Current Review

Small, Cysteine-Rich Proteins and Recognition in Fungal-Plant Interactions

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One of the most challenging problems facing plant pathology is to determine the molecular basis of the specificity between fungal plant pathogens and their host plants. This specificity is manifested both at the species level, where a given pathogen may attack only one or a few related plant species, and at the race-cultivar level, where, in a given pathosystem, races of a pathogen exist defined by their virulence on a unique subset of host plant cultivars (Keen 1990). Only in a few cases is the molecular basis of specificity well understood, and most of these involve the production of a host-specific toxin by the fungus (Yoder 1980; Meeley *et al.* 1992). There are now a number of examples where small, cysteine-rich proteins play a role in the specificity and pathogenicity of fungal pathogens. In this review, we point out some of the structural and functional similarities shared by these and other fungal proteins and comment on their possible evolutionary relationships. In addition, we compare the function of small, cysteine-rich proteins in fungi with similar proteins found in mammals, plants, and reptiles that have known functions.

Fungal avirulence genes and elicitins.

The genetics of race-cultivar specificity has been extensively studied, following Flor's gene-for-gene theory, which states that for each resistance gene identified in the plant there exists a corresponding avirulence gene in the pathogen (Flor 1971). In most cases, plant resistance genes and pathogen avirulence genes segregate as single dominant characters (Flor 1971; Ellingboe 1982), suggesting that resistance in the plant is due to the interaction of an avirulence gene product and a plant resistance gene product. This theory has evoked many models that postulate the nature of the avirulence and resistance gene products and how they might interact (Ellingboe 1981, 1982, 1984; Gabriel and Rolfe 1990; Keen 1982, 1990). Some suggest that proteins or glycoproteins on the surface of the pathogen interact with plant resistance gene products, resulting in a resistant reaction directly or via the induction of a resistance response.

Progress in identifying fungal avirulence gene products has been frustrated by the inability to purify molecules from liquid culture filtrates that retain race-cultivar specificity (Dixon *et al.* 1981; Rohwer *et al.* 1987; Darvill and Albersheim 1984). Only relatively recently has convincing evidence of the existence of race-specific proteins produced by plant pathogenic fungi been presented. A protein (AVR9), isolated from the intercellular fluids of tomato leaves infected with *Cladosporium fulvum* race 9, has been shown to elicit the hypersensitive response (HR) on tomato cultivars carrying the Cf9 gene (de Wit and Spikman 1982; de Wit *et al.* 1985; Higgins and de Wit 1985). This protein is of fungal origin (de Wit *et al.* 1984) and has been sequenced, its cDNA identified, and the gene cloned. The *avr9* gene encodes a 63 amino acid protein, including a secretory leader sequence, which is processed to a mature 28 amino acid peptide with six cysteine residues (Scholtens-Toma and de Wit 1988; van Kan *et al.* 1991; van den Ackerveken, *et al.* 1992). The transfer of *avr9* to other *C. fulvum* races makes them avirulent on tomato cultivars carrying the Cf9 gene. This confirms that this gene encodes the AVR9 phenotype (van den Ackerveken *et al.* 1992). Subsequently the *avr4* gene product from *C. fulvum* (Joosten *et al.* 1994) and a putative avirulence gene product from *Rhynchosporium secalis* (Wevelsiep *et al.* 1991) have been purified and sequenced. These three proteins are of low molecular weight (28–106 amino acids) and show no obvious sequence homology to each other or to other known proteins. They are, however, relatively cysteine-rich, containing between 6 and 10 cysteine residues spaced throughout the protein. A further small protein with eight cysteine residues, ECP1, has been isolated from the apoplastic space of *C. fulvum*–infected tomato leaves (Joosten and de Wit 1988; van den Ackerveken *et al.* 1993), but a role for ECP1 in pathogenesis or specificity has not yet been demonstrated.

Another group of proteins known as elicitins has been characterized from *Phytophthora* spp. (Ricci *et al.* 1989; Nespoulous *et al.* 1992; Pernollet *et al.* 1993; Kamoun *et al.* 1993). These are 10-kilodalton (98 amino acid) proteins that cause necrosis and trigger HR and the accumulation of pathogenesis-related proteins when applied to the nonhost tobacco. In this sense, they resemble the avirulence gene products isolated from *C. fulvum* by de Wit and colleagues. Although they are cysteine-rich proteins with three intramolecular disulfide bonds, they have no apparent amino acid homology with any of the *C. fulvum* avirulence gene products. Elicitins may be involved with specificity at the species level, at least in this *Phytophthora* pathosystem, in that the tobacco pathogen

MPMI Vol. 7, No. 3, 1994, pp. 320-325

Phytophthora nicotianae var. *nicotianae* does not produce an elicitin. The three-dimensional structure of an α-elicitin has been elucidated using multidimensional heteronuclear NMR, and the amino acid at position 13 is thought to be important in toxicity (Pernollet 1994).

Fungal hydrophobins.

A further group of relatively small cysteine-rich proteins has been characterized in a number of saprophytic and, more recently, pathogenic fungi. The hydrophobins (Wessels *et al.* 1991) are between 90 and 150 amino acids in length, contain eight cysteine residues, and are strongly hydrophobic. The amino acid sequence homology between hydrophobins from different species is low, and alignment relies heavily on the pattern of cysteine residues in the protein (Stringer and Timberlake 1993). Another common feature is that the second and third cysteines form a doublet and are usually followed by an asparagine residue. The best characterized hydrophobin genes are *eas* (*ccg-2*) from the ascomycete *Neurospora crassa* (Beever *et al.* 1979; Bell-Pedersen *et al.* 1992; Lauter *et al.* 1992), *rodA* from the ascomycete *Aspergillus nidulans* (Stringer *et al.* 1991), and *sc1, sc3,* and *sc4* from the basidiomycete *Schizophyllum commune* (Wessels *et al.* 1991; Wessels 1992). The genes *eas* and *rodA* encode proteins that polymerize into insoluble rodlet structures that form arrays in characteristic fascicles on the surface of conidia and other aerial structures and confer hydrophobic properties to these dry-spored species (Fig. 1; Beever and Dempsey 1978; Stringer *et al.* 1991). Similar rodlet structures have been observed on the aerial hyphae of the desiccation-tolerant *S. commune*. The SC3 protein probably forms this rodlet layer; indeed, it was recently shown that this protein can form such layers on the surface of air vesicles (Wösten *et al.* 1993). The less hydrophobic SC1 and SC4 proteins may be involved in binding hyphae together (Wessels 1992). These genes are expressed at high levels in the dikaryon only and have been implicated in fruiting body formation (Wösten *et al.* 1993). Rodlet fascicles closely resembling those encoded by characterized hydrophobin genes have been observed on spores and other aerial structures of many other fungi, including conidia of Deuteromycetes (Fig 1; Cole and Samson 1979), basidiospores of Gasteromycetes (Hess *et al.* 1972), teliospores of smuts (Gardiner *et al.* 1983), and sporangiospores of Zygomycetes (Hobot and Gull 1981; Latgé *et al.* 1986). Although chemical analyses of the protein component of these rodlet layers are only available in a few instances (e.g., Hashimoto *et al.* 1976; Hobot and Gull 1981), it is likely that these structures are also composed primarily of hydrophobins. Usually the rodlets form the outermost layer of the spore (Cole and Samson 1984). The external location of the rodlets means that they could mediate the initial contact in fungal interactions. It has been suggested, for example, that they may be of primary importance in fungal respiratory allergies (Latgé and Paris 1991).

The involvement of the fungal hydrophobins in plant-pathogen and insect-pathogen interactions is less clearly established. The phytotoxin cerato-ulmin, produced by the Dutch elm disease fungus *Ceratocystis* (*Ophiostoma*) *ulmi*, is a hydrophobin (Stringer and Timberlake 1993) located on aerial surfaces of the fungus (Takai and Hiratsuko 1980). This toxin alters membrane permeability and causes wilting of elm (Stevenson *et al.* 1979; Bolyard and Sticklen 1992). The chestnut blight fungus *Cryphonectria* (*Endothia*) *parasitica* also produces a hydrophobin (cryparin), which accumulates on the fungal aerial surfaces and shares some common characteristics with cerato-ulmin (Carpenter *et al.* 1992). While cryparin has not been found to be phytotoxic, at least to the nonhost plant apple, it is nevertheless down-regulated in hypovirulent strains carrying virus-like dsRNAs (Carpenter *et al.* 1992). The entomopathogenic fungus *Metarhizium anisopliae* produces a hydrophobin, SSGA, which is expressed during appressorial formation and conditions of starvation

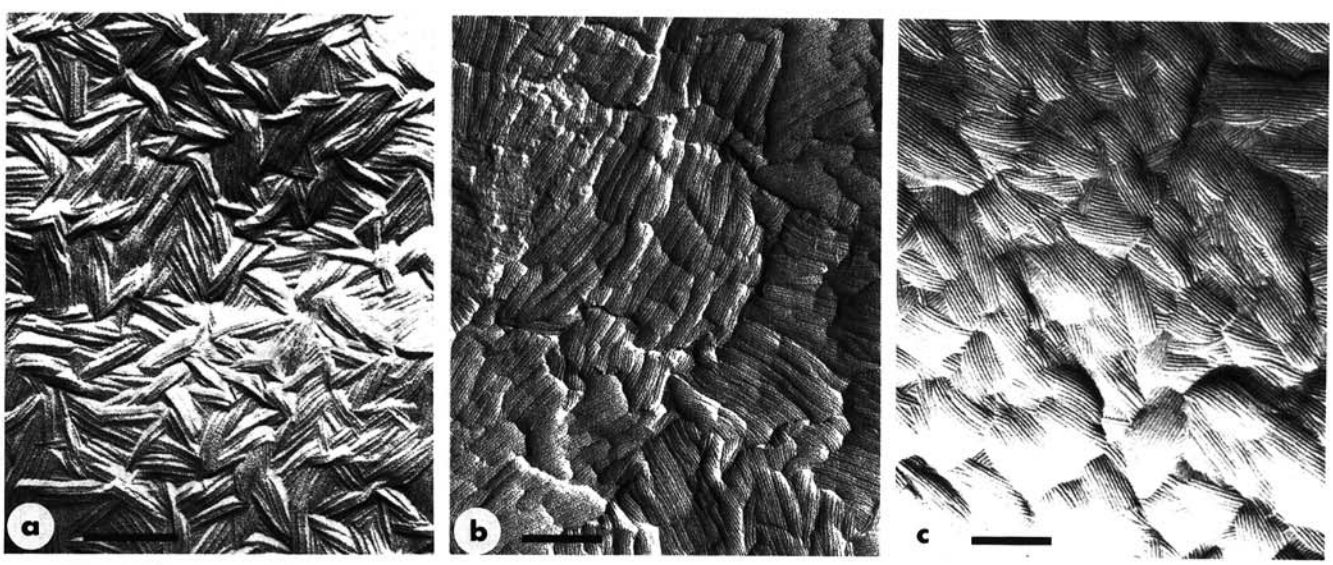

Fig. 1. Rodlet arrays on the spore surface of **A,** the saprophyte *Neurospora crassa* **B,** the saprophyte and secondary pathogen *Cladosporium cladosporioides* and **C,** the citrus-fruit-rotting pathogen *Penicillium digitatum*. Spores were dry shadowed with carbon-platinum and examined by transmission electron microscopy (Beever and Dempsey 1978); bars represent 200 nm. The rodlets of *N. crassa* are composed primarily of the hydrophobin encoded by *eas* (Bell-Pedersen *et al.* 1992; Lauter *et al.* 1992)

and may be involved in attachment to hydrophobic surfaces (St. Leger *et al.* 1992). Expression of the *ssgA* gene is synchronous with the *Prl* gene, which codes for a cuticle-degrading protease, suggesting that this hydrophobin may also be involved in pathogenesis. In the case of a hydrophobin recently isolated from the rice pathogen *Magnaporthe grisea*, gene disruption has demonstrated a significant role in pathogenesis, perhaps due to involvement of the hydrophobin in appressorial formation (Talbot *et al.* 1993).

Hydrophobins and avirulence gene products.

It is apparent from the above review that small cysteine-rich proteins (avirulence gene products, elicitins, hydrophobins) play a role in the pathogenicity and specificity of some fungi. We suggest that involvement of such proteins will prove to be widespread, and that these proteins, despite their lack of amino acid homology, may have similar functions. The only known function of avirulence gene products (and elicitins) is to limit the host range of a pathogen (Innes *et al.* 1993). This raises the question of why these proteins should be selected for. They may have other functions that contribute to the fitness of the fungus or they may have evolved from other important proteins that are structurally related, such as hydrophobins. A number of characteristics make hydrophobins attractive candidates for involvement in the plant-fungal interaction, in addition to their role in fungal morphogenesis. As proteins located on the cell wall, they may play a primary role involving direct contact with the recognition system that plants have developed for detecting pathogens. Therefore, they could potentially act as avirulence gene products, triggering the defense response in incompatible interactions. A mutation in the hydrophobin that altered the structure of the gene product so that it was no longer recognized by the plant would lead to virulence.

It was recently shown that, in the case of avirulence genes, mutations in the *avr4* gene of *C. fulvum* can lead to virulence on tomato cultivars carrying the Cf4 gene (Joosten *et al.* 1994). Homologues of the *avr4* gene were isolated and sequenced from seven different races of *C. fulvum* virulent on cultivars containing the Cf4 gene. Every *avr4* homologue contained a single base pair mutation in one of three different cysteine codons; in each case the mutation was from TGT to the tyrosine codon TAT (Joosten *et al.* 1994). This striking observation implies that either the mutated homologues containing tyrosine have some sort of common function (unlikely given that three different cysteines are involved) or that the fungal pathogen has developed a way of specifically mutating cysteine to tyrosine. Given the likely importance of cysteines in the tertiary structure of these proteins, such an ability would permit the fungus to actively mutate avirulence genes to avoid recognition by plant resistance genes. Furthermore the *avr9* gene, which contains seven cysteines (one in the putative signal peptide), contains a tyrosine adjacent to the second cysteine (van Kan *et al.* 1991; van den Ackerveken *et al.* 1992). If this tyrosine had mutated from a cysteine, the original protein would contain eight cysteines and a CCN motif identical to that found in most hydrophobins. With a mechanism for specifically mutating cysteines, the rate of evolution of virulence would be considerably accelerated and might explain the rapid appearance of new races in the field. It might also account for the lack of obvious amino acid homology between these proteins. Thus we postulate that, in addition to their role as pathogenicity factors, some hydrophobins may be involved in recognition and may share a common ancestry with avirulence gene products and elicitins.

The tertiary structure of small, cysteine-rich proteins from fungi.

Analysis of the three-dimensional structure of hydrophobins is limited. It is known that the eight cysteine residues in cerato-ulmin (Stevenson *et al.* 1979) and SC3 form disulfide bonds (de Vries *et al.* 1993), and this is probably the case in the other hydrophobins given the strong conservation of these residues. Assuming that the cysteine residues in hydrophobins form intramolecular disulfide bonds, the predicted structural features of hydrophobins resemble a class of toxin and agglutinin molecules (St. Leger *et al.* 1992). The three-dimensional structures of some of these functionally diverse protein domains, which include the lectin wheat-germ agglutinin and snake venoms, have been determined and the term "toxin-agglutinin fold" used to describe their topology (Drenth *et al.* 1980). Four disulfide bonds give these proteins a series of distinctive loops, the length and amino acid sequence of which vary between different proteins. The hydrophobin cryparin has been shown to have lectin-like activity (Carpenter *et al.* 1992), indicating a functional, and possibly structural, homology with proteins such as wheat-germ agglutinin.

The three-dimensional structure for an α-elicitin has been reported (Pernollet 1994), but there are no secondary or tertiary structural data yet for fungal avirulence gene products, and thus no structural data to link hydrophobins to either the elicitins or the avirulence gene products. We predict that the cysteine residues of avirulence gene products will form disulfide bonds. Significant changes in amino acid sequence (except at the cysteine residues) can occur without altering the overall toxin-agglutinin fold topology. This makes these

Table 1. Cysteine-rich proteins from nonfungal sources

Protein	Origin	Function	Reference
Defensins	neutrophils	antimicrobial	Lehrer *et al.* (1991)
P-proteins	mucin	unknown	Hoffmann and Hauser (1992)
Pore-forming peptide	*Entamoeba histolytica*	cell lysis	Leippe *et al.* (1992)
Neurotoxin-1	*Naja naja oxiana*	neurotoxin	Nickitenko *et al.* (1993)
Toxin 4	*Centruroides noxius*	Na^+ channel inhibitor	Vázquez *et al.* (1993)
Wheat germ agglutinin	*Triticum aestivum*	lectin	Drenth *et al.* (1980)
EP-2	carrot	lipid transfer	Sterk *et al.* (1991)
LPT	maize	lipid transfer	Sossountzov *et al.* (1991)
Hevein	rubber	allergen	Andersen *et al.* (1993)

proteins ideal for performing roles in recognition and specificity.

The function of small, cysteine-rich proteins.

The proteins that fold into the toxin-agglutinin domain and other cysteine-rich peptides have diverse functions (Table 1). They do not have known catalytic functions but tend to bind other molecules such as carbohydrates, proteins, and lipids. Many act as toxins or allergens and often bind to membrane-bound receptors (Drenth *et al.* 1980). These functions are consistent with most models that postulate that avirulence gene products interact with plant membrane–associated receptors.

Hydrophobins might play a more proactive role in pathogenesis and may be involved in establishing basic compatibility (Heath 1981) with the host plant. Two hydrophobins which could be playing such a role are cerato-ulmin (a toxin) and cryparin (since lectin activity is often associated with toxicity). Whether the activity of these hydrophobins determines specificity for their respective host plants remains to be resolved, but it has been suggested that cerato-ulmin may be host-specific (Sticklen *et al.* 1991).

In attempting to find a potential role for hydrophobins in the recognition and signal transduction of the HR, it may be useful to look for parallels in other signal transduction systems. A potential parallel is S-locus pollen self-incompatibility, which has been extensively analyzed in *Brassica* species (Nasrallah and Nasrallah 1989). One hypothesis is that self-recognition is mediated by the direct interaction of the cysteine-rich S-glycoproteins with a highly homologous cysteine-rich domain present on receptor proteins (Goring and Rothstein 1992). These receptor proteins contain an active serine-threonine kinase domain, which is linked to the cysteine-rich region by a single transmembrane domain. The recent cloning of the *Pto* gene from tomato, which confers resistance to *Pseudomonas syringae* pv. *tomato*, revealed that it also contains a serine-threonine kinase domain, which may be responsible for the HR signal transduction (Martin *et al.* 1993). Unlike the S-glycoprotein, however, the *Pto* gene contains no obvious transmembrane domain or extracellular domain that could function as the external receptor, although it has a potential myristoylation site, suggesting it could be membrane-associated. The exact nature of the interaction between the avirulence gene and the *Pto* resistance gene is therefore still uncertain. Given the tendency of hydrophobins to form aggregates, it is tempting to speculate that in fungi the recognition process might be mediated by an interaction between the cysteine-rich, hydrophobin-like domain of avirulence genes and similar domains present on the receptor. The signal could then be passed on via a transmembrane region to a serine-threonine kinase domain that would be able to initiate intracellular responses.

Alternatively, the cysteine-rich proteins, while necessary for pathogenicity, may play a more passive role during pathogenesis. They might, for example, be required to mask the highly elicitor-active β-glucans (Sharp *et al.* 1984a,b) and chitin (Roby *et al.* 1987) on the hyphal surface. Additionally, they may alter the hydrophobicity of the invading hyphae; this is important for the pathogenicity of some medically important fungi (Hazen and Hazen 1988).

Future work and directions.

Analysis of the three-dimensional structure of hydrophobins, elicitins, and avirulence gene products will definitively establish if there is a close structural relationship between these proteins. Advances in multidimensional NMR are such that it can be used to determine the tertiary structure of small proteins in solution. This technique has already been applied to some small cysteine-rich proteins of the toxin-agglutinin family (Andersen *et al.* 1993).

In the past, isolation techniques may have favored the recovery of more hydrophilic proteins; consequently, avirulence gene products found in more hydrophobic fractions (which might have properties, such as low solubility in water, similar to those of the known hydrophobins) may have been overlooked. Avirulence gene products are not often secreted in liquid culture, but hydrophobins are known to be produced on aerial hyphae, and the hyphal surface may provide a good source of proteins involved in pathogenesis. No hydrophobin has been characterized from *C. fulvum*, but related species have well-developed rodlet layers (Fig. 1). It would be interesting to know whether there is any amino acid sequence homology between hydrophobins from *C. fulvum* and its characterized avirulence gene products.

Determining the nature of avirulence genes is not just a point of academic interest. In addition to map-based cloning (Martin *et al.* 1993), it will provide new approaches for identifying the plant genes that interact with the avirulence gene products and provide leads in the isolation of plant resistance genes. Some groups are already attempting to identify plant receptors by affinity-based techniques using putative avirulence gene products. If these techniques are successful, they will provide additional means for isolating the receptor proteins encoded by plant resistance genes and may also shed some light on how they interact with avirulence gene products.

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