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Review

Arabidopsis pathology breathes new life into the necrotrophs-vs.-biotrophs classification of fungal pathogens

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SUMMARY

Fungal plant pathologists have for many decades attempted to classify pathogens into groups called necrotrophs, biotrophs and, more recently, hemibiotrophs. Although these terms are well known and frequently used, disagreements about which pathogens fall into which classes, as well as the precise definition of these terms, has conspired to limit their usefulness. Dogmas concerning the properties of the classes have been progressively eroded. However, the genetic analysis of disease resistance, particularly in the model plant *Arabidopsis thaliana*, has provided a biologically meaningful division based on whether defence against fungal pathogens is controlled via the salicylate or jasmonate/ethylene pathways. This mode-of-defence division distinguishes necrotrophs and biotrophs but it limits the biotroph class to pathogens that possess haustoria. The small number and limited range of pathogens that infect *Arabidopsis* means that several interesting questions are still unanswered. Do hemibiotrophs represents a distinct class or a subclass of the necrotrophs? Does the division apply to other plant families and particularly to cereals? and does this classification help us understand the intricacies of either fungal pathogenicity or plant defence?

INTRODUCTION

The urge to classify is at the heart of biology. Classification helps us organize our knowledge and suggests hypotheses for future study. Crucially, it enables us to make generalizations from a small number of specific observations. It is central to the notion that certain species can be regarded as 'model organisms', the study of which can be extrapolated to other (normally less-easy-to-study) members of the group. However, the validity of the generalizations generated in these studies is only as good as the classifications on which they are based.

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Fungal plant pathogens can be classified according to phylogeny or by mechanism of infection. Molecular data are currently being used to create phylogenetic trees that are believed to approximate to evolutionary history (see, for example, Berbee and Taylor, 2001). Pathologists have persistently attempted to classify pathogens on the basis of mechanisms of infection, and among the first lessons delivered to most novice plant pathologist will be the notion that fungal pathogens can be divided into two or three classes: necrotrophs, biotrophs and, more rarely, hemibiotrophs.

The definition of necrotrophy and biotrophy was perhaps best summarized by Lewis (1973) that

- biotrophs derive energy from living cells
- necrotrophs derive energy from killed cells

Hemibiotrophy has been defined by Perfect and Green (2001) as an 'initial period of biotrophy followed by "necrotrophic hyphae"', making it a subsidiary definition.

The value of a classification system lies in its ability to suggest robust generalizations. These terms have attracted many such interesting generalizations.

- Biotrophs:

1 are obligate (Scott, 1972);
2 possess haustoria (Gay, 1984; Mendgen *et al.*, 2000);
3 secrete limited amounts of lytic enzymes (Cooper, 1984; Lewis, 1973; Mendgen and Hahn, 2002);
4 cause little damage to the host plant (Cooper, 1984; Wood, 1986);
5 have a narrow host range (Lewis, 1973; Lucas, 1998);
6 induce hypersensitive cell death in incompatible interactions (Bailey, 1983);
7 are controlled by specific (gene-for-gene) resistance genes; examples include tomato leaf mould, the rusts, powdery and downy mildews;
8 are controlled by salicylate-dependent defence pathways (Hammond-Kosack and Parker, 2003).

- Necrotrophs:

1 are non-obligate;
2 have wide host ranges (Lucas, 1998);

Table 1 Classification of selected pathogenic fungi by different authors.

Pathogen	Disease	Haustoria	Class	Reference
Blumeria graminis	Powdery mildew	Yes	Biotroph	Hahn *et al.* (1997)
Uromyces fabae	Rust	Yes	Biotroph	Hahn *et al.* (1997)
Ustilago maydis	Maize smut	No	Biotroph	Perfect and Green (2001)
Cladosporium fulvum	Tomato leaf mould	No	Biotroph	Oliver *et al.* (2000), Mendgen and Hahn (2002)
			Hemibiotroph	Perfect and Green (2001)
Colletotrichum lindemuthianum	Anthracnose	Intracellular hyphae	Hemibiotroph	Perfect and Green (2001)
Phytophthora infestans	Potato late blight	Yes	Biotroph	Isaac (1992), Hammond-Kosack and Parker (2003)
			Hemibiotroph	Perfect and Green (2001), Mendgen and Hahn (2002)
			Necrotroph	Shaw (1988), Wheeler (1984)
Magnaporthe grisea	Rice blast	No	Hemibiotroph	Mendgen and Hahn (2002), Hammond-Kosack and Parker (2003)
			Necrotroph	Asiegbu *et al.* (2004)
Botrytis cinerea	Grey mould	No	Necrotroph	Prins *et al.* (2000)
Cochliobolus heterostrophus	Southern corn leaf blight	No	Necrotroph	Mendgen and Hahn (2002)
Mycosphaerella graminicola	Septoria leaf blight	No	Hemibiotroph	Rohel *et al.* (2001)

3 secrete copious cell-wall-degrading enzymes (Cooper, 1984; Kolattukudy, 1985; Lewis, 1973; Schafer, 1994);
4 produce toxins (Farrar, 1984; Walton, 1996);
5 are controlled by quantitative resistance genes, e.g. Septoria nodorum blotch caused by *Stagonospora nodorum*;
6 are controlled by jasmonate- and ethylene-dependent defence pathways (Hammond-Kosack and Parker, 2003).

In order to test these generalizations, we have to place organisms into the various classes (Table 1). Although there are some fungi that all authors agree upon, in a disturbingly large number of cases, different authorities have placed fungi in more than one class. For example, *Phytophthora infestans* has been placed in all three classes. With this level of agreement, discussion of whether each class shares any common features becomes problematic.

The classification of pathogens into these classes solidified between 20 and 30 years ago. The intervening years have revolutionized our understanding of pathogenicity genes, fungal gene expression, fungicide sensitivity, resistance genes and mechanisms of plant defence mechanisms. The purpose of this review is to investigate whether any of these areas supports a general and useful division of fungal pathogens into classes. We conclude that a division is supported and suggest directions in which future experiments might go in order to solidify the classification.

PATHOGENICITY GENES

The application of molecular genetics has provided a firm foundation for the study of fungal pathogenicity over the last 10–15 years. The genome sequences of *Magnaporthe grisea* and *Fusarium graminearum* are available as well as numerous expressed gene libraries (Lorenz, 2002; Soanes *et al.*, 2002; Tunlid and Talbot, 2002; Yoder and Turgeon, 2001). A number of studies have identified fungal genes expressed during infection (Kamoun *et al.*, 1999; Kim *et al.*, 2001; Rauyeree *et al.*, 2001; Skinner *et al.*, 2001; Thomas *et al.*, 2001; Trail *et al.*, 2003). These studies give largely correlative information (Solomon *et al.*, 2003).

The use of random and specific gene disruption techniques has given detailed functional information and has so far identified around 100 genes required for pathogenicity (as reviewed in Idnurm and Howlett, 2001). These studies have involved pathogens in all major classes, so it seems timely to ask whether any sets of orthologous genes are characteristic or diagnostic of any group of pathogens and whether such groups of pathogens correspond to any of the traditional classes of pathogen. The key technical advance that has allowed this progress is the development of techniques for gene disruption including transformation. We are hampered therefore in that no reproducible methods of transformation have been reported for any of the obligate (and biotrophic) fungi. All agree that all obligate pathogens are biotrophic. Therefore, for this class, we must rely on the correlative studies based on gene expression and other methods.

Genes expressed and required for pathogenicity in all groups

Recent studies in pathogenicity mechanisms have tended to emphasize commonalities between pathogens rather than differences. This is most noticeable in the case of genes encoding proteins involved in signal transduction processes, such as *PKA*, *MAP kinase*, *heterotrimeric-Gα* and *adenylate cyclase* (Table 2). These genes are not only present in all classes of pathogen, but in all fungi. Evidently, pathogens have recruited these ubiquitous genes to control pathogenicity. Other genes, such as *CAP20* and *gEgh16/MAS*, are also present in diverse pathogens but appear

Table 2 Pathogenicity genes identified from different pathogens. Table adapted from Idnurm and Howlett (2001) and COGEME website (http://cogeme.ex.ac.uk/).

Genes involved in pathogenicity	Organisms
Protein kinase A	*B. graminis*, *M. grisea*, *Mycosphaerella graminicola*, *B. cinerea*, *U. maydis*, *C. trifolii*
MAP kinase	*C. lagenarium*, *B. graminis*, *C. heterostrophus*, *B. cinerea*, *F. solani* f. sp. *pisi*, *F. oxysporum*, *P. teres*, *U. maydis*
Adenylate cyclase	*B. graminis*, *M. grisea*, *U. maydis*
Gα	*M. grisea*, *Cryphonectria parasitica*, *C. trifolii*, *U. maydis*
CAP20	*C. gloeosporioides*, *B. graminis*, *M. grisea*
Egh 16/MAS	*B. graminis*, *M. grisea*

to be absent from non-pathogens. Neither type of gene is able to distinguish pathogen classes.

Genes expressed only in some groups

The identification of genes that are specific to a given group of fungi is currently a difficult problem. We only have access to two pathogen genome sequences and there is no immediate prospect of a biotroph being fully sequenced. The absence of a gene from an EST database can only be regarded as preliminary evidence of its absence from the genome because such libraries only sample the more highly expressed genes. We can, however, ask whether genes regarded as characteristic of necrotrophs are present in biotroph EST databases. Extensive EST data are available for only one biotroph, *Blumeria graminis.* Cell-wall-degrading enzyme genes such as two beta-glucanases and a cutinase are present in *B. graminis*. Furthermore, several P450 genes, most similar to the sterigomatocystin biosynthesis cluster from *Aspergillus nidulans*, and several MFS-MDR genes are also present (Soanes *et al.*, 2002; Thomas *et al.*, 2001; and Cogeme.ex.ac.uk). We could therefore postulate that *B. graminis* is equipped to make and export toxins. Although this may be true, these genes are more likely to function in normal metabolism and growth.

It appears that it is easy to find similar genes expressed (and important for pathogenicity) in all groups of pathogens but difficult to find genes expressed only in certain groups. It is therefore questionable to base a classification scheme on current molecular genetic studies of pathogenicity.

Hemibiotrophy genes

The term 'hemibiotroph' appears to be used in two or three distinct contexts (Perfect and Green, 2001). The strict context involves temporally separate and non-overlapping biotrophic and necrotrophic fungal structures formed in different tissues in the plant. This first context is only consistently applied to members of the *Colletotrichum* genus and more rarely to species like *Phytophthora infestans*. The second use of the term hemibiotrophy is applied to species such as *Cladosporium fulvum*, *Mycosphaerella graminicolal Septoria tritici* and *Pyrenopeziza brassicae*, which have an extended (4–14 days) asymptomatic phase (which is assumed to correspond to the acquisition of nutrient from living cells) followed by the increasing development of plant tissue damage. These species do not appear to have differentiated infection hyphae. Neither of these criteria appears to apply to the rice blast pathogen *M. grisea.* The initially attacked cell is killed, leading quickly to a spreading necrotic lesion. Nonetheless, two recent papers have defined blast as a hemibiotroph (Hammond-Kosack and Parker, 2003; Mendgen and Hahn, 2002).

There is extensive structural evidence that the hemibiotrophic *Colletotrichum* species undergo two distinct phases during invasion (Perfect and Green, 2001). The first phase involves the generation of intracellular hyphae within intact plant cells—the biotrophic phase. Later, extracellular hyphae ramify between host cells—the necrotrophic phase. The suggestion that these fungi undergo a distinct metabolic switch from biotrophic to necrotrophic growth was boosted by the discovery of a gene that functions between the biotrophic and necrotrophic phases (Dufresne *et al.*, 2000). The gene *CLTA1* encodes a GAL4-like transcriptional activator, which is consistent with a role in reprogramming metabolism. It is clear that all pathogens are obliged to alter metabolic fluxes in numerous ways upon penetration to prepare for proliferation. The recent study of glycerol uptake in *Colletotrichum gloeosporioides* presents what appears to be the first (albeit indirect) evidence that the intracellular hyphae are involved in nutrient uptake (Wei *et al.*, 2004). This is a key postulated attribute of the hemibiotrophs and seems to be a priority subject for study.

FUNGICIDE SENSITIVITY

The study of fungal pathogenicity has a role to play in the discovery and characterization of fungicides. In the context of the classification of fungal pathogens, we may ask whether fungicide sensitivity differs systematically between either the nutrition classes, biotrophs, necrotrophs and hemibiotrophs, between taxonomic classes or between sites of infection. Hewitt (1998) has provided a valuable summary of publicly available disease spectrum data. Generally speaking, commercially available fungicides have very broad spectra and we should bear in mind that fungicides

with broad spectra are much more likely to progress through to commercial release than compounds with narrow spectra.

Different C14 DMI fungicides control all the listed ascomycete and basidiomycete species, but do not control the oomycete pathogens. The strobilurins control some ascomycetes, some basidiomycetes but also all the oomycetes. Metalaxyl only controls oomycetes. Among the fungicides listed it is not possible to find a single compound that specifically controls only biotrophs, necrotrophs or hemibiotrophs. Some fungicides have limited spectra. For example, quinoxyfen controls just *Erysiphe* (*Blumeria*) and *Magnaporthe* (but not other powdery mildews).

Analysis of the data does not support the idea that spectrum can be explained by modes of infection; there is no support for fungicide specificity between the biotrophs and necrotrophs. Some fungicides are specific to the very highest taxonomic levels (i.e. the oomycetes vs. the true fungi) but specificity even between basidiomycetes and ascomycetes is rare.

DEFENCE GENES AND DEFENCE RESPONSES

The cloning of disease resistance genes over the last decade has revealed that the many of the encoded proteins display a remarkable degree of structural similarity. Proteins that contain leucine-rich and either kinase, nucleotide-binding, Toll-interleukin repeat or coiled-coil domains control resistance to fungal, bacterial, viral and nematode pathogens (reviewed in Hammond-Kosack and Parker, 2003). Most of the cloned fungal resistance genes confer resistance to biotrophic pathogens, mainly because cultivar-specificity and simple genetics were important technical requirements in the cloning strategy. Two genes conferring resistance to necrotrophs (*Asc-1* and *Hm1*) are structurally distinct. The *M. grisea* resistance gene *Pi-ta* conforms to the majority class. Evidently, it is too early to say whether there is a clear difference in the structure of genes conferring resistance to biotrophic and necrotrophic pathogens. A more likely prediction is that genes that confer resistance to pathogens that rely on specific toxins will be of diverse structures, as they will be directed at detoxification or circumvention of the toxin; resistance that depends on recognition of macromolecular fungal components such as peptides or oligosaccharides will conform to a limited range of motifs that can be modified to form a vast range of detailed structures.

Following recognition of the pathogen, the plant mounts a defence response. The genetic dissection of the defence response has been particularly elaborate in *Arabidopsis*, for which various studies have identified several key genes and processes (reviewed in Hammond-Kosack and Parker, 2003). A consensus has emerged whereby *NPR1*- and salicylate-controlled processes provide protection exclusively against biotrophic pathogens, whereas *COI1* and EIN2 (via jasmonate and ethylene) control necrotrophic pathogens. The biotrophs include both powdery and downy mildews; the necrotrophs in question include *Pythium irregulare*, *Alternaria brassisicola*, *Botrytis cinerea* and *Plectosphaerella cucumerina* (Tierens *et al*., 2002).

The salicylate and jasmonate pathways are, to some extent, antagonistic defence responses (Spoel *et al*., 2003). This implies that defence against biotrophs and necrotrophs might likewise be antagonistic. There are few studies to test this but one such example is the relation between *B. graminis* and *M. grisea* resistance in barley (Jarosch *et al*., 2003). In this study it was shown that mildew resistance conferred by the *mlo* gene was accompanied by enhanced susceptibility to the necrotroph. A similar antagonistic resistance relationship occurs between the biotroph *Puccinia coronata* and the necrotroph *Cochliobolus victoriae* in oats (Wolpert *et al*., 1994).

As yet, *Arabidopsis* is the only plant species for which mutational analysis of defence has revealed defects in the response to a representative sample of both necrotrophic and biotrophic pathogens. However, the number of pathogens is still small and notably lacks hemibiotrophs, although a *Colletotrichum* pathogen has recently been described (Narusaka *et al*., 2003). Further studies in other plant species, such as *Medicago truncatula*, which harbour a much wider range of pathogens as well as *Rhizobium* and mycorrhizae, are eagerly awaited.

CONCLUSIONS

The classification of pathogenic fungi into a small number of divisions has been confounded by widespread disagreement regarding the boundaries of the classes. These disagreements reflect both the limited scope of the definitions of each class as well as a profound paucity of data on which the classifications were based. The purpose of this review has been to see whether the flood of new data can resolve this situation and we conclude there is reason for optimism. The notion of classifying fungal pathogens into groups based on nutritional mode does not appear to be supported either by studies of fungal genomics, genetics or fungicide sensitivity. However, a division based on (1) the presence/absence of haustoria is well supported by cell biological data and on (2) the genetics and biochemistry of defence, although at an early stage, is very promising.

The definition of biotrophy and necrotrophy as deriving energy from living or killed cells is seductively brief but it lacks precision. Does it mean all the energy or just the majority; does it mean throughout the life cycle or just at some stages? For example, a spore on a plant surface is very likely to be deriving most if not all of its energy from stored sources (Solomon *et al*., 2003). Only after it has penetrated the plant is it likely to derive its energy from plant resources. When we consider a powdery mildew colony, with its haustoria in the epidermal cell, we may feel confident that all the energy is derived from the living plant cell harbouring the haustoria. However, in the case of many pathogens, notably *Leptosphaeria maculans*, the fungus is found not only in the vicinity of the lesion, where some of the plant cells are

dead, but also well away down the petiole and into the stem. It is far from clear whether such 'scout' hyphae are being supplied with nutrient by the mycelium feeding off dead cells in the lesion. Indeed, it seems more likely that opportunistic sources of nutrient are used wherever the mycelium is growing. It is relevant to consider that studies of *C. fulvum* have shown that intercellular spaces are far more abundant sources of nutrient than was previously assumed (Solomon *et al.*, 2003). In the case of rust fungi, we have strong direct evidence that the haustoria take up nutrient (Voegele *et al.*, 2001). Such studies need to be extended to other fungal structures and species.

Molecular genetic and genomic studies of fungal pathogenicity genes have so far emphasized the similarities rather than the differences between species. This is likely to be an artefact of the still primitive state of our knowledge. Comparison of genomes has, however, revealed that some gene families encoding pathogenicity-related functions (concerned with toxin production) are amplified in pathogens (*Cochliobolus*, *Fusarium* and *Botrytis*) compared with non-pathogens (Yoder and Turgeon, 2001). It will be interesting to see if the rust and mildew genomes have gene family membership levels closer to the saprophytes than the toxigenic fungi. Given that *Blumeria* has been shown to possess cell-wall-degrading enzymes, we might expect that the key factors would be regulation and spatial expression.

The small sample of pathogens that are distinguished by reactions to *Arabidopsis* mutations is very tantalizing. A classification system for fungal pathogens based on plant response would be highly meaningful. It may be no coincidence that the fungal pathogens controlled by *NPR1* possess haustoria, whereas the species controlled by *COI1* and *EIN2* lack haustoria. Haustoria are the most specialized structures possessed by fungal pathogens. Cytological characteristics have been defined in great detail (Hahn and Mendgen, 2001; Perfect and Green, 2001; Mendgen and Hahn, 2002). The powdery and downy mildews, rusts and *P. infestans* share these structures. They are also present in VA mycorrhiza and some parasitic plants. Species such as *M. grisea*, *M. graminicola* and *C. fulvum* clearly fall outside this class.

Finally, we should note that division of fungal pathogens into two classes—haustorial/NPR1 and non-haustorial/COI1/EIN2—does not resolve fungicide spectrum any more effectively than the nutrition-based system. We should not be surprised at this as no current fungicides target the development of haustoria. The isolation of genes controlling haustorial development may be used in the screening of compounds that will interfere with this key process, although the spectrum of such compounds may not satisfy the commercial imperatives of fungicide marketing.

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