Identification and characterization of pathogenicity genes from the rice blast fungus Magnaporthe grisea

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Abstract. *Magnaporthe grisea* is an excellent experimental model for studying fungal pathogencity as it exhibits a very high level of pathogenic specialization and yet is also amenable to study by both classical and molecular genetics. Pathogenicity is a complex trait as it encompasses much of the life cycles of the fungus. This includes infection-related development, the capacity of the fungus to grow in planta, and its ability to debilitate plant metabolism. Identifying essential specialist 'pathogenicity' genes is therefore a significant challenge. A number of strategies have been devised to identify pathogenicity genes in *M. grisea*. Insertional mutagenesis has, for example, recently identified a number of pathogenicity mutants. Biochemical pathways believed to play key roles in the infection process have also been characterized including most notably the melanin biosynthesis pathway. Differential cDNA screening strategies, meanwhile, to identify genes expressed specifically during pathogenesis also offer a powerful technique to identify genes involved in the disease process.

We have used the latter strategy to identify a gene called MPG1 which plays a key role in appressorial formation in *M. grisea*. MPG1 was identified as a cDNA clone representing a fungal transcript expressed preferentially during growth in planta. Mpg1- null mutants were constructed by gene replacement and showed reduced pathogenicity on compatible rice cultivars. This defect was shown to be associated with a reduced ability to elaborate appressoria. The gene encodes a secreted hydrophobin-like protein and is regulated by nutrient starvation. It is highly expressed during appressorial development and may play a role in surface perception. The MPG1 gene product appears to share similar biochemical properties to class I hydrophobins and is known to form a rodlet protein layer on the surface of conidia. The identification of this gene and progress towards its functional characterization will be discussed.

To be a successful pathogen, a fungus has to pass through a well-defined series of physical and biochemical steps which together constitute the disease cycle. This high degree of pathogenic specialization is amply demonstrated by the rice blast fungus *Magnaporthe grisea*, a pathogen of considerable importance to global rice cultivation and of specific concern to rice growers throughout the Mediterranean basin.

Rice blast is a leaf spot disease which is spread by splash dispersal. Under conditions of heavy dew all aerial parts of the plant can be affected; leaf surfaces become speckled with oval lesions, plants are liable to lodging if stems are infected and if the panicle is infected then a severe yield loss results (Ou, 1985).

The infective life-cycle of *M. grisea* begins when an asexual spore lands on a rice leaf and attaches itself to the leaf surface. This is brought about by the release of spore tip mucilage from an apical compartment in the three-celled conidium (Hamer *et al.*, 1988). The conidium germinates, sending out a germ tube which then forms a terminal swelling called an appressorium. This specialised infection cell is the means by which the fungus enters the plant. Turgor pressure builds up inside the appressorium and this forces a penetration peg through the underlying rice cuticle. Once inside, the infection hyphae ramify throughout the epidermal and mesophyll cells. Some five to seven days following infection, conidiophores differentiate and thousands of new conidia are produced to perpetuate the cycle (for review, see Talbot, 1995).

In order to complete its disease cycle, the fungus has to be able to detect and respond to a number of cues. In the pre-penetration phase, for example, the conidium must sense that the conditions are favou-
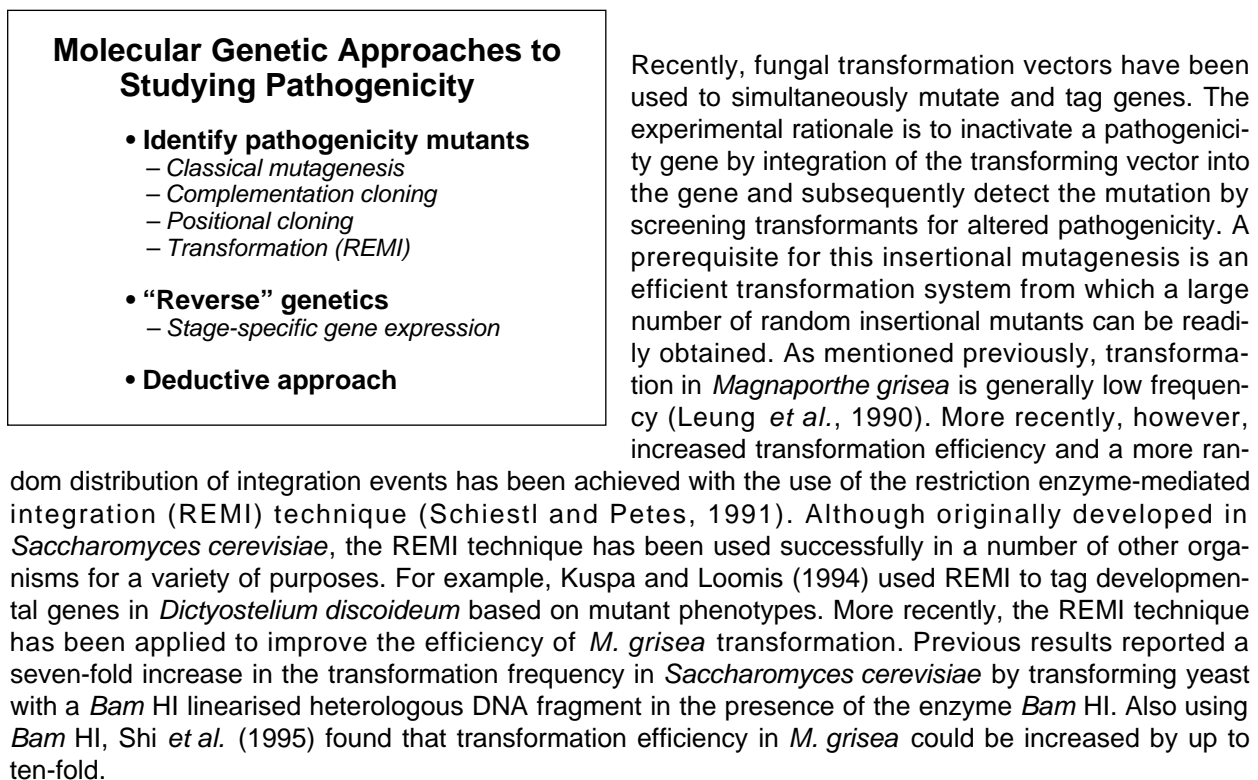
rable for infection; a conducive hydrophobic surface must be present, and conditions of nutrient deprivation to induce subsequent infection-related development. During appressorial development, the germ tube ceases polar growth and biosynthetic pathways direct the formation of melanin to strengthen the cell during the generation of turgor pressure. Once inside the host, polarity is re-established, nutrients are obtained from the plant and the fungus colonises the host tissues whilst remaining impervious to the plant's defence system. Each stage of the disease cycle therefore requires a combination of both structural and regulatory genes and it is because of this, that pathogenicity represents such a complex phenotype. Identifying pathogenicity genes is therefore likely to be a challenging and lengthy process. The whole life-cycle of the fungus must be considered and an attempt made to distinguish between genes which have a secondary effect on pathogenicity and those encoding specific deteminants which have evolved solely for pathogenesis.

I – Molecular genetic approaches to studying pathogenicity
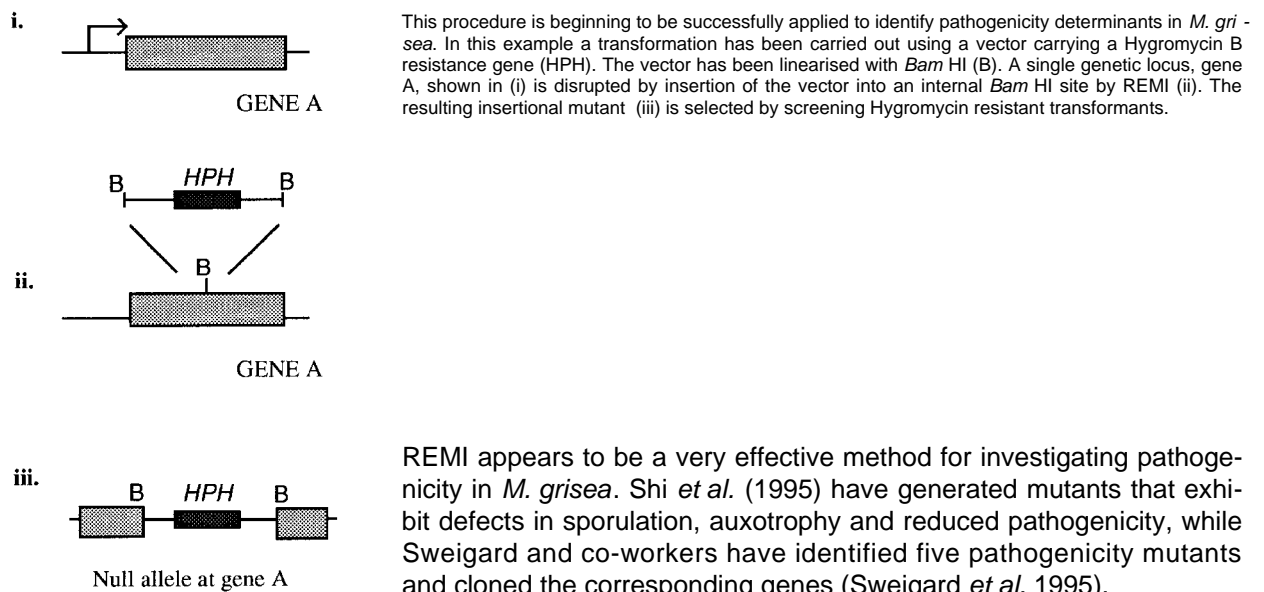
1. Identifying pathogenicity mutants

A wide range of techniques have so far been employed to identify pathogenicity genes and these are summarised in Figure 1. One of the earliest methods used and perhaps the simplest, is to isolate mutants (both naturally occurring ones and those generated by classical mutagenesis) and to test for differences in pathogenicity. The main benefit of this method is that it makes no assumptions about processes likely to be important in pathogenesis. The problem with this method in the past has been the reliance on complementation-cloning to isolate corresponding genes. *M. grisea* is easily transformable but frequencies are too low (20-50 transformants µg^{-1} DNA) to allow routine cloning by this method (Valent and Chumley, 1991). An alternative strategy is to use positional or map-based cloning, which has been used with some degree of success in *M. grisea* to clone avirulence genes (Valent *et al.*, 1994). This strategy uses RFLP and RAPD markers to identify mutant loci and direct chromosome "walks" using hybridisations from neighbouring linked DNA markers (Skinner *et al.*, 1994; Hamer and Givan, 1990; see accompanying chapter by Dioh *et al.*).

Figure 1. Emerging strategies for identifying pathogenicity genes in the rice blast fungus

Molecular Genetic Approaches to Studying Pathogenicity

- **Identify pathogenicity mutants**
  - *Classical mutagenesis*
  - *Complementation cloning*
  - *Positional cloning*
  - *Transformation (REMI)*

- **"Reverse" genetics**
  - *Stage-specific gene expression*

- **Deductive approach**

Recently, fungal transformation vectors have been used to simultaneously mutate and tag genes. The experimental rationale is to inactivate a pathogenicity gene by integration of the transforming vector into the gene and subsequently detect the mutation by screening transformants for altered pathogenicity. A prerequisite for this insertional mutagenesis is an efficient transformation system from which a large number of random insertional mutants can be readily obtained. As mentioned previously, transformation in *Magnaporthe grisea* is generally low frequency (Leung *et al.*, 1990). More recently, however, increased transformation efficiency and a more random distribution of integration events has been achieved with the use of the restriction enzyme-mediated integration (REMI) technique (Schiestl and Petes, 1991). Although originally developed in *Saccharomyces cerevisiae*, the REMI technique has been used successfully in a number of other organisms for a variety of purposes. For example, Kuspa and Loomis (1994) used REMI to tag developmental genes in *Dictyostelium discoideum* based on mutant phenotypes. More recently, the REMI technique has been applied to improve the efficiency of *M. grisea* transformation. Previous results reported a seven-fold increase in the transformation frequency in *Saccharomyces cerevisiae* by transforming yeast with a *Bam* HI linearised heterologous DNA fragment in the presence of the enzyme *Bam* HI. Also using *Bam* HI, Shi *et al.* (1995) found that transformation efficiency in *M. grisea* could be increased by up to ten-fold.

The theory behind REMI is a relatively simple one and is summarised in Figure 2. When chromosomal DNA is incubated with a restriction enzyme, e.g. *Bam* HI, the DNA breaks at several *Bam* HI sites leaving cohesive ends. Many of the breaks simply religate with DNA ligase but if vector DNA is cut with the same restriction enzyme and then mixed with the nicked chromosomal DNA, this vector DNA can become incorporated into the chromosomal DNA. This incorporation is possible because both the chromosomal and vector DNA have the same cohesive ends. The technique also makes use of the organism's natural DNA repair mechanism which is almost certainly stimulated by the restriction endonuclease treatment. It is the latter which is thought to increase the frequency of integration so dramatically (Sweigard *et al.*, 1995). As the restriction enzyme sites are restored, the DNA from the transformants can be digested with *Bam* HI and Southern hybridisation carried out to identify REMI events.

Figure 2. Schematic representation of restriction mediated insertion mutagenesis (REMI)

i.

GENE A

This procedure is beginning to be successfully applied to identify pathogenicity determinants in *M. gri - sea*. In this example a transformation has been carried out using a vector carrying a Hygromycin B resistance gene (HPH). The vector has been linearised with *Bam* HI (B). A single genetic locus, gene A, shown in (i) is disrupted by insertion of the vector into an internal *Bam* HI site by REMI (ii). The resulting insertional mutant (iii) is selected by screening Hygromycin resistant transformants.

ii.

GENE A

iii.

Null allele at gene A

REMI appears to be a very effective method for investigating pathogenicity in *M. grisea*. Shi *et al.* (1995) have generated mutants that exhibit defects in sporulation, auxotrophy and reduced pathogenicity, while Sweigard and co-workers have identified five pathogenicity mutants and cloned the corresponding genes (Sweigard *et al.* 1995).
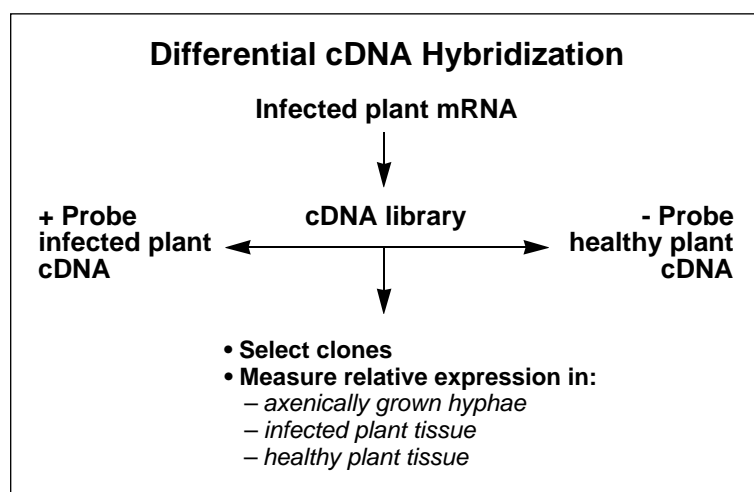
2. "Reverse" genetic approaches to identify stage-specific gene expression

Another powerful approach which has emerged for identifying pathogenicity genes relies on the induction of fungal gene expression during growth *in planta* or under conditions normally experienced during infection. Induced transcripts are detected by a differential cDNA screening procedure. Figure 3 highlights the stages involved in a differential screen to detect fungal genes expressed specifically during growth in host plant tissues (Talbot *et al.*, 1993). Total RNA was extracted from blast-infected rice leaves and poly(A)+ purified to enrich for mRNAs. A cDNA library was then constructed, representing both fungal and plant transcripts expressed during the infection process. A differential cDNA screen was performed by hybridising two different radio-labelled cDNA probes to the library, one derived from healthy rice plant mRNA and one derived from infected rice plant mRNA. Infection-specific cDNA clones were thus selected and counterscreened following plaque-purification with a cDNA probe derived from axenically grown *M. grisea*. In this way cDNA clones representing *M. grisea* genes expressed specifically during pathogenesis were selected. From an initial screen of 500 plaques, 120 infection-specific cDNAs were identified and were sorted into a group of 65 infection-specific rice genes and 25 infection-specific fungal genes. However, of the latter group only 3 were confirmed as being expressed solely during *in planta* growth, or at an elevated level (Talbot *et al.*, 1993). One of these cDNAs was selected and defined a new genetic locus, named *MPG1*. The gene was assayed for its role in pathogenesis by carrying out a one-step gene replacement (Rothstein, 1983). Null mutations were generated at the MPG1 locus in a rice pathogenic isolate, Guy-11. Two *mpg1:HPH* gene replacement mutants were thus selected and tested for their ability to cause disease on a susceptible rice cultivar, CO-39, using the wild type strain and two ectopic transformants from the experiment for control infections. A clear reduction in disease symptoms was apparent, characterised by a lesion deficiency phenotype. This was found to be due to a reduced ability to elaborate appressoria, as shown in Figure 4. The *MPG1* gene encodes a protein with

homology to the fungal hydrophobins, a group of proteins fulfilling diverse roles in fungal morphogenesis in a number of species. Deletion of the *MPG1* gene causes a reduction in cell-surface hydrophobicity, causing an 'easily wettable' phenotype consistent with its role as a hydrophobin. The gene appears to play a role either in surface adhesion or in surface perception with its presence being a rate-limiting step in effective transmission of the inductive signal for appressorial development (Talbot *et al.*, 1993). Interestingly, it was found that *MPG1* is expressed during both the early stages of infection—concomitant with appressorial formation—and during symptom development some 72-96 h later. Clearly the *MPG1* gene plays further, as yet unspecified roles, during pathogenesis. *MPG1* was also shown to be expressed during conditions of starvation. This suggests that general nutrient deprivation may be a key environmental cue for gene expression during early infection. This discovery has recently been used to identify a number of cDNA clones expressed during nitrogen starvation but also expressed during pathogenesis by *M. grisea* (McCafferty and Talbot, unpublished).

Figure 3. Schematic representation of the differential cDNA hybridization procedure used to identfy *M. grisea* genes expressed specifically during growth in rice tissue

Differential cDNA Hybridization

Infected plant mRNA

cDNA library

+ Probe infected plant cDNA

- Probe healthy plant cDNA

• **Select clones**
• **Measure relative expression in:**
– *axenically grown hyphae*
– *infected plant tissue*
– *healthy plant tissue*

A cDNA library constructed from rice leaves is hybridized with radiolabelled cDNA probes from uninfected rice leaf mRNA and infected rice leaf mRNA to identify infection-specific gene expression. Once isolated, fungal genes are identified by a third round of differential screening using a probe derived from axenically grown *M. grisea* mRNA. See text for details.

3. Deductive approaches to identifying pathogenicity genes

The final approach that has proved fruitful in identifying pathogenicity determinants from *M. grisea* is the deductive strategy, in which key processes which may play a role in pathogenesis have been identified and systematically tested to determine their importance.

The first of these to be targeted was the DHN-melanin biosynthetic pathway due to prevalence of melanin in the appressorium cell wall. A number of melanin biosynthetic mutants were isolated, identified by their failure to make the grey melanin pigment. Mutations in three genes *ALB1* (albino), *RSY1* (rosy) and *BUF1* (buff) were found to cause loss of melanization of appressoria and a complete loss in pathogenicity (Howard and Ferrari, 1989; Chumley and Valent, 1990). This is due either to a loss in rigidity of the cell, or due to an inability to generate turgor due to the loss of a compatible solute from the turgid appressorium (Howard *et al.*, 1991). The anti-penetrant fungicide, tricyclazole, which works by inhibiting appressorial melanization highlights the importance of these studies (Viviani *et al.*, 1993).

Conidial morphology mutants have also been found to have pleiotropic effects on pathogenicity. The Smo mutation (Spore Morphology) causes abnormally shaped spores which show a non-polarised shape. Smo mutants show reduced pathogenicity with smaller and fewer lesions present, due to the production of misshapen appressoria (Hamer *et al.*, 1989; Hamer and Given, 1990). Conversely mutations at a locus called CON1 produce highly polarised conidia which, although germinating efficiently, are incapable of forming appressoria, therefore being non-pathogenic (Shi and Leung, 1994). The latter examples show the probable close relatedness of the underlying molecular mechanisms that determine conidial formation and appressorial development (see Talbot, 1995).

II – Perspectives

A number of successful strategies have emerged for identifying pathogenicity genes in the rice blast fungus *Magnaporthe grisea*. These are broadly divided into identifying genes based on the production of a non-pathogenic mutant phenotype, or on the basis of a second criterion such as expression pattern or a defined role in a developmental process. The challenge ahead is twofold; to classify genes into those which are pathogenicity determinants and those which are fitness characters, and secondly, to translate fundamental information about the molecular mechanisms of infection into effective and durable disease control.

Figure 4. Mutations at the MPG1 locus cause a reduced ability to elaborate the infection cell, or appressorium. Light micrographs show detail of a conidium forming an appressorium on a Teflon membrane in the wild type strain Guy-11 and the mpg1- mutant , TM400-2 (Talbot *et al.,* 1993). Bar = 15cm.
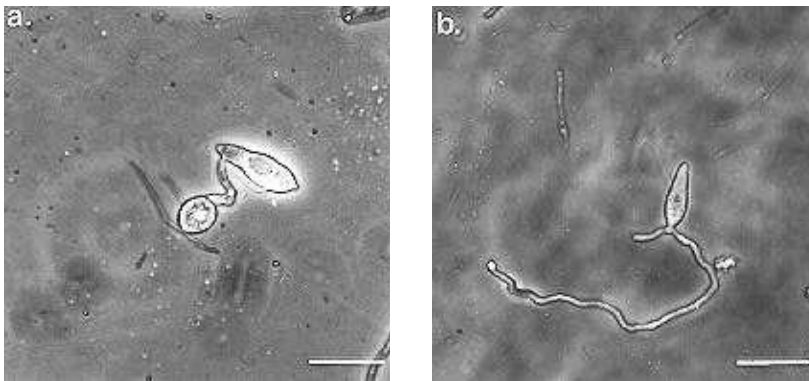
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