Molecular bases of fungal parasitism

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Introduction

My purpose is to develop a framework for hypothesis, predictions and experimentation concerning some of the major unanswered questions about fungal parasitism and resistance to it in plants. I shall do so by drawing upon general understanding from the literature and upon personal experience of three different types of fungal parasitism of plants. This experience has been with necrotrophic parasitism, as represented by *Botrytis fabae* Sard. on broad bean (*Vicia faba* L.), transiently biotrophic parasitism, as represented by *Colletotrichum lindemuthianum* Sacc. and Magn. on bean (*Phaseolus vulgaris* L.), and the totally biotrophic parasitism of *Puccinia* spp. on wheat (*Triticum aestivum* L.). Reflection about the parasitic habit leads to the conclusion that parasitism must have evolved on numerous occasions and independently in different groups of organisms. Some of its essential features among the fungi are possession of specialised structures, such as modified hyphal cells for infection as seen in the various types of appressoria and haustoria, the timing of life cycles to coincide with seasonal changes in hosts, the ability to overcome passive defensive hurdles in hosts and the ability to outmanoeuvre active defence in plant cells. The outmanoeuvring of active defence is my main theme but first I will draw attention to the nature of active defence.

Active defence and its elicitation

Plant cells respond rapidly to infection. Some of these responses can be seen by various forms of microscopy at the earliest stages of attempted parasitism (Aist 1983). An acceleration in the rate of cytoplasmic streaming and directional streaming to the point of infection are often observed. These may be non-specific responses unrelated to the final fate of attempted infection and carried out also after physical stimulation, such as with micro-needles or laser beams. Cell walls may become modified at infection sites. These modifications are sometimes seen as wall haloes around the sites, the haloes being zones of depletion or deposition and depositions including silicon, lignin and possibly callose and suberin. Other modifications are formation of papillae beneath infection sites, callose being a common component of papillae. Synthesis and depositions of these materials at and below attempted penetrations may represent forms of active defence. Hypersensitivity, meaning the rapid death of the first penetrated cell and perhaps its immediate neighbours (Stakman 1915), is a common response closely associated with many cases of defence against biotrophic fungi. Phytoalexins, being antimicrobial substances synthesised in response to infection (Paxton 1981), accumulate rapidly in many plants (Bailey and Mansfield 1982). Pathogenesis-related proteins, known for some years as accompaniments of local lesion formation following virus infections (Van Loon 1985), have recently been shown to accompany defence against fungal infections (Joosten and De Wit 1989; Kombrink *et al.* 1988) and to include chitinase, glucanase and proteinase among their activities (Kauffman *et al.* 1987; Legrand *et al.* 1987; Vera and Conjero 1988). Many of these responses seem likely to contribute to active defence, probably working together to localise invading organisms.

The response of phytoalexin formation has been studied intensively in recent years, findings about its elicitation in plant cells being particularly relevant to my theme. A wide range of treatments will elicit phytoalexin synthesis but I shall emphasise work on elicitors that could be involved during infection. Elicitors from pathogens grown in culture include glucans from *Phytophthora megasperma* (Ayers *et al.* 1976), carbohydrate-rich preparations from *Colletotrichum lindemuthianum* (Tepper and Anderson 1986), glycoproteins from *Cladosporium fulvum* (De Wit and Rozeboom 1980) and pectolytic enzymes from a number of organisms (Davis *et al.* 1984; Lee and West 1981). Elicitors from modified fungal and host cell walls include chitin oligomers (Barber *et al.* 1989) and pectic oligomers (Jin and West 1984; Nothnagel *et al.* 1983), the latter likely to be released from host walls by the action of pectolytic enzymes from pathogens. Many of these elicitors are highly active but most of them are non-specific in their action, not only with respect to host cultivars but also to plant species, an exception being the preparations from *C. lindemuthianum* which have some cultivar-specificity (Tepper *et al.* 1989). Although it remains to be demonstrated that all of the mentioned elicitors are involved during infection processes, there is one group of elicitors that seems particularly likely to be active *in planta*. These are the elicitors of hypersensitivity obtained from intercellular washing fluids of tomato leaves infected by *Cladosporium fulvum*, the elicitors also being specific in their action on resistant cultivars (De Wit and Spikman 1982). Some of the elicitors from fungal walls and culture fluids have been used in studies on the activation of pathways and enzymes involved in phytoalexin synthesis as described below.

The biosynthetic pathway from shikimic acid to the pterocarpanoid phytoalexins has been studied in order to characterise fully the intermediates and to examine the activation of some of its enzymes by infection and elicitors (Ebel 1986). The steps from phenylalanine to the phenolic acids and the enzyme phenylalanine ammonia lyase are important in the early part of this pathway and also as a source of precursors for lignin synthesis. The involvement of the enzyme chalcone synthase in a subsequent step from phenolic acids to chalcone has also received special attention because of its role in leading to the isoflavonoids and the pterocarpanoids.

Application of elicitors to cells in suspension culture causes rapid and substantial increases in the activities of phenylalanine ammonia lyase and chalcone synthase in legumes (Dixon and Lamb 1979; Lawton *et al.* 1983) and also in the amounts of the encoding mRNAs (Edwards *et al.* 1985; Ryder *et al.* 1984; Schmelzer *et al.* 1984). Infection by the pathogens from which the elicitors were derived also causes similar rapid activations in resistant tissues (Bell *et al.* 1984; Bonhoff *et al.* 1986; Ryder *et al.* 1984; Schmelzer *et al.* 1984). The high degree of localisation of these rapid responses related to defence in infected tissues is shown in the work of Hahn *et al.* (1985) on soybean and *Phytophthora megasperma* and of Cuypers *et al.* (1988) on potato and *Phytophthora infestans*. These results emphasise the early, rapid and localised activation of key components of defence when plant cells respond to elicitors and infection.

Outmanoeuvring active defence

The conclusion from earlier work and particularly from recent work on biochemical and cytological changes is that cellular responses leading to defensive molecules and structures are very readily switched on. How then do micro-organisms outmanoeuvre these responses in order to establish themselves as parasites on their particular host plants? One interpretation is that there are at least three ways by which parasites outmanoeuvre active defence. These are (1) by inactivating it, (2) by removing it and (3) by not provoking it. I shall consider each in turn, considering how it might be involved in the different types of parasitic habit among the fungi in particular.

1 Inactivating active defence I suggest that this is achieved by the necrotrophic parasites that kill host cells in advance of their hyphae. A much-studied parasite of this type is *Botrytis fabae* on its host, *Vicia faba*. An analysis of the attributes of numerous isolates revealed that the ability of germinating spores and their germ-tubes to kill host cells rapidly was most highly correlated with success in causing disease (Hutson and Mansfield 1980). Cells are killed as the spores penetrate the cuticle and wall, conceivably preventing synthesis of phytoalexins in the first affected cells. Phytoalexin synthesis does take place in surrounding live cells (Mansfield *et al.* 1974), but phytoalexin removal by the parasite probably then becomes highly important for its further development as discussed later. The nature of the cell-killing factor of *B. fabae* has not yet been revealed. Analyses of other parasites in the form-genus *Botrytis* indicates that each may bear a cell-killing factor specific for its particular host plant (Mansfield and Hutson 1980). These may be the primary determinants of their success as necrotrophic parasites.

Specialised parasites among the form-genus *Alternaria* secrete host-specific toxins, some of which have been characterised (Nishimura and Kohmoto 1983). The suggestion has been made that evolution of parasitism in *Alternaria* towards new hosts depends upon the production of an appropriate toxin. These toxins may have the role of inactivating defensive responses in the host cells.

Host-specific toxins are known as products of a number of other fungi, especially intensively studied being those of some *Cochliobolus* species (Macko 1983). Analyses of numerous isolates and progeny among crosses show that the toxin of *Cochliobolus victoriae*, victorin (Wolpert *et al.* 1985), is a primary determinant of parasitism on susceptible oat cultivars, being essential for the fungus to infect (Scheffer *et al.* 1967). This evidence suggests that victorin is an agent of inactivating host defence but the idea is difficult to sustain for two reasons. One is that cytological study of the infection process indicates no host cell death in the early phases of intracellular hyphal growth, implying biotrophy at this stage of parasitism (Yoder and Scheffer 1969). The other is that victorin is claimed as an elicitor of the oat phytoalexins, the avenalumins (Mayama *et al.* 1986). There is no doubt that victorin is a highly specific agent of damage to tissues of susceptible oat cultivars (Scheffer 1983), but the way in which it acts in order to establish parasitism remains uncertain and the term 'toxin' may be a misnomer in relation to the primary action in establishing infection. Further work is needed in order to assess the roles of these host-specific toxins in relation to the defence mechanisms of their host plants.

2 Removing the products of active defence The best examples of this are the detoxifications of phytoalexins, as demonstrated for a number of fungi that cause accumulation of these substances at early stages of parasitism and then remove them in the process of spreading further through tissues. *B. fabae* causes the synthesis of the wyerone group of phytoalexins in cells around infection sites but also has the capacity to reduce them to less antifungal compounds (Hargreaves *et al.* 1977). This reductive step, affecting the adjacent acetylenic and keto groups in the molecules, has been demonstrated both *in vitro* and *in vivo* (Hargreaves and Mansfield

1975; Mansfield and Widdowson 1973) and is correlated with pathogenic ability (Hargreaves *et al.* 1977). The enzyme kievitone hydratase seems likely to play a similar role in the pathogenesis of *Fusarium solani* f. sp. *phaseoli* towards bean by removing the phytoalexin, kievitone (Smith *et al.* 1982).

Evidence for the importance of this method of overcoming active defence is strongest for the pathogenesis of *Nectria haematococca* Berk. and Br. (*Fusarium solani* (Mart.) Sacc. f. sp. *pisi* (F.R. Jones) Snyd. and Hans.) towards pea (*Pisum sativum* L.). Infection causes the host to synthesise the phytoalexin, pisatin. A survey of a large number of isolates of the fungus revealed that all isolates, that could progress from infection sites and cause large lesions, had the ability to detoxify pisatin by demethylation (VanEtten *et al.* 1980). Conventional genetic crossing and analysis showed that pisatin demethylation was regulated at three genetic loci (Kistler and VanEtten 1984). Using the techniques of molecular biology, a gene for pisatin demethylation was isolated, cloned and transferred to the saprophytic fungus, *Aspergillus nidulans* (Weltring *et al.* 1988). This fungus was transformed so that it detoxified pisatin. With appropriate plasmid vectors, genes encoding for pisatin demethylation were used to transform *A. nidulans* and also *Cochliobolus heterostrophus*, a parasite of corn (*Zea mays*) but not of pea, and transformants with high copy numbers of the plasmid were selected for further experiments (Schafer *et al.* 1989). These transformants were highly active in pisatin demethylation. The transformed *A. nidulans* remained non-pathogenic on pea but the transformed *C. heterostrophus* had acquired the ability to cause significant disease on pea stems. Of all the work on parasitism and resistance, this work is the most complete in confirming the importance of an enzymatic process in parasitism and of a phytoalexin in the process of resistance by a plant.

I suggest that capacity to remove barriers produced through active defence is one of the keys to the success of some parasites.

3 Avoiding provocation of active defence I suggest that not provoking defensive responses in host cells is essential to a biotrophic parasite. By definition, biotrophs feed from live cells. Observations of their interfaces with susceptible cells suggest a high degree of compatibility in the relationships. There are no cytological or physiological signs of defensive responses in at least the early stages of infection and colonisation of the host. Considering how non-provocation might be achieved leads me to an hypothesis for biotrophic parasitism.

The hypothesis proposes a mechanism for achieving basic compatibility and then superimposing the action of single resistance genes in host cultivars against strains of parasites bearing complementary avirulence genes (Heath 1982). The hypothesis involves three steps (Table 1).

The first step in the success of a biotrophic species in infecting a new host species is envisaged to be the evolution of a suppressor molecule specifically capable of preventing responses to invasion in that host. This step has been proposed also by Ellingboe (1976). The suppressor is envisaged to act, for example, where intracellular hyphae of *Colletotrichum lindemuthianum* grow beside the protoplasts of the first cells infected in susceptible bean tissues (Skipp and Deverall 1972) or, for example, where haustoria of powdery mildew or rust fungi invaginate the plasmamembranes in their fully susceptible hosts (Littlefield and Heath 1979). A suppressor is postulated in order to explain recent findings about parasitism of rust fungi in wheat. The suppressor is envisaged to be involved in preventing the action of the non-specific elicitors produced by rust fungi and present in intercellular washings of infected wheat leaves (Deakin and Deverall 1985; Sutherland *et al.* 1989; Sutherland and Deverall 1989). These elicitors are present regardless of the virulence or avirulence of the particular strains of rust fungi to cultivars of wheat. The hypothetical suppressor is suggested to have evolved to enable the rust fungi to grow compatibly in wheat. Experimental evidence for a suppressor has been obtained in

Table 1 An hypothesis for the evolution of basic compatibility in, and complementary gene resistance towards, biotrophic parasitism

Step 1	— the evolution of a suppressor molecule to prevent the host from reacting to the invading organism
	— this suppressor is active at the interface between the invader and the host protoplast
	— basic compatibility is created
Step 2	— the evolution or incorporation of a resistance gene into the host
	— this gene encodes for a receptor of a pre-existing product of those strains that are recognised as avirulent invaders
	— the product is effectively a gene-specific elicitor
Step 3	— a mutation occurs in the invader causing a loss of the gene-specific elicitor
	— a strain virulent with respect to the resistance gene now exists

work with infection structures and intercellular fluids of the bean rust fungus, *Uromyces appendiculatus* (Heath 1980); the suppressor prevented cell wall depositions in bean inoculated with the cowpea rust fungus, *Uromyces vignae*, which was enabled thereby to progress further than it normally did in bean (Heath 1981). Thus, a suppressor is conceived of as the agent for creating basic compatibility between a biotroph and its host.

The second step is the consequence of adding a resistance gene to the host. In natural populations of plants, the addition would occur by evolution. In cultivated plants, the addition would be done normally by the plant breeder. The phenotypic expression of the resistance gene is the lesser development of the parasite. In many cases, this lesser development is accompanied by signs of some incompatibility, such as hypersensitivity, in the host cells and tissues. I suggest that the resistance gene acts by encoding for a host receptor of a pre-existing product of those strains of the parasite that are now resisted. In effect, these strains are now termed 'avirulent' with respect to the resistance gene. Their pre-existing product is what may be termed a gene-specific elicitor. This product is encoded for by what is now termed the avirulence gene complementary to the resistance gene. I suggest that the recognitional event, involving the interaction between the host receptor and the gene-specific elicitor of the parasite, switches on active defence resulting in signs of incompatibility and the lesser development of the parasite.

This part of the hypothesis predicts that gene-specific elicitors of the action of resistance genes should be isolatable. Although many elicitors have been found as described earlier, gene-specific elicitors have been isolated to date in only one parasite–host interaction, that between *Cladosporium fulvum* Cooke and tomato, *Lycopersicon esculentum* Mill. The main features of this interaction are the entry of the fungus via stomata in leaves and its growth only in the intercellular spaces of the mesophyll where resistance is expressed through the action of complementary genes in the fungus and the host (De Wit 1977). Sampling of washings from the intercellular spaces of infected plants has yielded elicitors that induce hypersensitive-like responses only when reintroduced to leaves of resistant cultivars (De Wit and Spikman 1982). The elicitors are specific to avirulent strains of the fungus and the complementary resistant cultivars. One of the elicitors specific for action towards the *Cf*9 resistance gene has been isolated and characterised as a 3049 D peptide (Schottens-Toma and De Wit 1988). Others specific for the *Cf*4 and *Cf*5 resistance genes have been isolated as low molecular weight proteins (De Wit *et al.* 1988). Success in isolating gene-specific elicitors in this case may derive from sampling the micro-site where the fungus intercommunicates with the host and where resistance is expressed. One implication for seeking gene-specific elicitors in interactions between other biotrophic fungi, such as rust and powdery mildew fungi, and their hosts is that the haustorium/plasmalemma interface should be sampled, this being the common site of resistance expression in these cases.

The third step is the consequence of mutation to virulence in the parasite. Mutations of certain avirulence genes to virulence are quite common in nature and can be brought about readily in the laboratory (Luig 1979; Pryor 1987; Watson and Luig 1968). Mutation to virulence at the locus of an avirulence gene in a strain of a parasite results in loss of the resistance encoded for by the complementary gene in the host. The hypothesis proposes that the mutation causes a change in the gene-specific elicitor, rendering it ineffective. The prediction is that slightly modified molecules should be detectable at, and isolatable from, the appropriate microsite in the parasite/host interaction where the virulent strain is now growing.

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