pv. *tomato* has been isolated.¹⁶⁶ These may be the first examples of dominant major resistance gene to be characterized at the molecular level.

The two-component sensor system proposed by de Wit¹⁶⁷ is an interesting strategy for engineering of disease resistance utilizing a cloned avirulence gene. The suggested strategy was to create a transgenic tomato line carrying both the *Cf9* resistance gene and the corresponding avirulence gene *avr9* of *Cladosporium fulvum* under a pathogen-inducible promoter. The rationale behind this strategy is that once the *avr9* gene is induced, the product of this gene should recognize the product of the *Cf9* gene and cause hypersensitive cell death, and thereby a resistant response to all virulent races of the pathogen. Timely expression and correct targeting of the gene product of *avr9* are two crucial requirements for this strategy. If it works, the resistance obtained may be durable because there will be a selection pressure against *avr9* mutation. The same strategy could in principle be applicable to other crop species in which the corresponding avirulence genes of the pathogen have been cloned.

As the functions of most avirulence genes and all dominant resistance genes that exhibit gene-for-gene correspondence are currently unknown, prediction of optimal strategies for improvement of crop plants by manipulation of disease-resistance genes is not easy. However, plant transformation protocols override the problem of interspecific or intergeneric incompatibility; thus, nonhost disease-resistance genes, once isolated, can be easily transferred to desired host species. In addition, isolation of a series of disease-resistance genes for a particular pathogen would facilitate the construction of multigenic isogenic lines rather than multigenic near isogenic lines in a short period of time. A series of multigenic isogenic lines each of which carries different combinations of resistance genes produced through plant transformation would be an invaluable genetic resource from which to construct desirable multiline cultivars (by mixing isolines) with durable disease resistance.

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Since the time of writing this article, five disease resistance genes have been cloned and characterized at the molecular level. They are from tomato (Martin et al., *Science*, 262, 1432, 1993; Jones et al., *Science*, 266, 789, 1994), *Arabidopsis* (Bent et al., *Science*, 265, 1856, 1994; Mindrinos et al., *Cell*, 78, 1089, 1994), tobacco (Whitham et al., *Cell*, 78, 1101, 1994), and flax (Moffat, *Science*, 265, 1804, 1994).

Also, the first example of increased disease resistance in transgenic plants due to the accumulation of an engineered phytoalexin was published. Tobacco plants producing the stilbene resveratrol due to the introduction of the stilbene synthase genes from grapevine were more resistant to a tobacco fungal pathogen (R. Hain et al., *Nature*, 361, 153, 1993).

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