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MOLECULAR BASIS OF RECOGNITION BETWEEN *PHYTOPHTHORA* PATHOGENS AND THEIR HOSTS

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■ **Abstract** Recognition is the earliest step in any direct plant-microbe interaction. Recognition between *Phytophthora* pathogens, which are oomycetes, phylogenetically distinct from fungi, has been studied at two levels. Recognition of the host by the pathogen has focused on recognition of chemical, electrical, and physical features of plant roots by zoospores. Both host-specific factors such as isoflavones, and host-nonspecific factors such as amino acids, calcium, and electrical fields, influence zoospore taxis, encystment, cyst germination, and hyphal chemotropism in guiding the pathogen to potential infection sites. Recognition of the pathogen by the host defense machinery has been analyzed using biochemical and genetic approaches. Biochemical approaches have identified chemical elicitors of host defense responses, and in some cases, their cognate receptors from the host. Some elicitors, such as glucans and fatty acids, have broad host ranges, whereas others such as elicitins have narrow host ranges. Most elicitors identified appear to contribute primarily to basic or nonhost resistance. Genetic analysis has identified host resistance (R) genes and pathogen avirulence (Avr) genes that interact in a gene-for-gene manner. One *Phytophthora* Avr gene, *Avr1b* from *P. sojae*, has been cloned and characterized. It encodes a secreted elicitor that triggers a system-wide defense response in soybean plants carrying the cognate R gene, *Rps1b*.

INTRODUCTION

Recognition is the earliest step in any direct plant-microbe interaction. At the whole-organism level, we may infer recognition by the response of one organism to a substance produced by another organism, i.e., a signal, in the broadest sense of the term. At the molecular level, we may define recognition as including interaction of the substance with a receptor of some kind and transduction of the signal generated by the receptor within and outside the recipient cell; these are distinct from the responses of the cell or tissue triggered by recognition.

The term recognition could imply that the role of the recognizing or responding organism is an active one, while the role of the recognized organism is passive. However, for the purpose of this review we use a broader definition of recognition that is agnostic of activity or passivity, since such definition of roles is usually a

matter of perspective. For example, is a nitrogen-fixing *Rhizobium meliloti* bacterium actively recognizing a flavone molecule that happens to be released by alfalfa roots, or is it passively responding to a stimulant actively released by alfalfa roots to promote colonization by the bacteria? An agnostic definition of recognition therefore encompasses the intricate series of signal-response exchanges that is required for development of highly evolved interactions including mutualistic associations and infection by biotrophic pathogens. It also includes, for example, the series of events by which a necrotrophic pathogen detects the presence of plant host tissue, adjusts its physiology to the particular species and particular tissue encountered, adjusts further to counter whatever defense responses the plant may present, and finally adjusts to the range of nutrients created by the destruction of the host tissue.

Phytophthora and related phytopathogens such as *Pythium* species, downy mildews, and white rusts are oomycetes, a diverse group of heterotrophic organisms that morphologically and physiologically resemble fungi, but are phylogenetically distant from them. Oomycetes fall within the Stramenopiles, one of the major radiations of crown eukaryotes that are distinct from plants, animals, and fungi (50). This group also includes diatoms, brown algae such as kelp, and golden-brown algae.

The approximately 60 species of *Phytophthora* are all destructive pathogens, causing rots of roots, crowns, stems, leaves, and fruits of a huge range of agriculturally and ornamentally important plants. Some species such as *P. cinnamomi*, *P. parasitica* (syn. *P. nicotianae*), and *P. cactorum* each attack hundreds of different plant host species. Others, such as *P. sojae* (syn, *Phytophthora megasperma* f.sp. *glycinea*) and *P. infestans*, have narrow host ranges, infecting just a few host plant species. The economic damage overall to crops in the United States by *Phytophthora* species is estimated in the tens of billions of dollars, including the costs of control measures, and worldwide it is many times this figure (47). Late blight of potato caused by *P. infestans* resulted in the Irish potato famine in the nineteenth century and continues to be a difficult and worsening problem for potato and tomato growers worldwide. *P. sojae* is an ongoing problem in soybean-growing areas around the world, particularly the United States.

Phytophthora species grow primarily as coenocytic hyphae, with no septa (46). They are heterotrophic and saprophytic, and grow readily in culture. Three kinds of asexual spores are commonly produced, sporangia, zoospores, and chlamydospores. In some species such as *P. infestans*, sporangia are released freely from aerial hyphae and serve as agents of dispersal, often by the wind or by arthropod vectors. Sporangia can germinate directly to produce hyphae, or else can differentiate to produce 10–30 zoospores. Zoospores are aquatic, lack a cell wall, and have two flagella for swimming. Zoospore production is typically triggered by flooding. Zoospores are generally short-lived (hours) and quickly differentiate to form adhesive cysts, which in turn germinate to produce hyphae. Zoospores are the most important route of infection of roots, especially when the soil is flooded. The zoospores encyst on the root surface from where the hyphae penetrate the root

directly from the cyst. Zoospores, as well as sporangia, can also be spread to the upper plant by splashing.

Research into recognition between *Phytophthora* species and their hosts currently is separated into recognition of the host by the pathogen and recognition of the pathogen by the host's defense machinery. There is as yet little research into complex cascades of signal exchange occurring throughout the infection process. Recognition of the host by the pathogen includes detection by the pathogen of chemical, electrical, and physical properties of host tissue, including chemicals diffusing from the plant tissue surface. Research in this area has concentrated substantially on tactic responses by the motile zoospores. Recognition of the pathogen by the host has focused primarily on identifying and isolating pathogen substances that directly trigger a defense response in the host, i.e., elicitors, and on genetically identifying pathogen genes responsible for the production of such substances, i.e., avirulence genes.

RECOGNITION OF THE HOST BY *PHYTOPHTHORA* PATHOGENS

Motile zoospores are an important means of initiating infection by *Phytophthora* (20). Zoospores do not divide, however, but differentiate into adhesive cysts (a process called encystment), which in turn germinate to produce hyphae that are actually responsible for invading the host tissue (20, 34). Accordingly, taxis of the zoospores toward host tissue, encystment, germination of the cysts, and tropism of the hyphae toward host tissue all represent behaviors that can be affected by plant signals, and thus are a point at which recognition of the host may be effected.

Zoospore Chemotaxis

Phytophthora zoospores swim along a helical path at speeds of 100–200 μm/sec depending on temperature and species (20). However, since zoospores turn frequently, their linear progress is typically much less. Over a period of hours *P. cryptogea* zoospores swam 2.5–3.5 cm over flooded soil, while *P. cinnamomi* zoospores swam up to 6 cm through coarse flooded soils (20), though the fraction reaching this distance prior to encystment was very small (0.1–0.2%), comparable to the number expected for diffusion of a small molecule. Under conditions of low nutrient and low Ca^{2+} concentrations, encysted zoospores often differentiate a sporangium, which releases a single new zoospore, called a secondary zoospore. This process can be repeated a number of times, with the result that a single motile zoospore can progress a substantial distance by means of these repeated incarnations. The motility of zoospores also greatly increases the distance that they can be carried by a flow of water through a particulate matrix such as soil (20). Zoospores of most *Phytophthora* and *Pythium* species show a relatively nonspecific attraction to amino acids, particularly aspartate, glutamate, asparagine, glutamine, arginine,

and methionine (20, 34). Most are also attracted by 0.2–20 mM ethanol, which may promote infection of flooded roots (20, 104).

Attraction to these compounds may account for the nonspecific attraction of many *Phytophthora* and *Pythium* zoospores to root exudates of many plants. However, some species of *Phytophthora* and *Pythium*, especially those with restricted host ranges, appear to exhibit more specificity in their attraction toward root exudates. For example, Mitchell & Deacon (98) showed that zoospores from *Py. graminicola* and *Py. arrhenomanes*, which characteristically infect graminaceous hosts, preferentially accumulated behind root tips of grasses compared to dicots, whereas zoospores of the broad host range species *Py. aphanidermatum* and *Py. ultimum* did not show preference for grass roots. Similarly, zoospores of *Py. dissotocum* (a cotton pathogen) were attracted to cotton roots but zoospores of *Py. catenulatum* (not a cotton pathogen) were not. P. F. Morris (personal communication) showed that zoospores of *P. sojae* were attracted only to roots and root exudates of legumes but not to those of non-legumes, and of these, strong attraction was shown only to exudates of soybean and chickpea; these exudates showed attraction even at 500-fold dilution, whereas attraction to exudates of other legumes occurred at dilutions of 1 to 100.

The question of specificity in chemotaxis is an important one as it relates to the contribution of chemotaxis, and subsequent steps of infection by zoospores, to host selection and host specificity. There are several examples in which specific attraction of oomycete zoospores to plant signals has been characterized. For example, isovaleraldehyde, valeraldehyde, and ante-isovaleraldehyde are chemoattractants down to concentrations of 1 μM for *P. palmivora* zoospores (19). Prunetin (4′,5-dihydroxy-7-methoxy-isoflavone) and related compounds are potent attractants (down to 10 nM) for zoospores of *Aphanomyces euteiches* (68, 137, 138), while the zoospores of *A. cochlioides* are attracted to cochliophilin A (5-hydroxy-6,7-methylenedioxy-flavone) from the roots of its host, spinach, at concentrations down to 1 nM (68, 137, 138).

By far the best-characterized example of specificity is the attraction of *P. sojae* zoospores to the isoflavones daidzein and genistein (Figure 1), which are present in soybean seeds and exuded by the roots (104, 149). *P. sojae* zoospores are attracted to concentrations of these compounds down to 0.1 nM, but the zoospores from six other species of *Phytophthora* and one species of *Pythium* were not attracted even at 30 μM (104). Therefore Morris & Ward (104) suggested that the sensitive attraction of *P. sojae* zoospores to soybean isoflavones may account for the specificity of their attraction to soybean roots. The specificity of *P. sojae* zoospores for isoflavones has been defined using a wide variety of compounds with various levels of structural similarity to isoflavones (149). Phenolic hydroxyl groups corresponding to the 7′ and 4′ positions on isoflavones were the most important determinant of attractiveness. For example, 4,4′ dihydroxy stilbene (Figure 1) was an excellent attractant. In contrast, a wide variety of flavones showed no attraction, and methylation of the 4′ hydroxyl, commonly found on isoflavones released by most legumes other than soybean, reduced attractiveness 30-fold (149). An

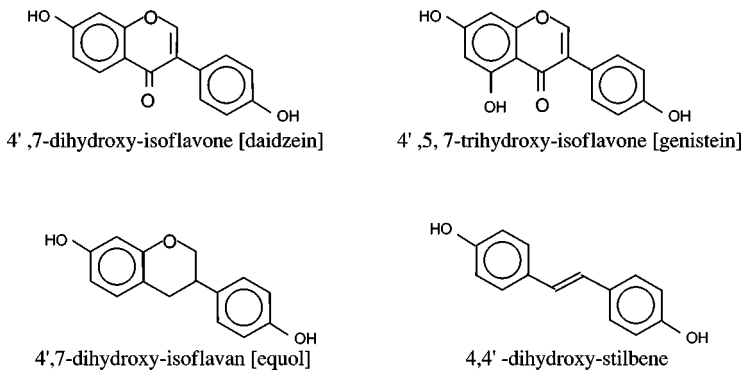

Figure 1 Isoflavone attractants of *Phytophthora sojae* zoospores, and active analogs.

important finding of this study was that *P. sojae* zoospores could respond to a very wide variety of phenolic compounds, albeit at significantly higher concentrations (1 μM) than for the isoflavones (149). Furthermore, in some cases the response observed was repulsion rather than attraction. These observations raise the possibility that *P. sojae* zoospores can integrate a large amount of information about their chemical environment, over and above their attraction to isoflavones. Substantial levels of genetic variation were found in the attraction of zoospores of different *P. sojae* genotypes to isoflavones (149). For example, an isolate of genotype IV could be attracted by 0.25 nM genistein, whereas an isolate of genotype I required 10 nM for attraction. Genetic crosses between the isolates showed that a single gene was responsible for the difference in attraction to genistein and other isoflavones. However, the genetic differences in the responses to non-isoflavone phenolics were determined by at least six additional independently segregating genes (156), supporting the notion that *P. sojae* has an extensive array of receptors capable of sensing the phenolic environment of the zoospores. Detailed mapping of these genes may provide a route to cloning the *P. sojae* receptors responsible for detecting isoflavones and other phenolic compounds.

Zoospores of many oomycetes, including *Phytophthora* and *Pythium*, display species-specific chemotaxis toward previously encysted zoospores, resulting in large clumps of encysted zoospores, especially at high zoospore densities (34, 128). This phenomenon may increase the likelihood of infection as a large population of cysts collects at a single site of potential infection. The substance(s) responsible for auto-attraction are not known, though one candidate is calcium, which is released by encysting zoospores (70, 128).

Electrotaxis

In addition to integrating information about chemical concentrations, zoospores may also integrate information about their electrical environment. Plant roots

develop weak electric fields, especially at the tips, at wounds, and branch points (102). In vitro, zoospores of *Py. aphanidermatum* and *Phytophthora palmivora* exhibit electrotaxis at electrical field strengths comparable to those generated by plant roots (c. 0.5 V/m) (102). Electrotaxis of zoospores of *Py. aphanidermatum* was toward the cathode, whereas *P. palmivora* zoosproes swam toward the anode. The specificity of the electrotaxis correlated with the charge observed on the anterior and posterior flagella. The surface charge of the posterior flagella of anodotactic *P. palmivora* zoospores was positive, while the posterior flagella of cathodotactic *Py. aphanidermatum* zoospores were negative (102). In *P. palmivora*, swimming velocity and especially turning frequency were increased in an electric field. It was suggested that electrotaxis might enable zoospores to distinguish living roots from dead ones, or contribute to selection of an infection site on the root surface (102).

Docking Behavior

When zoospores encyst on root surfaces, they almost invariably do so with the ventral side (bearing the flagella) against the root surface [reviewed in (34)]. The hypha of the germinating cyst emerges from the site from which the flagella were resorbed during encystment. Therefore when the zoospore encysts in the manner described above, the hyphae will penetrate the root surface directly. In addition to swimming behavior that enables zoospores to reach the root surface, there must be an additional program of behavior to enable the zoospores to detect the close proximity of the root surface and orient themselves accordingly in preparation for encystment. Hardham & Gubler (67) found that *P. cinnamomi* zoospores swam along the root surface with the ventral side down prior to encystment. More commonly, however, rapid circular swimming is observed prior to encystment. Circular swimming and encystment can be triggered in vitro by high concentrations of chemoattractants, calcium, or electric fields (38, 102). Some combination of these factors, all of which occur close to the root surface, could thus be responsible for signaling the proximity of the root surface to the approaching zoospore and triggering the switch from seeking behavior to docking behavior.

Encystment and Germination

The transition from swimming zoospore to growing hypha capable of invading host tissue requires two rapid developmental steps—encystment and cyst germination. These two steps are potential control points in determining the likelihood of attempted infection of a given host by a zoospore of a particular species. Zoospore encystment involves resorption or shedding of the flagella, rounding up and rapid extrusion of a temporary cell wall, and is complete in around 10 minutes (20). The cyst is initially quite adhesive, enabling it to attach firmly to a root surface or other physical substrate. As the cyst matures it loses its adhesiveness, allowing it to be transported by water currents.

Encystment is triggered by a very wide range of factors including agitation, crowding or physical obstruction, or the presence of calcium or nutrients

[reviewed extensively in (34)]. Physical obstruction and the presence of calcium and nutrients are of course diagnostic of the presence of a plant root. One remaining question is whether there is any degree of host selection at this step. *P. sojae* zoospores can be stimulated to encyst by high concentrations (1 μm) of soybean isoflavones (104, 164), suggesting that the presence of a soybean root may be more likely to trigger encystment of a *P. sojae* zoospore than the root of a nonhost plant. Donaldson & Deacon (39) found some specificity in the ability of particular polysaccharides to stimulate encystment by zoospores of *Py. aphanidermatum* and *Py. dissotocum*, suggesting the different components of root slime may contribute to specificity.

Germination of cysts to form hyphae also is stimulated by nutrients and calcium, typical of the rhizosphere [reviewed extensively in (34)]. Fewer cysts germinate in distilled water and those that do are more likely to produce a secondary zoospore. Again the question is whether germination is a control point that contributes to host selection. In *P. sojae*, germination of cysts is stimulated by isoflavones (103, 104). The percentage of cysts germinating can be very high, but the percentage of germinated cysts that can expand into macroscopic colonies can be very much lower. In *P. infestans*, for example, as few as 0.1% of germinated zoospores may produce a colony, even on an optimal medium (27). This point is overlooked by many authors who report only germination rates. It suggests that in some species such as *P. infestans*, there may be an additional developmental step required for transition from a cyst germling to a continuously growing mycelium and that this transition may require specific chemical or even physical signals, perhaps from the plant.

Chemotropism

Zoospores may encyst and germinate some distance from the root surface. In this context, the ability of the hyphae to grow chemotropically toward the root is very important. Hyphae of *P. palmivora* and *P. cinnamomi* have been reported to grow chemotropically to host extracts or exudates (20) but the attractive signal was not identified in these cases. There have been some reports of chemotropism toward amino acids (20). Detailed studies with the nonpathogenic oomycete *Achlya bisexualis* reveal that chemotropism toward amino acids is very complex, generally requiring the presence of mixtures (106). If the same is true of pathogenic oomycetes there may be some basis for specificity in the attraction to specific amino acid mixtures. *P. sojae* hyphae exhibit chemotropism toward soybean roots (103), and this could be accounted for by the attraction of the hyphae to pure isoflavones (103). In this case, isoflavones appear to have the potential to guide *P. sojae* hyphae specifically towards roots of their host, soybean.

Thigmotropism

Infection of a root or leaf involves intimate contact between the pathogen and the surface of the host tissue. Specific responses of the pathogen to features of the tissue surface have been well documented in fungi (57, 58, 127), especially rust fungi (2).

Phytophthora sojae hyphae preferentially penetrate the root surface at the base of a depression that corresponds to the junction between the anticlinal walls of the epidermal cells (45). One possibility is that this site is preferred because of a locally higher concentration of chemical attractants. However, the primary signal appears to be thigmotropic (103). *P. sojae* hyphae emerging from cysts on the surface of a porous PET membrane grew tightly appressed to the membrane even when bathed in a solution of isoflavones. When the hyphae encountered a pore, they invariably grew through the pore, even against a gradient of isoflavones. However, once a hypha had passed through a pore, it grew away from the surface and into the medium on the other side of the membrane, indicating that a developmental switch had occurred as a result of passing through the pore (103). This switch occurred even in the complete absence of isoflavones. The presence of isoflavones diffusing through the pores did not appear to increase the frequency with which hyphae detected pores. Therefore, on the PET membrane surface, the dominating signal appeared to be thigmotropic. The behavior triggered by the membrane appeared consistent with the physical environment of the root surface in which *P. sojae* hyphae seek the depression characteristic of the presence of an epidermal cell junction.

Summary

In summary, each of the developmental stages by which infection by zoospores proceeds, zoospore taxis, encystment, germination, and hyphal tropism, provides opportunities for specific recognition of a potential host by *Phytophthora* pathogens, through specific combinations of signals. The best evidence for specific host recognition at this stage of infection comes from *P. sojae*, in which host isoflavones specifically stimulate all four infection stages. Many questions remain, however. For example, how many other *Phytophthora* species exhibit responses to specific host signals? Is isoflavone recognition required for infection by *P. sojae*, or does it simply increase the chance that infection will be attempted; this problem could be addressed if isoflavone-insensitive mutants of *P. sojae* could be obtained. Another important question relates to the true sphere of influence exerted by specific chemicals released by plant roots. Can compounds such as isoflavones attract zoospores or hyphae from distances of centimeters, or do the combined effects of diffusion, water movement, adsorption to soil particles, and microbial degradation reduce the effective range of the chemicals to only a few millimeters? Do zoospores primarily respond to individual compounds such as isoflavones, or does recognition involve integrating a large amount of information about the chemical environment including phenolic compounds, amino acids, sugars, calcium, polysaccharides, electric fields, pH gradients, etc? How important are the contributions of other rhizosphere microorganisms to the chemical environment? For example, an isoflavan, equol (Figure 1), is 10–100 times more attractive to *P. sojae* zoospores than the native soybean isoflavones daidzein and genistein (149). Equol is a product of anaerobic metabolism of isoflavones by bacteria (23). Does this mean that in anoxic flooded soils, or perhaps in microaerophilic nitrogen-fixing

root nodules, genistein and daidzein are converted to equol, resulting in greatly magnified attraction to *P. sojae* zoospores? Finally, biochemical and genetic characterization of chemotaxis receptors would greatly expand our knowledge of the contribution of recognition processes to pathogenicity by *Phytophthora* species, and could perhaps enable novel control measures targeted against the earliest steps in infection. We currently have absolutely no information about the nature of *Phytophthora* chemotaxis receptors or other receptors for plant signals. One possible class of candidate receptors for chemotaxis are seven-transmembrane-spanning-domain receptors, which ubiquitously mediate detection of environmental signals in other eukaryotes, and include the chemotaxis receptors of slime molds and of cells of the mammalian immune system (22). On the other hand, histidine kinase two-component systems are responsible for chemotaxis reception in bacteria (1) and mediate the detection of soybean isoflavones by the nitrogen-fixing bacterium *Bradyrhizobium japonicum* (90). Two-component systems are also found in eukaryotes (1); although they normally seem to mediate osmotic sensing, could they also be recruited as chemotaxis receptors in oomycetes?

RECOGNITION OF *PHYTOPHTHORA* PATHOGENS BY THE HOST

Inducible plant defense responses require detection of invading pathogens. A very large body of research has been gathered over the past 20 years characterizing those responses [e.g., reviewed in (32)], and to some extent characterizing the pathogen signals responsible for triggering those responses [e.g., reviewed in (62, 111). In *Phytophthora* pathosystems, as in other pathosystems, both biochemical and genetic approaches to identifying pathogen signals have been undertaken. Biochemical approaches have identified specific pathogen-derived compounds, elicitors, that have the ability to trigger defense responses in host and nonhost species. In some cases, biochemical approaches have also identified the receptors of the elicitors or their components. Genetic approaches have identified "avirulence" genes in the pathogen that interact in a gene-for-gene manner with host resistance genes with a specificity that suggests recognition of a specific pathogen molecule by the plant. Key questions in all cases are: What are the actual contributions, if any, of biochemically identified elicitors to host or nonhost resistance, and what are the functions of avirulence genes in pathogens?

Elicitors in *Phytophthora* Infection

A wide variety of *Phytophthora*-derived elicitors have been identified, including carbohydrates, proteins, and small molecules (62). With few exceptions, these molecules are found in all isolates of the relevant species, and in some cases are found in all *Phytophthora* species, and they trigger a defense response on both susceptible and resistant varieties of the host plants. As a result, the biological relevance of these compounds to plant-microbe interactions has sometimes been

questioned. However, even plant varieties that appear susceptible in greenhouse or field tests are not irredeemably susceptible. Under less favorable infection conditions (temperature, humidity, reduced inoculum density, etc.), large numbers of "susceptible" plants will survive infection. It seems plausible therefore that some of the elicitor responses observed contribute to this base level of resistance. Elicitor responses may also be viewed from the perspective of nonhost resistance. Because most plants are resistant to most pathogens, some or all of the elicitor responses observed may contribute to this basic level resistance to the plant. Ultimately, the contributions of these elicitor responses can be evaluated once the genes encoding corresponding plant receptors are cloned and expression of those receptors eliminated.

CELL WALL FRAGMENTS Cell wall preparations of varying degrees of complexity have been reported to be effective elicitors of defense responses in many *Phytophthora* host interactions including soybean with *P. sojae* (86), potato with *P. infestans* (121), tobacco with *P. parasitica* (13), pepper with *P. capsici* (105), rose with *Phytophthora cinnamomi* or *Phytophthora megasperma* (10), and *Cinchona robusta* with *P. cinnamomi* (125). However, only in the case of *P. sojae* (see below) and *P. infestans* (4, 12, 121) have the active components been identified as cell wall glucans. In other cases, the active component has been identified as a cell wall protein (see below).

The interaction of *P. sojae* cell wall β-glucans with soybean has been extensively characterized [reviewed in (42, 62)]. The minimum elicitor unit is a branched (1,3–1,6) hepta-β-glucoside (Figure 2). The heptaglucan has been purified to homogeneity and synthesized chemically, and the structural requirements for its activity (Figure 2) have been detailed using a large variety of modified forms of the heptaglucan (24). In vivo, glucans with elicitor activity are released by germinating cysts of *P. sojae* (154) and also by the action of soybean β-1,3 glucanases on mature *P. sojae* cell walls (63, 64). Since these soybean glucanases are induced during infection, it has been suggested that the release of elicitor-active glucan fragments during infection may contribute to host defense (63, 64). It has been further proposed that *P. sojae* secretes glucanase inhibitors in order to block release of the elicitor-active glucans (65). In addition to soybean, the following nonhosts of *P. sojae* have been reported to respond to purified *P. sojae* cell wall glucans: chickpea, broad bean (*Vicia faba*), French bean (*Phaseolus vulgaris*), pea, white lupin (*Lupinus albus*), *Lotus japonicus*, alfalfa, *Medicago truncatula*, *Lycopersicon peruvianum*, and sunflower (29, 31, 140, 143).

A hepta glucan binding activity has been identified in soybean plasma membrane fractions. Evidence that this is the functional receptor for the hepta glucan elicitor comes from an elegant correlation of the binding constants of a series of hepta glucan analogs with the elicitor activity of the analogs (24, 24a). The binding activity has been solubilized and extensively purified by affinity chromatography (30, 100). It consists of a 240-kDa multiple subunit complex, and the glucan binding subunits have been identified by using radiolabeled glucan. A cDNA clone

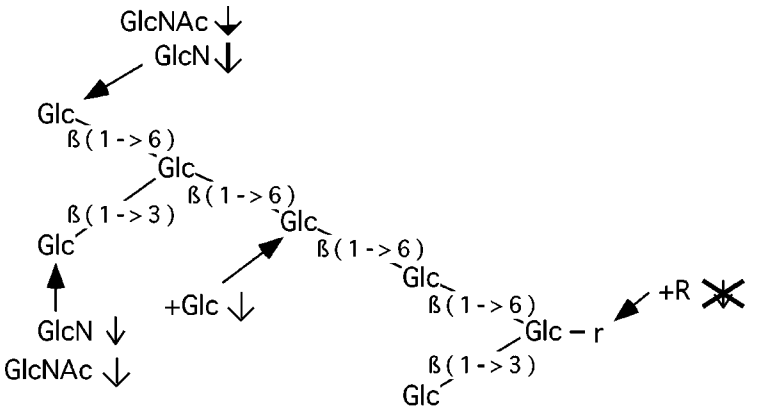

Figure 2 Structure-function determinants of the hepta glucan elicitor from *Phytophtora sojae* cell walls. Glc = glucose. GlcN = substitution of glucosamine. GlcNAc = substitution of N-acetyl-glucosamine. +Glc = addition of glucose. +R = addition of any derivative. −r = reducing end. The width of the downward arrows indicates the reduction in elicitor activity on soybean and soybean membrane receptor binding caused by the modification, from 10-fold to 100,000-fold. The crossed arrow indicates a negligible reduction.

for the 75-kDa subunit has been cloned (74, 99, 150). Expression of the cDNA in *Escherichia coli* cells results in a protein with glucan binding activity. Somewhat surprisingly, the protein is hydrophilic, has no obvious transmembrane domain, and has no secretory leader sequence. Nevertheless, expression of the cDNA in tobacco (74) or tomato (99) cells results in reconstitution of the binding activity in membrane fractions with an affinity of 4.5 nM, comparable to that in soybean (1–3 nM). In addition, antibodies directed against the *E. coli* synthesized protein inhibited hepta glucan induction of phytoalexins in soybean cells (74, 150). Presumably, the 75-kDa subunit is posttranslationally modified or interacts with other proteins in order to be localized to the plasma membrane. Expression of the 75-kDa protein in tomato did not confer a hepta glucan response on the tomato cells (99). In contrast, Kakitani et al. (74) report that tobacco suspension culture cells transformed with the cDNA exhibit a calcium influx response to glucans characteristic of the soybean response, whereas leaves from tobacco plants transformed with the cDNA produce phytoalexin in response to glucans. The tobacco plants expressing the cDNA showed increased resistance against *Rhizoctonia solani* and especially against *Phytophthora parasitica* (74). These results suggest that the cloned 75-kDa subunit is a key component of the glucan receptor from soybean and that the receptor has the ability to mediate resistance against *Phytophthora* infection.

ELICITINS Elicitins are conserved 98-amino acid sterol-binding proteins secreted in culture by all *Phytophthora* species tested and by some *Pythium* species.

Elicitins trigger a wide range of defense responses in most *Nicotiana* species tested [reviewed extensively in (78, 120, 129, 133)], and in some cultivars of turnip and radish (84). This defense response is sufficient to protect against infection not only by *Phytophthora* (84, 130), but also by bacteria (84), fungi (87), and viruses (40, 76, 129). Isolates of *P. parasitica* that are most virulent on tobacco do not produce elicitins (130a); transcription of their elicitin genes is turned off (79). Other tobacco pathogenic isolates that produce elicitins in culture downregulate elicitin expression following infection and produce milder symptoms (28, 83). On the basis of these observations, elicitins have been proposed to be a major determinant of the nonhost resistance of *Nicotiana* species against *Phytophthora* infection (83, 130). The most direct support for this hypothesis comes from silencing of the elicitin gene *Inf1* in *P. infestans*, which resulted in *P. infestans* gaining the ability to infect *Nicotiana benthamiana*, though not *N. tabacum* (82).

Elicitin proteins have been sequenced from many species [reviewed in (120)], and elicitin genes have been cloned from *P. parasitica* (40, 79), *P. sojae* (7, 93, 124), *P. infestans* (77, 80), *P. cryptogea* (117), and *P. cinnamomi* (41). These sequences are highly similar and define the canonical or class I elicitins. In most of these species the genes are present in large gene families. Extensive sequencing of cDNA clones (expressed sequence tags, ESTs) in *P. infestans* (77) and *P. sojae* (124) have indicated that, in addition to the canonical elicitins described above, these species express a diverse superfamily of elicitin-like proteins. Genes encoding divergent elicitins have also been cloned from *P. parasitica* (40), *P. cryptogea* (117), and *P. cinnamomi* (41). Some members of the superfamily are secreted, whereas others have short N-terminal extensions and/or serine- and threonine-rich cell wall anchor sequences attached to their C terminus (40, 81). Some appear to have phospholipase activity (109).

The role of elicitins in the biology of *Phytophthora* species is not yet clear. Since canonical elicitins are sterol carrier proteins (9, 97), and since *Phytophthora* species do not synthesize their own sterols, one likely role is that they are sterol scavengers. Elicitins are small enough to pass freely across the cell wall and so could readily transport captured sterols to the plasma membrane. Strains of *P. parasitica* and *P. infestans* that no longer express canonical elicitins in culture are apparently normal. However, it has been shown that in the case of *P. infestans*, these strains express high levels of other members of the elicitin superfamily (77, 81). Kamoun et al. (79) identified a strain of *P. cryptogea* in which all elicitin genes that could be detected by hybridization to a canonical elicitin gene probe were deleted. This strain was completely nonpathogenic, sexually sterile, and failed to produce vegetative zoospores. However, it was not determined that the phenotype was due to the loss of the elicitin genes. Probably the other members of the elicitin superfamily also bind lipids, but it is not clear if their role differs from that of the canonical elicitins. Some evidence suggests that lipid binding may be involved in the ability of elicitins to trigger defense responses in *Nicotiana* species (116).

It is not clear whether elicitins contribute directly to the pathogenicity of *Phytophthora* species. Elicitins have the ability to spread systemically throughout the

plant (36). In the case of one subclass of canonical elicitins, the beta elicitins, this results in patches of necrosis distal to the site of elicitin exposure (130). Alpha elicitins also spread throughout the plant but do not trigger necrosis (84, 130). The ability to systemically spread in the plant might be consistent with a role in pathogenicity. There is also evidence that elicitins may enter inside tobacco cells in the absence of the pathogen, a property that hints of a positive role in pathogenicity. Expression of an elicitin gene in the viral vector potato virus X (PVX) triggers a defense response even when the leader signal for secretion is missing from the gene (75, 76; A. McClean & B. M. Tyler, unpublished). Furthermore, when an elicitin gene, including the secretory leader, was fused to a C-terminal endoplasmic reticulum (ER) retention signal (-KDEL), the expressed protein could still trigger an HR in tobacco leaves when introduced via an *Agrobacterium* leaf infiltration transient expression system (agroinfiltration) (A. McClean & B. M. Tyler, unpublished). To rule out the possibility that elicitin protein was being secreted despite the ER retention signal, the same gene was introduced into *Nicotiana sylvestris*. *N. sylvestris* responds only very weakly to exogenously applied elicitin. Furthermore, it does not display an HR when a normal elicitin gene (including the secretory leader) is introduced into leaf cells by agroinfiltration, whereas tobacco leaf cells do respond strongly. When the elicitin-KDEL gene was introduced into *N. sylvestris* by agroinfiltration, a strong HR was observed (A. McClean & B. M. Tyler, unpublished). Taken together, these observations suggest that *N. sylvestris* does not respond normally to elicitins because elicitins cannot enter inside the cells of *N. sylvestris*, but when elicitin is delivered into the cells by expression inside the cells, a normal HR ensues (Figure 3). Similar results were obtained with certain elicitin mutants on tobacco (see below). Agroinfiltration or stable transformation of tobacco with elicitin genes lacking the secretory leader does not result in an HR (81, 87, 145; A. McClean & B. M. Tyler, unpublished), presumably because either most of the protein is misfolded when synthesized in the cytoplasm instead of the ER, or the elicitin receptor is located in the ER. A final hint that elicitin may have a role in pathogenicity comes from our observation that elicitins bind tightly to the dimerization domain of a tobacco transcription factor closely resembling gt-3a of *Arabidopsis thaliana* (C. Mau, L. Yu & B. M. Tyler, unpublished). This observation was made in the course of screening for the elicitin receptor using the yeast two-hybrid system (see below). Gt transcription factors bind to the promoters of light-regulated genes and also to the promoters of many defense-related genes. Binding of elicitins to transcription factors involved in expression of defense-related genes could contribute to pathogenicity.

The three-dimensional structure of several elicitins has been determined by X-ray crystallography and NMR (8, 9, 14, 15, 49, 56). The structure consists of five alpha helices stabilized by three highly conserved disulfide bridges, surmounted by an omega loop and a two-strand beta sheet. The core is hydrophobic and has many highly conserved residues. The bound sterol molecule is entirely encapsulated in the core. The external residues are mostly hydrophilic and contain most of

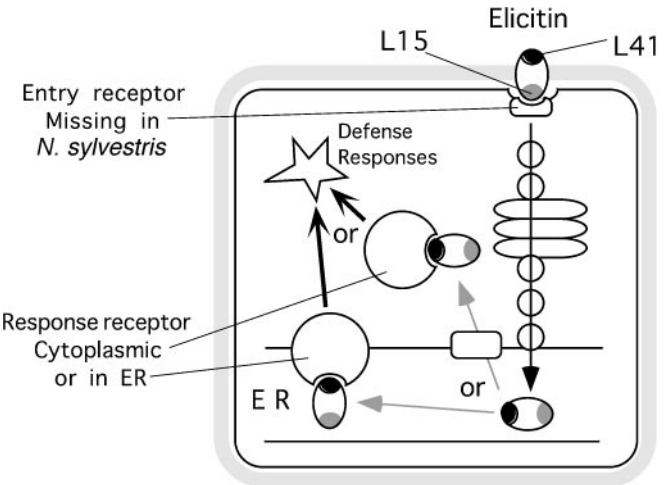

Figure 3 Model for entry of elicitins into tobacco cells by receptor-mediated endocytosis and interaction with an intracellular receptor. The intracellular receptor could be in the endoplasmic reticulum, or else elicitin could enter the cytoplasm by retrograde translocation and interact with a cytoplasmic receptor. L15 and L41 indicate distinct regions on the elicitin molecule surface (see text).

the variation among elicitins. However, there is an external hydrophobic pocket centered on leucine 15 that is highly conserved and is thought to be the entry point for sterols into the inside of the molecule (35). Site directed mutagenesis has established that the disulfide bonds are essential for elicitor activity (40; A. McLean & B. M. Tyler, unpublished) and that residue 13 is a primary, though not sole, determinant of the difference between alpha and beta elicitins (115, 119). Doyle (40) carried out a systematic mutagenesis of the elicitin from *P. parasitica* (parasiticein) using alanine scanning and also targeted mutagenesis of conserved surface residues. She found that two sites on the surface of the elicitin molecule centered on leucine 15 and leucine 41 were important for elicitor activity, but did not affect sterol binding. Mutations in the region of L15 could be rescued if the mutant elicitin was expressed inside the plant cell, but mutations in the region of L41 could not (E.A. Doyle, A. McClean & B. M. Tyler, unpublished), leading to the hypothesis that the region of the elicitin molecule centered on L15 was required for entry of elicitins into the cell, whereas the region centered on L41 was involved in receptor binding (Figure 3).

Identifying the receptor responsible for the elicitin response is of considerable interest because all *Phytophthora* species produce elicitins and therefore the elicitin receptor should confer broad-spectrum *Phytophthora* resistance (166). Also, coexpression of elicitin and its receptor in a plant could confer resistance against a wide range of pathogens (87, 145, 166). Since elicitin is an extracellular protein, it would seem most intuitive that the receptor would be located in the plant plasma

membrane. An elicitin binding activity in membrane preparations has been described with a *K*d of ~10 nM (16, 158). However, our results described above suggest that the elicitin receptor responsible for triggering the defense is internal to the cell, especially in the case of *Nicotiana sylvestris*. Since we observed that elicitins trigger an HR in tobacco and in *N. sylvestris* when directed to the endoplasmic reticulum, we speculate that elicitins enter the cell via the endomembrane system, for example, via receptor-mediated endocytosis (60, 69, 96, 165), as illustrated in Figure 3. This is the same route by which many bacterial endotoxins such as diphtheria, pertussis, and cholera toxins enter mammalian cells (91). If elicitins enter the cell by receptor-mediated endocytosis, we would expect that there are two receptors for elicitins, one on the plasma membrane that mediates cell entry, and one inside the cell that is responsible for generating the signal to the plant's defense machinery. This speculation is supported by the observation (elaborated above) that there are two distinct locations on the surface of elicitin molecules that are required to trigger HR, and that mutations at one of the sites can be rescued by expression of the mutant elicitins inside the plant cell (40). In this context, the membrane-located elicitin binding proteins (16, 158) might be the receptor that mediates entry. This possibility could be tested by assaying for the binding protein in *N. sylvestris* and by assaying for binding to the elicitin mutants described in the previous paragraph. The ability of elicitins to bind lipids could also play a role in cell entry. Many other elicitors from fungi and oomycetes likely interact with their receptors inside plant cells, namely those encoded by avirulence genes that interact with an intracellular class of resistance gene (see following section on Avr gene–R gene interactions).

We (C. Mau, L. Yu, E. A. Doyle & B. M. Tyler, unpublished) have used the yeast two-hybrid system to isolate tobacco cDNAs encoding elicitin binding proteins. One class of cDNAs encoded a protein containing nucleotide-binding-site and leucine-rich-repeat motifs similar to but distinct from those found in major disease resistance genes (R genes) encoding intracellular proteins. Silencing of the cognate tobacco genes using the cDNAs resulted in a 1000-fold reduction in the elicitin response of the tobacco plants, suggesting that protein encoded by the cDNAs is required for the elicitin response in tobacco. Expression of the cDNAs in tomato or petunia did not result in gain of an elicitin response in those plants, even when elicitin was expressed intracellularly, suggesting that additional proteins were required for the elicitin response.

P. sojae 42-kDa ELICITOR OF PARSLEY *P. sojae* cell wall preparations are active as elicitors against cultured parsley cells. The active elicitor component in this case has been shown to be a 42-kDa glycoprotein that is a component of the *P. sojae* cell wall (118). *P. sojae* is not a pathogen of parsley, so this system is normally considered a model for nonhost resistance. *P. parasitica* is, however, a pathogen of parsley (48) and contains a cell wall protein immunologically cross-reactive with the *P. sojae* protein (118). Therefore, the responsiveness of parsley to the *P. sojae* protein may derive evolutionarily from its exposure to *P. parasitica* infection.

The gene, *gpe*-1, encoding the 42-kDa protein has been cloned from *P. sojae* (131). The gene encodes a transglutaminase whose function is likely to crosslink proteins in the cell wall via glutamine residues (134). The gene is present in 13 of 14 *Phytophthora* species examined, including *P. parasitica*, but not in any *Pythium* species (134). The overall sequence identity among genes from the different species was 70–90% (134). Only a 13-amino acid peptide from the C terminus of the protein, VWNQPVRGFKVYE (called PEP-13), is required to trigger defense responses in parsley cells (113, 131). This peptide sequence is perfectly conserved in the other *Phytophthora* species (134), supporting the hypothesis that recognition of this protein contributes to nonhost resistance against diverse *Phytophthora* species. Within this peptide, substitution of W2 or P5 with alanine abolishes elicitor activity, but substitution of Y12 with alanine does not (134).

The responses of parsley cells to PEP-13 have been extensively characterized [reviewed in (111, 114)]. A candidate receptor polypeptide has been identified by using radiolabeled PEP-13 (108, 111, 112). A single 100-kd polypeptide has been identified that binds to PEP-13 with a *K*d of 2–11 nM. Binding to the 100-kd polypeptide also is affected by mutations W2A and P5A in PEP-13, but not Y12A, supporting the hypothesis that the 100-kd protein is a component of the functional receptor (112). The polypeptide has been solubilized in several nonionic amphipathic detergents and purified 5000-fold by affinity chromatography with PEP-13 (108). However, further purification and sequencing of the polypeptide have been hampered by the very small amounts of the polypeptide present and by its instability.

OTHER ELICITORS A 34-kDa glycoprotein (CBEL) from the cell wall of *P. parasitica* has been isolated that triggers defense responses in tobacco (136). A cDNA clone encoding the protein moiety of CBEL has been isolated (94). It encodes a 268-amino acid protein containing two direct repeats of a cysteine-rich domain characteristic of the cellulose binding domain of fungal glucanases (94). The protein binds to fibrous cellulose and to plant cell walls. Presumably, it is anchored to the cellulose cell wall of *P. parasitica* via this domain. Sequences highly similar to CBEL are abundant in the *P. infestans* and *P. sojae* EST databases (77, 124). The CBEL protein triggers defenses responses in tobacco at a concentration of 150 nM, which is sufficient to protect against subsequent infection with a virulent isolate of *P. parasitica* (136).

A 28-kDa protein (NPP1) has been identified in both *P. parasitica* and *P. sojae* that triggers necrosis on a wide variety of plant species. The *P. parasitica* protein was identified through biochemical analysis of elicitor activities in the cell walls, purified, and then sequenced (T. Nuernberger, personal communication). The *P. sojae* protein was identified from an EST database by its similarity to a protein (NEP1) previously characterized from the fungus *Fusarium oxysporum* f.sp *erythroxyli* that triggered necrosis and ethylene production in a variety of plants (123, 124). The cDNA clone was inserted into a potato virus X vector and expressed in *Nicotiana benthamiana* cells, where its ability to trigger necrosis on several plant

species was confirmed (124). Homologs of NPP1 also occur in *P. infestans* (77), *P. medicaginis* (Genbank accession # AW559250; identified by BLAST search), and *Py. aphanidermatum* (153). The *Pythium* protein (called PaNie) triggered programmed cell death in carrot, *Arabidopisis*, tomato, and tobacco cells, but not in three monocots (153). The *Fusarium* protein triggered necrosis or ethylene production in 22 dicots but no monocots. The very broad specificity of this elicitor raises interesting questions as to the nature of its receptor or target. Interestingly, homologs of NPP1 occur outside the oomycetes only in *Fusarium oxysporum* f.sp *erythroxyli* and in the bacteria *Streptomyces coelicolor*, *Vibrio sp*. CH-291, and *Bacillus halodurans*, but not in any other sequenced fungal or bacterial genomes (including *Bacillus subtilis*). Immunological analysis showed it to be present in only three of seven *Fusarium* species tested (5). This scattered distribution outside the oomycetes suggests that the gene for the protein might have been transferred horizontally to other organisms from the oomycetes. The *Vibrio* protein was identified as a hemolysin (72), suggesting that the NPP1 proteins may have pore-forming activity.

Arachidonic acid, which is released from *Phytophthora infestans* during infection, and probably by other *Phytophthora* species as well, elicits defense responses from a wide variety of plants, especially solanaceous plants such as potato (11), *Datura* (163), tomato (88, 168), eggplant (21), and pepper (53). The response to arachidonic acid has been extensively characterized in potato (26, 169). It has been proposed that elicitation by arachidonic acid is mediated by oxylipin signal compounds produced as a result of metabolism of arachidonic acid by lipoxygenases (11, 88, 122). Silencing of an elicitor-induced lipoxygenase in tobacco enabled *P. parasitica* to infect even in the presence of a resistance gene (126). The induction of defense responses by a *P. parasitica* cell wall preparation also was blocked, but induction of defense responses by arachidonic acid was not tested.

Genetically Defined Recognition Events: Avirulence Genes and R Genes

Genetic analysis of disease resistance by plant breeders has identified many single dominant genes that confer resistance (R genes). R genes typically confer resistance against a subset of pathogen isolates. Genetic analysis of pathogens has shown that single dominant genes called avirulence (Avr) genes are responsible for whether a particular R gene will be effective against a given isolate. Since in general each R gene is specific for a given Avr gene, R genes and Avr genes are said to have gene-for-gene specificity (33). The molecular basis for gene-for-gene specificity has been proposed to result from recognition of a pathogen molecule encoded by an Avr gene by a receptor encoded by an R gene. This has been directly confirmed in the case of a bacterial (135, 144) and a fungal pathogen (71) (the *AvrPto*/*Pto* and *AvrPi-ta*/*Pi-ta* gene pairs, respectively). Many R genes and Avr genes have now been cloned and characterized from a variety of pathosystems [reviewed in (32, 44, 52, 66, 89, 162)].

Single dominant R genes against *Phytophthora* infection have been described in soybean against *P. sojae* (18), in tomato and potato against *P. infestans* (43, 101), in pepper against *P. capsici* (155), in tobacco against *P. parasitica* (107), in strawberry against *P. fragariae* (151), in cowpea against *P. vignae* (6), and in pigeonpea against *P. cajani* (61). However, only in *P. sojae* (55, 148, 159, 160) and *P. infestans* (141, 152) have matching avirulence genes been defined genetically in the pathogen. Five R genes against downy mildews, which are also oomycetes, have been cloned (25, 32, 44). However, no R genes against *Phytophthora* pathogens have been cloned, nor until recently have any genetically defined *Phytophthora* Avr genes been cloned.

P. infestans ON POTATO AND TOMATO At least 11 R genes in potato (43) and 2 in tomato (101) have been described that protect against *P. infestans* infection. Of the potato R genes, *R3*, *R6*, and *R7* are located in a cluster (43) while others have unique or unmapped locations (54). Efforts are under way to clone DNA regions spanning *R1* and the *R3*, *R6*, *R7* cluster (www.bakerlab.usda.gov/BakerLab/research6.html). Avirulence genes corresponding to six R genes (*Avr1*, *Avr2*, *Avr3*, *Avr4*, *Avr10*, and *Avr11*) have been genetically defined and placed on a genetic map of the pathogen (152). Unlike avirulence genes in true fungi, some clustering of Avr genes is observed in *P. infestans*; *Avr3*, *Avr10*, and *Avr11* occur in a tight cluster (152). BAC contigs near to or spanning the *Avr4* and *Avr11* genes have been identified (161).

P. sojae ON SOYBEAN At least 14 R genes have been described that protect soybean against *P. sojae* (3, 17). Six are clustered at the *Rps1* locus (*Rps1a*, *Rps1b*, *Rps1c*, *Rps1d*, *Rps1e*, and *Rps1k*) and three at the *Rps3* locus (*Rps3a*, *Rps3b*, and *Rps3c*) (3, 17). Genetic analysis in the pathogen has defined single dominant avirulence genes corresponding to ten of the *Rps* genes (*Avr1a*, *Avr1b*, *Avr1d*, *Avr1k*, *Avr3a*, *Avr3b*, *Avr3c*, *Avr4*, *Avr5*, and *Avr6*) (95, 148, 159, 160). As in *P. infestans*, some clustering of Avr genes is observed in *P. sojae*. *Avr1b* and *Avr1k* are inseparable genetically (160; W. Shan & B. M. Tyler, unpublished), as are *Avr4* and *Avr6* (55, 160). *Avr3a* and *Avr5* are only 5 cM apart (160).

Efforts to clone *Rps* genes are well advanced in the case of *Rps2* (59, 85) and *Rps1k* (132; M. K. Bhattacharyya, personal communication). In both cases, a BAC contig spanning the *Rps* gene has been isolated and a number of R gene paralogs have been identified within the region. In both cases, the R gene paralogs are of the nucleotide binding site leucine-rich-repeat (NBS-LRR) class. For the *P. sojae* avirulence genes, a BAC contig spanning *Avr1a* has been identified (92) and a cosmid contig spanning *Avr4* and *Avr6* has been identified (147).

CLONING AND CHARACTERIZATION OF *P. sojae Avr1b* A single 60-kb BAC clone spanning the *Avr1b* and *Avr1k* genes of *P. sojae* has been isolated and the *Avr1b* gene has been identified within this region (W. Shan & B. M. Tyler, unpublished). The *Avr1b* gene encodes a secreted protein that is specifically expressed during

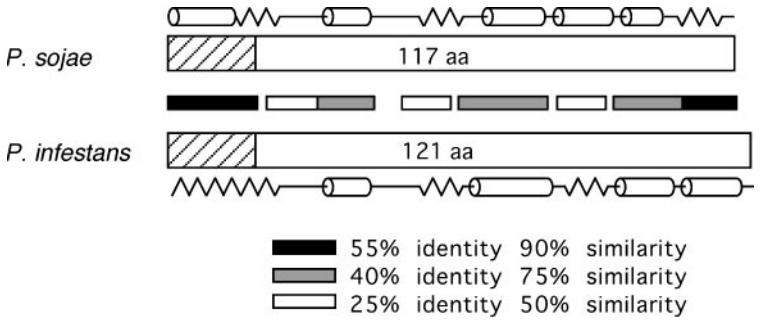

Figure 4 Structural comparison between Avr1b protein from *Phytophthora sojae* and its homolog from *P. infestans*. The hatched boxes indicate secretory leader sequences. The bars in the center indicate the degree of sequence similarity in different regions of the molecules. Predicted secondary structures are: tube, alpha helix; zig-zag, beta strand; straight line, coil.

infection. Expression of the *Avr1b* gene in the yeast *Pichia pastoris* resulted in secretion of a protein that triggered a vigorous defense response on soybean plants containing *Rps1b* but not on plants lacking *Rps1b* (W. Shan & B. M. Tyler, unpublished). Interestingly, prolonged exposure of *Rps1b* soybean plants to higher concentrations of Avr1b protein resulted in complete collapse of the plant, suggesting that the Avr1b protein was spreading systemically throughout the plant (though spread of a plant signal could not be ruled out) (W. Shan & B. M. Tyler, unpublished). The Avr1b protein is 117 amino acids long with a secretory leader of 21 amino acids. It shows no similarity to any sequences in the public databases, except for a homolog from *P. infestans* that shows weak to moderate similarity to the *P. sojae* protein, especially at the C terminus (Figure 4). The protein is hydrophilic and contains no disulfide bonds (W. Shan & B. M. Tyler, unpublished), unlike both the elicitins and the extracellular avirulence gene products that have been characterized to date from fungi. The predicted secondary structure of Avr1b is rich in alpha helices (Figure 4) of which three are also predicted for the *P. infestans* homolog, suggesting that for those three helices the prediction is a robust one. Structural similarity searches suggest that the tertiary structure of Avr1b is most similar to trihelical bundle DNA and RNA binding proteins, and to cytochrome *c*. The significance of this similarity remains to be determined.

Isolates of *P. sojae* that no longer express the Avr1b phenotype, and hence can infect soybean plants containing *Rps1b*, fall into two categories with respect to the mechanism by which *Avr1b* expression is lost (W. Shan & B. M. Tyler, unpublished). In some isolates, there are large numbers of substitution mutations. The pattern of mutations in these strains indicates that there has been strong divergent selection on *Avr1b*, as all but one of the base substitutions in these strains result in an amino acid substitution. Other *Avr1b*$^-$ isolates show no sequence differences whatsoever with the *Avr1b*$^+$ isolates. In these *Avr1b*$^-$ isolates, *Avr1b*

mRNA no longer accumulates, suggesting that a transcriptional or posttranscriptional mechanism is responsible for negating *Avr1b* expression (W. Shan & B. M. Tyler, unpublished). Loss of transcription was also responsible for loss of elicitin expression in isolates of *P. parasitica* virulent on tobacco (79). The mechanism by which *Avr1b* mRNA expression is lost is not yet clear. Preliminary evidence suggests that a second gene, located about 100 kb from *Avr1b*, is responsible for this loss of expression (W. Shan & B. M. Tyler, unpublished). This second gene, which complements *Avr1b* genetically, has been termed *Avr1b*-2. The isolates that have substitution mutations in *Avr1b* appear to have inherited them from one of two relatively ancient "progenitor" genotypes of *P. sojae* (51). Isolates in which *Avr1b* mRNA is no longer present appear to have lost *Avr1b* expression by more recent "mutations," since their genetic background is identical to that of avirulent isolates.

OUTSTANDING ISSUES At least two issues are of great interest with regard to *Phytophthora* avirulence genes. The first is whether the interaction between *Phytophthora* avirulence gene products and the corresponding host R gene products occurs at the plasma membrane or inside host cells. Viral avirulence gene products are naturally produced inside the cell, and many bacterial avirulence gene products appear to be delivered inside plant cells by the type III secretion system (52, 162). R gene products that are involved in detection of secreted avirulence gene products such as Avr4 and Avr9 of *C. fulvum* (Cf4 and Cf9 of tomato, respectively) have a structure expected for a component of a membrane-spanning receptor (32, 89). In contrast, resistance genes that protect against bacterial and viral pathogens typically have NBS-LRR structures consistent with an intracellular location, as expected (32, 44). However, many resistance genes that protect against fungal and oomycete pathogens also have NBS-LRR structures, for example, the L, M, N, and P families of rust resistance genes of flax (44); the *Pib* (157) and *Pi-ta* (71) rice blast resistance genes of rice; the *Rpp1*, *Rpp5*, *Rpp8*, and *Rpp13* resistance genes of *Arabidopsis* that protect against the oomycete *Peronospora parasitica* (32, 44); and the *Dm3* resistance gene of lettuce that protects against the oomycete *Bremia lactucae* (25). The predicted intracellular locations of these resistance gene products imply that fungal and oomycete pathogens have mechanisms for introducing proteins inside the cells of their hosts. In the case of Avr1b, in which the secreted protein can trigger defense responses in the absence of the pathogen (W. Shan & B. M. Tyler, unpublished), the intuitive expectation would be that the receptor would be on the plasma membrane and that *Rps1b* would resemble tomato R genes Cf9 and Cf4 (32). However, the BAC clone that has been identified as spanning the k allele of *Rps1* (*Rps1k*) contains polymorphic NBS-LRR class resistance genes (M. Bhattacharyya, personal communication), raising the intriguing possibility that both the k and b alleles of *Rps1* encode intracellular NBS-LRR proteins and that Avr1b protein has the ability to enter host cells in the absence of the pathogen. The large number of fungal and oomycete resistance genes that encode intracellular receptors, together with the evidence that elicitins, and possibly

also Avr1b, can enter cells, suggest that fungal and oomycete pathogens also have evolved mechanisms for infiltrating proteins inside the cells of their hosts.

The second interesting question, related to the first, is whether the avirulence gene products play a positive role in infection, i.e., do they contribute to pathogenicity as many bacterial avirulence gene products appear to do (52, 162)? In the case of *Avr1b*, we have several hints that it may aid infection (W. Shan & B. M. Tyler, unpublished). First, it is expressed specifically during infection. Second, it may spread systemically through the plant. Third, it may interact with the *Rps1b* product inside soybean cells. We are currently testing this hypothesis.

GENOMIC APPROACHES TO *PHYTOPHTHORA*-HOST RECOGNITION

Until now, molecules involved in *Phytophthora* host recognition have been identified by biochemical or genetic analyses. However, genomics approaches have great potential to assist in identifying additional molecules. Genetic approaches to the identification of *Phytophthora* receptors involved in plant signal recognition will be greatly aided by integrated physical and genetic maps of the organism, and eventually a complete genome sequence (147). The availability of extensive collections of *Phytophthora* EST sequences (77, 124, 147) will also facilitate identification of new candidates for molecules involved in plant recognition.

As an example of the kinds of novel approaches that genomics will enable, Kamoun (146, 147) used the SignalP algorithm (110) together with bioinformatic identification of the startpoint of translation to screen for *P. infestans* ESTs encoding small secreted proteins similar to elicitins or Avr1b. Of 2147 *P. infestans* ESTs screened in this way, 261 (12.2%) were predicted to be secreted (146). Among these was the *P. infestans* homolog of *P. sojae Avr1b*. To test the function of these ESTs, the ESTs were expressed in the host plants *N. benthamiana* and tomato using a systemic viral expression system (potato virus X), then the plants were assayed for changes in susceptibility to *Phytophthora* infection. Several cDNAs were identified that induce necrosis in plant tissue and alter the tomato response to *P. infestans* (146).

On the plant side, genomics can greatly aid in cloning R genes by identifying R gene paralogs in sequences of targeted regions. Identification of genes involved in synthesis and release of signal compounds can also be used to dissect the contributions of signal recognition. For example, *Arabidopsis*, tobacco, and other non-legumes have been engineered to produce isoflavones using an isoflavone reductase gene from soybean (73, 142, 167). What will be the response of *P. sojae* to these plants? Similarly, now that the complete biosynthetic pathway to isoflavones has been elucidated in *Medicago* (37), and soybean homologs of the cloned biosynthetic enzyme genes have been identified from the vast soybean EST database (142; www.tigr.org/tdb/gmgi/), it will soon be possible to manipulate the levels and structures of isoflavones produced by soybean and other legumes, and

to test the effects of these changes on infection by various *Phytophthora* species that are or are not natural pathogens of those plants.

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