**REVIEW
ARTICLE****Molecular dissection of fungal
phytopathogenicity**Richard Oliver¹ and Anne Osbourn²

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¹Norwich Molecular Plant Pathology Group, School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, UK²Sainsbury Laboratory, John Innes Centre, Norwich Research Park, Norwich NR4 7UH, UK**Keywords:** fungal phytopathogenicity, targeted gene disruption, toxins, black box approaches**What is pathogenicity?**

Fungi constitute the most diverse and damaging group of plant pathogens and have attracted a considerable amount of interest as challenging and important subjects for research in plant disease. Attention has focused on identifying factors that are required for pathogenicity. Pathogenicity determinants are generally regarded as being necessary for disease development but not for normal growth on artificial media, although this division is clearly arbitrary and is not applicable to obligate pathogens. This review is concerned with the progress that has been made towards elucidating the role of 'basic pathogenicity determinants' in plant infection. A detailed knowledge of such determinants will be invaluable in developing our understanding of the way in which these organisms interact with their plant hosts. It should also enable us to identify key processes that may be targeted in the search for durable, effective and environmentally benign strategies for disease management. Some fungi exhibit host-cultivar or species specificity involving classical gene-for-gene interactions between fungal avirulence genes and plant resistance genes. This area has been reviewed recently by De Wit (1992). Avirulence can be regarded as being superimposed on the fundamental ability of the fungus to infect plants and will not be considered further here.

Different mechanisms for different fungi

Great diversity exists among pathogenic fungi in their lifestyles and the symptoms which they cause, and so it is likely that different attributes will be important for different fungi to cause disease. For example, infection may be initiated from germinating spores or resting structures, or directly by mycelium surviving on plant debris or in the soil. Many foliar pathogens invade their hosts through naturally occurring openings or wounds. Others penetrate the intact plant surface directly, either by mechanical force, or by enzymic degradation of the

cuticle. Some fungi appear to show very little differentiation during infection, while others form runner hyphae, infection hyphae, appressoria, infection pegs or haustoria. In general the more differentiated structures are associated with the more refined lifestyle of the biotrophs. Biotrophic fungi such as the rusts and mildews parasitize living tissue, and inflict little damage. Although macroscopic symptom expression is often limited to sporulation of the pathogen, serious yield losses may still ensue. In contrast, necrotrophic fungi kill or debilitate host cells, giving a spectrum of symptoms which may include lesions, rotting, wilting or hypertrophies. The production of cell wall degrading enzymes and toxins has often been postulated to be important in diseases caused by necrotrophic fungi.

During growth and differentiation within the plant, the fungus is likely to encounter various hurdles presented by the host. Many plants contain pre-formed inhibitors of fungal growth, or produce antibiotics (phytoalexins) in response to attack. The nature and level of the substances which are encountered will depend on the host plant, its age, on the mode of infection and on the tissue specificity of the pathogen. There is a considerable body of literature proposing that the ability to detoxify or tolerate these molecules is essential for pathogenicity of some fungi. Other plant defence molecules such as PR proteins (which include chitinases and 1,3- β -glucanases) are also produced in response to infection, and some of these have been demonstrated to have antifungal properties (Cutt & Klessig, 1992). The ability to suppress or avoid such plant responses has been postulated to be important in some interactions (Shiraishi *et al.*, 1992).

Testing gene function by targeted disruption

Biochemical, immunocytochemical and classical genetical approaches have implicated various fungal products in pathogenesis. Much of this evidence is circumstantial. Classical genetic analysis of mutants can provide evidence

Table 1. Testing gene function by targeted disruption – is there a role in pathogenicity?

Nature of process	Fungus	Molecular determinant	Reduction in pathogenicity?	Reference
Overcoming physical barriers	<i>Nectria haematococca</i>	Cutinase	No	Stahl & Schäfer (1992)
	<i>Magnaporthe grisea</i>	Cutinase*	No	Sweigard <i>et al.</i> (1992b)
	<i>Cochliobolus carbonum</i>	Endo-xylanase*	No	Apel <i>et al.</i> (1993)
		Endo-polygalacturonase	No	Scott-Craig <i>et al.</i> (1990)
		Exo- β -1,3 glucanase*	No	Schaeffer <i>et al.</i> (1994)
	<i>Penicillium olsonii</i>	Polygalacturonase*	No	Kusserow & Schäfer (1994)
Detoxification of:				
(a) Pre-formed inhibitors	<i>Gaeumannomyces graminis</i>	Avenacinase	Yes	Osbourne <i>et al.</i> (1994)
	<i>Gloeocercospora sorghi</i>	Cyanide hydratase	No	Wang & VanEtten (1992); VanEtten <i>et al.</i> (1994)
(b) Phytoalexins	<i>Nectria haematococca</i>	Pisatin demethylase	No?	VanEtten <i>et al.</i> (1994)
Toxin production:				
(a) Non-specific	<i>Gibberella pulicaris</i>	Trichodiene synthase	Yes	Desjardins <i>et al.</i> (1992)
(b) Host-specific	<i>Cochliobolus carbonum</i>	HC-toxin synthetase 1	Yes	Panaccione <i>et al.</i> (1992)
Other:	<i>Ustilago maydis</i>	Mating type	Yes	Banuett, 1992
		<i>sid1</i> (siderophore biosynthesis)	No	Mei <i>et al.</i> (1993)
		Chitin synthase*	No	Gold & Kronstad (1994)
	<i>Magnaporthe grisea</i>	MPG1 (a hydrophobin?)	Yes	Talbot <i>et al.</i> (1993)
	<i>Cladosporium fulvum</i>	Extracellular protein ECP1	No	Honée <i>et al.</i> (1994)
		Extracellular protein ECP2	No	Marmeisse <i>et al.</i> (1994)

* Mutants still had residual enzyme activity.

for association of a particular phenotype with pathogenicity but, as with biochemical and immunocytochemical evidence, does not unequivocally prove that this phenotype is required for the ability to cause disease. Furthermore, mutants generated using chemicals or radiation may harbour multiple mutations; in principle this problem can be resolved by genetic analysis, but in practice the sexual stages of many plant pathogenic fungi cannot be obtained in the laboratory.

The relatively recent development of DNA-mediated transformation techniques for phytopathogenic fungi has allowed direct testing of the importance of putative pathogenicity determinants (Leong, 1988; Fincham, 1989). Gene disruption by transformation with cloned DNA sequences has enabled the construction of specific, defined mutants which are defective only in the gene of interest. The genes to be tested have been isolated using a range of approaches which include reverse genetics (Soliday *et al.*, 1989; Scott-Craig *et al.*, 1990; Panaccione *et al.*, 1992; Wang & VanEtten, 1992; Apel *et al.*, 1993; Van den Ackerveken *et al.*, 1993; Osbourne *et al.*, 1994; Schaeffer *et al.*, 1994), complementation of mutants with DNA libraries (Mei *et al.*, 1993), use of heterologous DNA probes (Desjardins *et al.*, 1992; Sweigard *et al.*, 1992a) or of oligonucleotide probes or primers based on conserved regions of genes (Gold & Kronstad, 1994; Kusserow & Schäfer, 1994), identification of differentially expressed genes (Talbot *et al.*, 1993), or by heterologous expression (Kronstad & Leong, 1989; Weltring *et al.*, 1989; Schulz *et al.*, 1990; Froeliger & Leong, 1991; Bölker *et al.*, 1992). Comparison of the pathogenicity of

mutants generated by gene disruption with that of the wild-type allows a stringent assessment of the role of the gene in plant infection. Since transformation may induce unexpected changes in chromosome structure or complement, it is prudent to evaluate the phenotype of a number of independent transformants, including those which have not undergone disruption at the gene of interest. Using targeted gene disruption, a number of properties of different fungi have been tested for a role in pathogenesis (Table 1). However this approach is not readily applicable to diploid or polyploid fungi such as potato late blight (*Phytophthora infestans*) because homologous recombination of all copies of the resident gene must be achieved. Also our inability to transform the obligate fungi such as the rusts and mildews remains a critical barrier to the testing of gene function in these fungi.

Factors involved in overcoming physical barriers

Cutinase

Cutinase has been implicated in facilitating enzymic degradation of the plant cuticle by foliar fungal pathogens which directly penetrate the leaf surface. The best-studied cutinase is that of *Nectria haematococca* (*Fusarium solani* f.sp. *pisi*) (Schäfer, 1993). Until recently the evidence supporting a role for this cutinase in fungal pathogenicity was highly persuasive (Kolattukudy, 1985). Cutinase activity is associated with spores of *N. haematococca* prior to germination, indicating that the enzyme is likely to be

important for the early stages of infection. Using immunological techniques cutinase was observed at the site of infection. When isolates with low cutinase activity were supplemented with exogenous cutinase at inoculation their pathogenicity was increased; co-inoculation of pathogenic isolates with anti-cutinase polyclonal antisera or serine hydrolase inhibitors resulted in a decrease in pathogenicity (although the specificity of the inhibitors and antisera for cutinase has not been adequately demonstrated; Schäfer, 1993). A single undefined cutinase-minus mutant generated by UV showed an 80–90% reduction in cutinase activity and an associated reduction in pathogenicity (Danzig *et al.*, 1986). All this evidence, while supportive of a role for cutinase in pathogenicity, is circumstantial.

More recently, the cloning of a cutinase gene from *N. haematococca* and the development of a transformation system for this fungus have paved the way for gene disruption experiments (VanEtten & Kistler, 1988; Soliday *et al.*, 1989). The *N. haematococca* strain used in the disruption experiment contained a single cutinase gene, as judged by homology to a cutinase gene from another *N. haematococca* isolate. Disrupted mutants had no detectable residual activity when grown under inducing conditions *in vitro* and were clearly reduced in ability to grow on cutin. However loss of this cutinase activity had no obvious effect on pathogenicity to pea (Stahl & Schäfer, 1992). The possibility remains that there is an additional uncharacterized cutinase activity in *N. haematococca* which is not synthesized under the inducing conditions used in these studies. If so, the gene encoding it does not share homology with the cloned cutinase gene. Alternatively cutinase may not be important for pathogenicity to pea (Schäfer, 1993). More recently, however, Rogers *et al.* (1994) report that in their experiments *N. haematococca* mutants disrupted in the cutinase gene were significantly reduced in pathogenicity. These observations contradict those of Stahl & Schäfer, and so the role of cutinase in pathogenicity of *N. haematococca* remains controversial.

A gene encoding cutinase activity in the rice blast fungus *Magnaporthe grisea* has also been cloned and disrupted (Sweigard *et al.*, 1992a, b). The mutant had wild-type pathogenicity but also had residual cutinase activity, and so the role of cutinase activity in pathogenicity of *M. grisea* has not yet been definitively tested.

Plant cell wall degrading enzymes

Many plant pathogenic fungi produce a diverse range of plant cell wall degrading enzymes. During pathogenesis these enzymes may be needed for fungal nutrition and/or to facilitate spread of the fungus through the host tissue. The polymer-degrading enzymes which are important for disease are likely to vary between different fungus/plant interactions and at different stages of infection (Cooper *et al.*, 1988). Plant cell wall degrading enzymes are also of considerable interest because the enzymes themselves (in the case of xylanases) or the products of their action (in the case of pectin-degrading enzymes) may trigger plant defence responses (Walton, 1994).

So far, genes encoding four plant cell wall degrading enzymes have been disrupted (endo-xylanase, endo-polygalacturonase and exo-1,3- β -glucanase genes from *Cochliobolus carbonum*, and polygalacturonase from *Penicillium olsonii*; Table 1) (Scott-Craig *et al.*, 1990; Apel *et al.*, 1993; Kusserow & Schäfer, 1994; Schaeffer *et al.*, 1994). With the exception of endo-polygalacturonase from *C. carbonum* the mutants all had residual enzyme activities. No effect on pathogenicity was demonstrated for any of the disrupted mutants including the *C. carbonum* endo-polygalacturonase mutant. The results suggest that endo-polygalacturonase is not required for pathogenicity of *C. carbonum*. These experiments illustrate the problems associated with testing the roles of these enzymes by gene disruption. The existence of multiple enzyme forms encoded by different genes means that disruption experiments may only eliminate a component of the enzyme activity under investigation. However multiple isoforms are not always an indication of genetic complexity, and may arise from post-translational modifications (Caprari *et al.*, 1993) or multimer formation.

Most studies of plant cell wall degrading enzymes have focused on *defined* enzyme activities induced in culture by model substrates or plant cell wall preparations. However it is likely that a more comprehensive knowledge of the full spectrum of enzymes which are produced *in planta* will be needed in order to identify enzymes which may be important for pathogenesis. In theory the ideal starting point for characterizing such enzymes is infected plant material, but this is complicated by the presence of enzymes of plant origin. A complementary approach suggested by Walton (1994) involves the characterization of *all* genes which are induced *in vitro* by plant cell wall material. This should allow the identification of genes encoding enzymes that are biochemically intractable, i.e. those which are not induced by, or active on, model substrates, or which are difficult to purify. The role of these differentially expressed sequences in pathogenicity can then be tested by gene disruption.

Detoxification of plant substances by fungi

Tolerance to, or detoxification of, antifungal substances produced by plants has been predicted to be a requirement for pathogenicity of certain fungi to some plants. The importance of detoxifying enzymes has been characterized in detail for three fungi, *Gaeumannomyces graminis* var. *avenae* and *Gloeocercospora sorghi* (which both detoxify pre-formed inhibitors of fungal growth) and *N. haematococca* (which detoxifies the pea phytoalexin pisatin).

Detoxification of pre-formed antifungal molecules

Oat-attacking isolates of the soil fungus *G. graminis* (*G. graminis* var. *avenae*) are relatively insensitive to the toxic effects of the pre-formed oat root saponin, avenacin, while var. *tritici* isolates are unable to attack oats and are avenacin-sensitive. The resistance of var. *avenae* isolates to the saponin is attributable to their ability to produce the enzyme avenacinase, which deglycosylates avenacin to less toxic forms (Turner, 1961; Crombie *et al.*, 1986).

Clones encoding avenacinase were obtained by screening a cDNA expression library with polyclonal antisera raised against the purified enzyme, and avenacinase-minus mutants were generated by targeted disruption. The mutants were unable to infect oats, but were still fully pathogenic to the non-saponin-containing host, wheat. This is the first genetic proof that saponin detoxification determines host range in a plant pathogenic fungus (Osborn *et al.*, 1994). The roles of other saponin-detoxifying enzymes, such as the tomatine-degrading enzymes produced by a number of fungal pathogens of tomato, remain to be tested genetically (Roddick, 1974; Osborn *et al.*, 1994).

G. sorghi (a soybean pathogen) produces the enzyme cyanide hydratase (CHT), which converts cyanide to formamide. Production of this enzyme has been associated with the ability of plant pathogenic fungi to infect cyanogenic plants and so CHT has been implicated as a determinant of pathogenicity for such hosts (Fry & Evans, 1977). The *cht* gene was cloned from *G. sorghi* by a reverse genetics approach, and mutants disrupted in this gene were highly sensitive to cyanide *in vitro* (Wang *et al.*, 1992; Wang & VanEtten, 1992). However pathogenicity was unaffected, indicating that CHT is not likely to be a key factor in facilitating fungal invasion of cyanogenic plants (VanEtten *et al.*, 1994).

Detoxification of phytoalexins

Isolates of *N. haematococca* which are pathogenic to pea are able to detoxify the pea phytoalexin pisatin by the production of the substrate-inducible cytochrome P-450 monooxygenase, pisatin demethylase (PDA) (VanEtten *et al.*, 1989). The genetics of pisatin demethylation in *N. haematococca* are complex. A total of six genes encoding PDA activity have been identified, although field isolates rarely have more than two of these genes. Classical genetic studies indicated that only those isolates which can quickly and efficiently detoxify pisatin are highly pathogenic to pea. However isolates do exist which are highly active against pisatin but are only weakly pathogenic, indicating that factors other than pisatin demethylation are also important for pathogenicity. More recently, studies of the electrophoretic karyotype of *N. haematococca* have revealed that all of the genetically characterized *pda* genes are on dispensable chromosomes or on dispensable portions of chromosomes and can be lost during meiosis, making the observed genetic linkage of pathogenicity and PDA activity less clear-cut than it initially appeared (Miao *et al.*, 1991).

One of the genes conferring a high level of PDA activity was cloned by expression in *Aspergillus nidulans* (Weltring *et al.*, 1989). Introduction of the cloned gene into Pda⁻ isolates of *N. haematococca* with a low level of pathogenicity to pea conferred PDA activity and increased pathogenicity (Ciuffetti *et al.*, 1988). However, *N. haematococca* mutants disrupted in the *pda* gene did not show the anticipated reduction in pathogenicity (VanEtten *et al.*, 1994). These experiments have led VanEtten and co-workers to propose that other determinants of pathogenicity to pea are also located on dispensable chromo-

somes which can be lost along with PDA activity during meiosis. The existence of such determinants is currently being investigated by detailed characterization of the dispensable chromosomes.

Toxins produced by fungi

Non-specific toxins

Many fungi produce non-specific toxins that damage many plant species. Among these are the trichothecenes, sesquiterpene epoxides produced by a number of *Fusarium* species including *F. sporotrichioides*, *Gibberella pulicaris* (*F. sambucinum*) and *Gibberella zeae* (*F. graminearum*). The gene encoding trichodiene synthase (*tox5*), which catalyses the first unique step in trichothecene biosynthesis, was originally cloned from *F. sporotrichioides* by a reverse genetics approach (Hohn & Beremand, 1989). The equivalent *tox5* gene was isolated from *G. pulicaris* by homology, and *tox5* *G. pulicaris* mutants were generated by gene disruption (Desjardins *et al.*, 1992, 1993). The mutants were reduced in pathogenicity to parsnip but not to potato, indicating that trichothecenes are important for some interactions and not for others. Similar results have been obtained for *tox5* mutants of *G. zeae*; the mutants showed reduced pathogenicity to seedlings of the wheat cultivar 'Wheaton', but wild-type pathogenicity to other wheat cultivars, corn, oats and rye (Proctor *et al.*, 1993).

Host-selective toxins

The best characterized instances of production of host-selective toxins by plant pathogenic fungi involve members of the genus *Cochliobolus* (reviewed recently by Panaccione, 1993). Within this group of fungi, toxin production has been implicated in determining: (a) virulence of race T of *C. heterostrophus* to maize lines with Texas type cytoplasmic male sterility (*cms-T*) (T-toxin); (b) pathogenicity of *C. carbonum* race 1 to certain maize varieties bearing the dominant allele of the *Hm* gene (HC-toxin) – these varieties are resistant to non-toxin producing races; (c) the ability of *C. victoriae* to cause blight on Victoria oats (victorin). The three toxins are structurally distinct. T-toxin is a linear polyketide, HC-toxin is a cyclic tetrapeptide, and victorin is a chlorinated cyclic pentapeptide. Toxin production is controlled by a single gene in each of these three species (*tox1* in *C. heterostrophus*, *tox2* in *C. carbonum* and *tox3* in *C. victoriae*).

(a) T-toxin production by *C. heterostrophus*. Characterization of the *tox1* locus of *C. heterostrophus* has involved the generation of near-isogenic Tox⁻ mutants using a variety of strategies. Chemically induced mutants have been isolated using a procedure designed to enrich for Tox⁻ mutants, which incorporates the target of the toxin, the T maize mitochondrial protein, URF 13 (Yang *et al.*, 1994). Transformation-mediated generation of mutants has also been achieved using restriction enzyme mediated insertion of plasmid DNA (Kuspa & Loomis, 1992; Yoder *et al.*, 1994). A third strategy was based on the prediction that a polyketide synthase is required for T-toxin biosynthesis. Oligonucleotide primers to conserved regions of known polyketide synthases from other

organisms were used to generate a PCR product from *C. heterostrophus* DNA. After transformation with a plasmid bearing this DNA fragment a Tox^- mutant was isolated which had undergone a deletion event (Yoder *et al.*, 1994). These combined approaches have yielded a total of nine mutants. For all nine, the Tox^- mutations map to the genetically defined *tox1* locus, and the Tox^- phenotype is associated with a corresponding reduction in the ability to cause disease. Crosses between mutants suggest that the *tox1* locus is a large gene (as would be predicted if it were a polyketide synthase) or a gene cluster. Interestingly one of the mutations, while mapping to *tox1*, is not linked to the other eight mutations. This has been attributed to irregular recombination events associated with a reciprocal translocation breakpoint that is at, or very close to, the *tox1* locus. These mutants should now permit fine structure mapping of the *tox1* region and enable the *tox1* gene to be isolated.

(b) HC-toxin production by *C. carbonum*. The enzyme HC-toxin synthetase 1 (HTS-1), which is involved in HC-toxin biosynthesis in *C. carbonum*, was purified and antibodies raised against it were used to screen a cDNA library. The *Hts-1* gene was located on a duplicated region of 22 kb which segregated with the *tox2* locus in crosses. This region was absent from Tox^- strains and other *Cochliobolus* species which do not produce HC-toxin (Panaccione *et al.*, 1992). Disruption of both copies of the *Hts-1* gene gave Tox^- mutants which were non-pathogenic when tested on maize varieties with *Hm*, which are normally susceptible to toxin-producing (race 1) isolates. It is now known that *Hm*-specific resistance of maize to infection by *C. carbonum* race 1 is due to production of the detoxifying enzyme HC-toxin reductase (Meeley *et al.*, 1992).

(c) Victorin production by *C. victoriae*. The study of biosynthesis of victorin by *C. victoriae* is not as far advanced as for T-toxin and HC-toxin biosyntheses. Attention has focused on the nature of sensitivity of Victoria oats to the toxin (encoded by the nuclear gene *Vb*) because this gene is closely linked to, or the same as, the gene which confers resistance to specific races of the crown rust fungus *Puccinia coronata*. Wolpert & Macko (1989) have demonstrated specific binding of victorin to a 100 kDa protein in susceptible oats only. Recent evidence indicates that this protein is the P-protein component of glycine decarboxylase (Wolpert *et al.*, 1994). Other workers have reported the binding of victorin to a 100 kDa protein in both susceptible and resistant oat lines, and also to additional proteins, and have questioned whether the interaction of victorin with the 100 kDa protein is the specific component of the interaction (Akimitsu *et al.*, 1993).

Mating type genes and pathogenicity in *Ustilago maydis*

The maize smut fungus *Ustilago maydis* has a complex life cycle involving alternation of a non-pathogenic haploid yeast-like phase and a pathogenic diploid filamentous phase. The rapid development of molecular genetic

technology has contributed to spectacular progress in understanding the genetic basis of pathogenicity in this fungus (reviewed by Banuett, 1992). Fusion of haploid cells is under the control of two mating type loci called *a* and *b*. There must be different alleles at both *a* and *b* for successful fusion to take place. The *a* locus encodes the components of a pheromone response pathway (Spellig *et al.*, 1994), while the *b* locus encodes a regulatory protein belonging to the homeodomain class of DNA-binding proteins (Gillissen *et al.*, 1992). In addition to allowing fusion and filamentous growth the *b* alleles also control pathogenesis. Consequently the functions of the mating type loci are essential for *U. maydis* to cause disease. Mutants defective in enzymes involved in siderophore biosynthesis, or in chitin biosynthesis, have been generated by gene disruption, but were unaffected in pathogenicity (Mei *et al.*, 1993; Gold & Kronstad, 1994). Elucidation of the roles of other genes whose expression is regulated by the *b* locus, and which may be important for the pathogenic phase of the life cycle, is likely to be a fruitful area for future research.

'Black box' approaches – towards a more comprehensive understanding of pathogenesis

While considerable progress has been made in testing the roles of specific properties of fungi, it is unlikely that we will be able to predict all pathogenicity determinants. Use of so-called 'black box' approaches, which can identify factors involved in pathogenesis without any a priori knowledge of their function, will be essential if we are to draw up a comprehensive list of factors which are required for disease development. These approaches have proved very valuable in the understanding of bacterial pathogenesis (Daniels *et al.*, 1988). 'Black box' approaches can generally be subdivided into: (a) the characterization of mutants, and (b) the analysis of fungal genes which are differentially expressed during pathogenesis.

(a) Mutants

The generation of mutants and the characterization of their defects presents a powerful strategy for identification of pathogenicity determinants. Until recently such experiments have involved the use of chemicals or radiation as mutagens, and have employed simple mutant screens which can be performed *in vitro* (e.g. for mutants affected in production of plant cell wall degrading enzymes, for melanin biosynthesis or for appressorial development). These experiments have permitted association of protease with pathogenicity of *Pyrenopeziza brassicae* to oil seed rape (Ball *et al.*, 1991), and of melanin biosynthesis and of other factors affecting appressorial development with pathogenicity of *Colletotrichum lagenarium* (Katoh *et al.*, 1988; Kubo *et al.*, 1991; Perpetua *et al.*, 1994). Complementation of the lesions in these mutants with wild-type DNA from genomic libraries has allowed isolation of the cloned genes of interest. Confirmation of the roles of the genes by targeted gene disruption and characterization of the

cloned DNA by sequence analysis is required in order to confirm these associations.

In principle, transformation-mediated gene disruption is an ideal strategy for mutagenesis because it allows a mutated gene to be cloned simply by isolating the DNA which flanks the insertion (Kuspa & Loomis, 1992). In the past this approach has suffered from problems associated with variation in regenerated protoplasts in the absence of transforming DNA, mutations at sites unlinked to the insertion, non-randomness of insertion and multiple insertions. Despite these problems, recent unpublished observations indicate that this approach, particularly when coupled with restriction-enzyme-mediated integration of plasmid DNA, is proving highly effective in identifying pathogenicity factors in *M. grisea* and *U. maydis* (B. Valent & R. Kahmann, personal communications). Improvements in transformation frequencies and the development of suitable screens to identify mutants defective in pathogenicity may enable the approach to be applied to a number of phytopathogenic fungi.

(b) Differential expression of fungal genes during pathogenesis

Identification of fungal genes which are expressed exclusively or preferentially during growth in the plant may identify genes which are important for pathogenicity, and should also provide a picture of the metabolic requirements of the fungus *in planta*. This approach has the advantage that it can be applied to fungi which cannot be cultured or are not amenable to DNA-mediated transformation, and promises to generate a rapidly expanding catalogue of differentially expressed sequences for a range of fungi with varied lifestyles. The large numbers of DNA sequences which are present in the databases, and the great rate at which new sequences are being added, means that determination of the DNA sequences of the cloned genes may give some clue to their function. Differential screening has allowed the isolation of nine *in-planta*-induced (*ipi*) genes from the potato blight fungus *P. infestans*, and DNA sequence analysis suggests that two of these may encode polyubiquitin and calmodulin, respectively (Pieterse *et al.*, 1991, 1993a, b). Two further genes, one encoding a glycine-rich protein and the other a putative cell adhesion protein, have also been characterized in detail (Pieterse *et al.*, 1994a). Convincing evidence of a role for these genes in pathogenicity requires a test involving gene disruption or some other alternative method of specific interference with gene expression. While gene disruption in *P. infestans* is problematic because the fungus is diploid, the development of techniques for anti-sense RNA inhibition for this fungus should enable the roles of the *ipi* genes in pathogenicity to be investigated (Judelson *et al.*, 1993). The *M. grisea* gene *mpg1*, also identified by virtue of its differential expression *in planta*, shows significant homology to a group of fungal proteins called hydrophobins (Talbot *et al.*, 1993). Disruption of *mpg1* reduced the ability of the fungus to form appressoria, and hence to cause disease. These examples demonstrate the success of 'traditional' differential

screening approaches. More sophisticated strategies such as differential display (Liang & Pardee, 1992; Bauer *et al.*, 1993) may also be valuable in the identification of fungal genes which are differentially expressed *in planta*.

Expression of some *P. infestans ipi* genes, the *mpg1* gene of *M. grisea*, and also the *Cladosporium fulvum* avirulence gene *avr9*, has been demonstrated to be induced *in vitro* by starvation conditions (Talbot *et al.*, 1993; Pieterse *et al.*, 1994b; Van den Ackerveken *et al.*, 1994), suggesting that starvation may be experienced by pathogenic fungi during development in the plant. Conversely, a study of *C. fulvum* starvation-induced genes revealed that some were also plant-induced. DNA sequence analysis indicated that two of these genes are likely to encode alcohol and aldehyde dehydrogenase, respectively (Oliver *et al.*, 1994). Characterization of subsets of fungal genes which respond to different starvation conditions, and studies of the expression of these genes during plant infection, should enable us to make predictions about the conditions under which fungi grow *in planta*.

Differential expression can also be studied at the protein level. For instance, the growth of *C. fulvum* is restricted to the intercellular spaces of tomato leaves, making the characterization of apoplastic proteins from infected plants a fertile source of information about the *C. fulvum*/tomato interaction (Joosten & De Wit, 1988; De Wit, 1992). Two *C. fulvum* extracellular proteins, ECP1 and ECP2, were purified from apoplastic fluid of diseased tomato plants, and the corresponding genes were cloned by reverse genetics. However, mutants generated by disruption of either of these genes were not reduced in pathogenicity (Marmeisse *et al.*, 1994; Honée *et al.*, 1994). Immunological strategies involving monoclonal antibodies specific for components of the haustorial complex in the *Erysiphe pisi*/pea interaction offer the potential for molecular characterization of putative pathogenicity determinants in *E. pisi*, although transformation-mediated gene disruption is not yet possible for this fungus (Mackie *et al.*, 1993).

Where do we go from here?

Targeted gene disruption has allowed the generation of specific mutants defective in various properties which have been implicated in pathogenesis. Some of these properties have been demonstrated to be required for disease development, while others have not (Table 1). Cloning and disruption of genes encoding other factors which have been predicted to be important will further clarify our understanding of the nature of fungal pathogenicity to plants. Complementary approaches for identification of pathogenicity genes involve the generation of pathogenicity mutants by random mutagenesis with subsequent characterization of the induced mutations, and also the analysis of fungal genes which are expressed *in planta*. These approaches do not require a priori knowledge of gene function, and are likely to be of great value in the molecular dissection of phytopathogenicity.

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