CHAPTER 3

MOLECULAR CHARACTERISATION OF MGL1 DISRUPTANTS FROM
THE RICE BLAST FUNGUS, Magnaporthe grisea
INTRODUCTION

Over the last decade, the application of molecular genetic approaches has dramatically improved the understanding of many basic fungal processes such as cell cycle regulation, metabolic gene expression, sporulation, mating and meiosis. By comparison, the understanding of fungal interactions with the plant hosts remain sketchy and preliminary.

*Magnaporthe grisea* executes a series of developmental and metabolic pathways, from the time that spores land on the waxy leaf surface until production of sporulating lesions. A successful invasion results in intracellular pathogen growth, invasion of adjacent epidermal cells as well as underlying mesophyll cells. The pathogen can account for up to 10% of the biomass of infected leaf tips by three days after inoculation (Talbot et al., 1993). Sporulating lesions appear and release thousands of new conidia per day to reinitiate the disease cycle. The interactions between the cells of *M. grisea* and the host, involve a host of complex biological influences, which finally leads to rice blast disease. The initial infection steps during pathogenesis, involve a sequence of critical events, disruption of any of which, may result in failure of the pathogen to colonise host tissue.

Developing a well-defined, efficient system for the introduction of DNA sequences into *M. grisea* is critical for the analysis and understanding of genes that govern features of pathogenesis and host specificity. Transformation of filamentous fungi occurs by insertion of donor DNA into a chromosomal locus if the donor DNA shares genetic homology with the recipient genome, integration can occur by homologous recombination. Recombination of nonhomologous sequences ('illegitimate recombination') can lead to the integration of donor DNA at ectopic sites in the genome (Fincham, 1989). The relative frequencies of integration via homologous or
nonhomologous recombination vary according to the extent of genetic homology shared by donor and recipient, the type of donor DNA molecules (covalent closed circular and linear), and the intrinsic properties of the organism being transformed. Homologous recombination permits the replacement of genomic segments with sequences that have been altered in vitro, generating transformants that are isogenic with the parental recipient strain, except for the changes engineered at the target locus. A two-step (Scherer and Davis, 1979) and one-step (Rothstein, 1983) gene disruption was first developed in *Saccharomyces cerevisiae*, wherein the function of the gene of interest was blocked by the insertion of foreign sequences into the gene. Gene disruption techniques have been extended to several filamentous fungi including *Aspergillus nidulans* (Miller et al., 1985), *Neurospora crassa* (Paietta and Marzluf, 1985), and *Ustilago maydis* (Fotheringham and Holloman, 1989, Kronstad et al., 1989). Genes that play critical roles in pathogenesis, encode proteins that are potential targets for the design of novel, environmentally safe fungicides. Knowledge concerning the set of gene products that is expressed or modified specifically during spore formation, appresorial development and infection have been studied. In *M. grisea*, three genetic loci that play a role in appresorium development have been identified. Mutations at the *SMOl* locus, which result in an abnormal spore morphology, also caused abnormally shaped appresoria (Hamer et al., 1989). Pathogenicity towards rice was reduced in *Smo*− with regard to both the number and the size of lesions formed. *MPG1*, a gene that encodes a small, secreted cysteine-rich protein with characteristics of a fungal hydrophobin, was found to be differentially expressed during infection (Talbot et al., 1993). *mpg1* exhibited a easily wettable
phenotype, indicating a change in the hydrophobicity of the fungal surface. Germ tubes of mpgl were blocked in normal appresorial morphogenesis when grown on normal inductive surfaces. Another gene, a mitogen-activated protein (MAP) kinase was identified which regulates appresorium formation in M. grisea (Xu and Hamer, 1996). Disruption of the MAP-kinase gene (PMKI) inhibited appresorium formation and resulted in pathogenicity. This type of phenotype appeared to be somewhat similar to that of cpkA mutants (Mitchell and Dean, 1995). Cyclic AMP (cAMP)-dependent kinase (CPKA) disruption mutants suggested that CPKA is dispensable for appresorium formation and invasive hyphal growth in plants, but may be essential for appresorial penetration. However, null mutations in a protein kinase A (PKA) catalytic subunit gene, CPKA, have a pleiotropic effects on growth, conidiation, sexual development, and appresorium formation. Another candidate gene, the mitogen-activated protein kinase MPSI, has been shown to be essential for appresorium formation. Null mutants of mpsI, display sensitivity to cell wall digesting enzymes, reduced sporulation and fertility (Jin-Rong Xu et al., 1998). Hence, it is clear from knock-out experiments of various genes from M. grisea, that these genes govern directly or indirectly morphogenetic events such as sporulation and appresorium formation.

In the context of the several genes that have been characterised for their functional roles mentioned above, it was important to ascertain the role of the M. grisea laccase in the pathogenesis of rice blast disease. Several extracellular enzymes such as cutinase (Sweigard et al., 1992) and xylanases (Wu et al., 1995) from M. grisea have been reported. The biochemical activity profiles of these enzymes revealed their expression pattern very late during the infection cycle of the blast disease. It was significant, that M. grisea laccase is expressed extracellularly very early on in its growth cycle, which
suggested that it may play a role in the infection process. Laccases have been found to be present in certain phytopathogenic fungi. Although its exact role in plant pathogenesis is not known, its presence during the infection and its effects on certain morphological and biochemical phenomena have been recorded in these plant pathogens.

**Laccase in plant pathogens**

*Botrytis cinerea* causing soft rot infection in many horticultural crops such as carrot and cucumber, produces extracellular laccases that are involved in the pathogenic process (Bar-Nun & Mayer, 1989, 1990). Repression of laccase synthesis in the pathogen, mediated by, cucurbitacins and tetracyclic triterpenoids produced by the plant (Viterbo et al., 1993) has been shown to protect the plant from infection. Evidence for a role of laccase in pathogenesis has also been obtained from the American chestnut blight fungus *Cryphonectria parasitica* (Rigling & Van Alfen, 1991).

In hypovirulent strains of this fungus, the diminution of virulence is correlated with repressed laccase synthesis by the prevention of accumulation of laccase mRNA. In addition to reduced virulence, these strains also exhibit a number of distinguishing characteristics that are collectively referred to as hypovirulence-associated traits (Hillman et al., 1990). The constellation of traits varies among different hypovirulent strains, but central to these traits are suppressed sporulation, altered colony morphology, reduced pigmentation, diminished oxalate accumulation and reduced laccase activity (Anagnostakis, 1982, Anagnostakis, 1984, Elliston, 1985, Rigling et al., 1989, Hillman et al., 1990). In order to address the functional role of laccase in *M. grisea* and its effect on pathogenesis, it was important to knock-out the gene *in vivo* and analyse its subsequent role in the rice blast disease.
In *M. grisea*, following the identification of the gene for laccase, the function of laccase in the context of plant pathogenesis as well as in the growth cycle of the fungus was evaluated by gene disruption experiments.
Materials and Methods

Bacterial Strains

Escherichia coli strains JM101 (F' traD36 lacI4 Δ (lacZ) M15 pro A'B/ SupE thi Δ (lac-pro AB)) and DH5α (F'/ endA1 hsdR17 (rK- mK+) supE44 thi-1 recA1 gyr A (NalR) relA1 Δ (lacZYA-argF) U169 deoR (r80 dlac Δ (lacz) M15) were used for bacterial transformation and plasmid propagation.

The E. coli strains were grown at 37 °C on either Luria-Bertani (LB) agar medium (10 g bacto-tryptone, 5 g yeast extract, 10 g NaCl, 16 g agar, H2O to 1 l, pH 7.0), or M9 minimal agar medium (12.8 g Na2HPO4, 3 g KH2PO4, 0.5 g NaCl, 1 g NH4Cl, 4 g glucose, 16 g agar, H2O to 1 l, pH 7.0) supplemented with thiamine. E. coli transformants carrying plasmid vectors were grown on LB agar containing ampicillin at a concentration of 100 μg/ml. Bacterial strains were maintained at 4 °C as slants or stab cultures on LB agar or M9 minimal agar medium. Long term preservation was under 8% DMSO solution at -70 °C.

Fungal Culture

Magnaporthe grisea (IC9), were grown and maintained on either potato medium (250 g of peeled potatoes were boiled for 1 h, the extract was filtered through cheesecloth and diluted to 1 l with distilled water, 1% sucrose was added as carbon source) or YEG agar medium (yeast extract 3 g, peptone 10 g, glucose 15 g, H2O to 1 l). For infection experiments the fungi were grown on sterile rice straw.

To maintain a homogeneous pathogen population, the blast fungus M. grisea, was purified by isolating a single conidium from the disease lesions. Disease lesions were incubated overnight at room temperature under moist condition. The lesions were
scraped lightly using a blunt ended glass rod, and streaked on an agar (1%) surface in a petridish

Individual conidia were observed under a light microscope and picked up using a finely drawn glass capillary An individual conidium was thus purified and cultured by transferring to a potato agar slant and grown at 28 °C for 7-9 days This served as a master culture for further analysis

For preservation and storage, cultures were allowed to colonise pieces of Whatmann 3MM Chr paper discs (5 mm x 5 mm) placed on agar medium Upon complete colonisation by the fungus, the paper discs were removed, dried in a lyophiliser and stored with silica gel at -20 °C

**Transformation of M. grisea**

Transformation was accomplished by a procedure modified from Parsons et al (1987) Mycelium for protoplast formation was produced by macerating several 4 cm² of mycelium from oatmeal agar plates with a glass rod All subsequent manipulations were performed aseptically The macerated mycelium was added to 100 ml of Potato Sucrose Broth containing 2% sucrose and incubated at room temperature at 200rpm for 1-2 days The resulting mycelium was harvested by filtration, washed with distil water, weighed, and resuspended in 30 ml of 1M Sorbitol Novozym 234 (20mg/ml in 1M Sorbitol) was added at a rate of 1.5ml of Novozym 234 solution per 3 g of mycelium The enzyme/mycelium mixture was incubated at room temperature on a shaker at 80 rpm After 60-90 minutes, protoplast were harvested by filtering through cheese-cloth and mira-cloth (Calbiochem) respectively The protoplast suspension was centrifuged in a swinging bucket rotor (3000 x g, 10 min), and the pellet was resuspended in 10 ml of 1M sorbitol This step was repeated and was resuspended in 10ml of STC (1 M sorbitol, 50mM Tris-HCl pH 8 0, 50mM CaCl₂)
The protoplasts were counted using a hemacytometer, centrifuged as before and resuspended at $5 \times 10^7$ / ml. Protoplasts (0.2 ml) were mixed with DNA (1-5 μg in 5-10 μl of 10 mM Tris-HCl pH 8.0, 1mM EDTA). After 15 minutes of incubation at room temperature, 1.25 ml PTC (40% polyethylene glycol 8000 in STC) was added. After additional incubation for 20 minutes, 3 ml of PSB with 1 M sorbitol was added, and the protoplasts were incubated at room temperature for 12-14 h at 80 rpm on a shaker. The protoplast suspension was then centrifuged as before, and the pellet was resuspended in 0.1 ml of STC. Molten regeneration medium [10 ml PSB with 1% low melting agarose (Bethesda Research Labs) at 50°C] was added, and the protoplasts were poured onto PSB agar plates containing 200 μg hygromycin B/ml. After 5-7 days, transformants were picked to PSB plates and allowed to sporulate. Single conidia were then isolated from each transformant to ensure that the strain was derived from a single nucleus. Transformants which were unable to sporulate were checked under the light microscope and 1 cm² blocks were transferred to PSB agar plates containing 200 μg/ml hygromycin.

**Infection of Rice Plants with *M. grisea***

**Rice Cultivars**

Rice cultivars, HR-12 and Tadukan plants were grown in peat moss and vermiculite (1:2) in plant growth chambers (Percival, U.S.A.) for 14 h day (28 °C, light 30,000 lux) and 10 h night (26 °C).

For infection assays with *M. grisea* twenty day-old rice plants were inoculated with $10^6$ spores/ml of *M. grisea* and the plants were incubated in a dew chamber (Percival, U.S.A) at 26 °C, 90% relative humidity for 12-14 h followed by incubation in growth chambers (Percival, U.S.A) for 14 h day (28 °C, 30,000 lux, 90% relative humidity).
and 10 h night (26 °C, 90% relative humidity) till disease symptoms developed. In case of susceptible plants, lesions developed within 3–4 days and spread rapidly, followed by death of the plant within 10 days of inoculation. In case of resistant plants, hypersensitive lesions developed within 3–4 days of infection and remained localised.

**Microscopy**

**Assay for Appresorium Development**

Appresorium development was assayed by germinating conidia on the hydrophobic surface of GelBond (a polyester film from FMC Bio-products) and coverslips treated with Sigmacote. Ten 10μl drops of spore suspension were placed on the hydrophobic side of GelBond film and coverslips, in a sterile petri dish containing moistened Whatman No 1 filter. Petridishes were wrapped with Parafilm and incubated at 24° C overnight. Following incubations for designated time periods pertaining to the individual experiment), the germinated conidia were observed under a light microscope (Nikon Optiphot-2). The percentage of germinating conidia induced to form appresoria was determined by direct microscopic examination of 100 conidia per 10μl drop, with a minimum of three 10μl drops counted in each experiment. Experiments were repeated a minimum of three times.
RESULTS

Disruption of *MGL1*, the *M. grisea* Laccase Gene

Construction of the Laccase Gene Disruption Vector

The *M. grisea* isolate B157 was engineered to disrupt the function of *MGL1* gene. A plasmid vector was constructed, designed to function as a one step gene disruption vector (Rothstein, 1983). Figure 18 details the construction of the disruption vector designated as pLM21. A 3.5 kb *BglII-HindIII* DNA fragment comprising the *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase promoter driving the hygromycin phosphotransferase gene from *E. coli* with the terminator *trpC* from *A. nidulans* from pAN7-1 was isolated and cloned into a unique *BglII* site to give rise to pLM21 (Figure 19). Altogether, pLM21 contained 2.1 kb of *M. grisea* DNA, with 0.9 kb and 1.2 kb of *M. grisea* DNA flanking both sides of the inserted hygromycin resistance cassette. In *S. cerevisiae* transformation, homologous recombination events leading to the incorporation of a disruption marker, occur more frequently if a linear donor molecule is homologous to the genome at both ends (Orr-Weaver and Szostak, 1985). No convenient restriction sites were available to generate recombinogenic ends in disruption vector, pLM21. Hence, pLM21 was restriction digested with *SalI*, to generate linear donor molecules for *M. grisea* transformation.

Transformants were selected on plates containing 200 µg/ml of hygromycin B to obtain a frequency of 2.77%. Laccase disruptants were scored by the absence of pink color on mycelial tips upon addition of 0.5 mM syringaldazine. Transformants showing near-normal vegetative growth in culture but defective in a variety of aspects of growth, conidiation or pigmentation were also examined using a light microscope. Disruption of the native gene with the *MgI1* *Hph* construct was expected to result in replacement of the 4.8 kb *HindIII* fragment with a 8.3 kb fragment (Figure 20). Of 72
Figure 18 Strategy for the construction of the disruption vector containing the *M. grisea* laccase (*MGL*) gene. A 3.5 kb BglII containing the entire cassette PgpD-hph-trpC from pAN7-1 was blunt-end ligated into a unique BglII site of pLAC resulting in a 8.5 kb vector termed as pLM21.
Figure 19 Analysis of the construct pLM21 on a 1% agarose gel electrophoresed in 0.5X TBE. Lane 1, parent plasmid pLM21 containing the laccase gene. Lane 2, 1 kb molecular weight marker. Lane 3, plasmid pAN7-1 containing the 3.5 kb hygromycin resistance gene cassette. Lane 4, Disruption vector pLM21 digested with KpnI which released the 0.9 kb region from the left flank with the entire 3.5 kb hygromycin cassette. The hygromycin gene was driven by the gpd (glyceraldehyde 3-phosphate dehydrogenase) promoter. The promoter was cloned in the opposite orientation as opposed to the predicted translation of the laccase gene product.
Figure 20 Diagrammatic representation of the targeting knockout vector and event. The black arrow indicates the positions and transcriptional directions of the MGL1 and HPH genes, respectively. The stippled region indicates the coding region of the MGL1 gene. Restriction enzyme cleavage sites are abbreviated as follows: A, AatII, B, BgIII, K, KpnI, M, Msel, P, PstI, X, XhoI. Integration by homologous recombination produces a mutated gene in which the coding region containing the four copper binding sites, necessary for its enzyme activity, are deleted. The positions of the two probes for DNA gel blot analysis are depicted by dotted lines.
transformants screened, two exhibited the expected pattern and were designated B157-4 and B157-34 respectively.

Southern Analysis of Disruption Event in MGL1 Transformants

HindIII digests of genomic DNA from hygromycin resistant MGL1 transformants were probed independently with MGL1 and the HPH resistance gene. Transformant No 11 in (Figure 21A, B) and transformant No 12 in (Figure 22A, B) indicated that disruption had taken place at the MGL1 locus. These transformants were designated as B157-4 and B157-10, respectively. MGL1 probe hybridised to a higher molecular weight (8.3 kb) in the Mgl1 Hph disruption transformants, than in the recipient B157 (4.8 kb) and the bands that hybridised with MGL1 also hybridised with the HPH gene in B157-4 and B157-10 at the same position of 8.3 kb. In transformants where integration occurred at non-homologous sites, a fragment of 6.2 kb was consistently observed along with resident MGL1 locus at 4.8 kb (Figure 21B, 22B). Hence, DNA gel blot analyses from these transformants, indicated that the Mgl1 Hph construct had disrupted the resident gene MGL1 gene (Figure 21 and 22 A,B). The putative transformants (B157-4 and B157-10) were reconfirmed independently by DNA gel blots. These transformants were found to have undergone homologous recombination replacing the MGL1 gene with the hygromycin resistance gene. Figure 23A, B shows the result of HindIII digested genomic DNA transformants B157-4, B157-10, B157-34 probed with the pLM21 HindIII-EcoRI insert (probe 1 shown in Figure 23A). These two transformants a hybridised band at 8.3 kb, consistent with the size expected from a gene disruption event. Transformant B157-34 exhibited bands at 8.3, 4.8 and 2.4 kb, suggesting a single crossover integration event or integration at an ectopic site. To confirm disruption of MGL1 in B157-4 and B157-10, the blot was stripped and reprobed with an internal sequence of HPH gene, a 998-bp AatII-BamHI fragment of
Figure 21(A). DNA gel blot analysis of hygromycin-resistant transformants containing the expected disruption event. DNA was extracted, digested with HindIII, fractionated on a 0.8% agarose gels, blotted and hybridised with a 2.1 kb-HindIII- EcoRI fragment of the Mgl1 gene (Probe 1).
Figure 22(A). DNA gel blot analysis of hygromycin-resistant transformants containing the expected disruption event. DNA was extracted, digested with HindIII, fractionated on a 0.8% agarose gels, blotted and hybridised with a 2.1 kb-HindIII- EcoRI fragment of the \textit{MGL1} gene (Probe 1), was stripped and reprobed with 10 kb-AatII-BamHI fragment containing the \textit{HIPH} gene (Probe 2).
Figure 23(A). DNA gel blot analysis of hygromycin-resistant transformants containing the expected disruption event. DNA was extracted, digested with HindIII, fractionated on a 0.8% agarose gels, blotted and hybridised with a 2.1 kb-HindIII-EcoRI fragment of the MGl.1 gene (Probe 1), and (B) stripped and reprobed with with 10 kb-AatII-BamHI fragment containing the HPH gene (Probe 2). Two transformants, B157-4 (lane 2) and B157-10 (lane 3) had undergone the desired disruption event at MGl.1. Confirmation of transformants was shown by hybridisation of the HPH gene to a 8.3 kb-genomic restriction fragment.
pAN7-1 (probe 2 shown in Figure 23B). As expected, the 8.3-kb fragment that previously hybridised to probe1, hybridised to probe2, indicating that integration of \textit{HPH} gene, had indeed taken place in the \textit{MGL1} gene. The 4.8 kb and 2.4 kb fragment and the 4.8-kb fragment was absent in lanes containing DNA from B157-34 and wild type B157 respectively (Figure 23B). The size of the \textit{HindIII} fragment containing the \textit{Mgll::Hph} construct in B157-34 (Lane 4) where integration had occured at non-homologous sites was different from the size of the fragment generated in the gene disruption transformants, B157-4 (Figure 23A, B).

\textbf{MGL1 Transcript is Abolished in Disruptant B157-4}

The changes in the abundance of \textit{MGL1} mRNA, when wild type B157 and mutant B157-4 was grown in complete medium, was monitored by Northern analysis. MGL1 mRNA was detected in the wild type B157 which also served as a positive control. However, no transcript was detected in disruptant B157-4. Hybridisation to the \textit{M. grisea} rDNA gene showed equal intensities of signal (as detected by densitometeric studies) for this message in all the lanes for the same blot (Figure 24 A, B). We conclude that \textit{MGL1} transcript is not expressed in the disruptant B157-4 due to the integration of the hygromycin resistance gene cassette in \textit{MGL1} gene.

\textbf{MGL1 Gene Product is Absent in Disruptant B157-4.}

Total protein from the \textit{MGL1} transformant B157-4 was assayed for laccase activity spectrophotometrically. Recipient strain B157 and the \textit{Mgll::Hph} disruption strain B157-4 were grown in complete medium with 1% glucose as the sole carbon source. In the wild type strain B157, activity of the laccase enzyme was detected to be highest at 24 h after conidia germination. Correspondingly, no enzyme activity was detected in the B157-4 mutant.
Figure 24. Northern hybridisation of total cellular RNA from the wild type B157 and disruptant B157-4 (∅ mgl::Hph) For each strain, 20 μg of total cellular RNA isolated from complete medium was electrophoresed on a 1.2% agarose gel containing 0.6 M formaldehyde, blotted on to nylon membrane (Hybond N+), and probed with the pLM21 kb EcoRI-HindIII fragment containing the MGL1 gene. RNA from the wild type fungus at 24 h after spore germination was used as a positive control. Hybridisation to rDNA from *M. grisea* was used to ensure equal amount of RNA in all lanes.
Time after inoculation (h)

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A

B
Figure 25  Non-denaturing gel electrophoresis and laccase activity staining of extracellular proteins from wild type B157 and mutant B157-4. Proteins were electrophoresed on 8% polyacrylamide gel and stained with ABTS and incubated at 20°C for 30 minutes. The amount of protein per lane was 25 μg.
Qualitative Assessment of Gene Disruption Transformants

i) Activity Gel Electrophoresis by ABTS Staining

Extracellular protein samples of \( mg/l \) were collected at different time intervals after inoculation in complete medium. Total protein was electrophoresed on non-denaturing polyacrylamide gels and stained with 1% ABTS. A 24 h protein sample from wild type B157 was used as a positive control. A dark green band, indicating laccase activity, was detected in the lane corresponding to 24 h (Figure 25). However, no laccase activity was detected in the B157-4 protein samples. This result confirmed that the disruption of \( Mg/l \) was absolute as no residual enzyme activity was detected upon specific staining by ABTS. The absence of oxidative products of ABTS in the case of B157-4 was further confirmed by western analysis.

ii) ABTS Assay

A 6.0 (diameter) mm inoculum from a 9-day old YEG plate from B157, B157-4 was cultured on YEG plates containing 1% ABTS. After 2 days of growth, B157 displayed a light green halo which gradually turned to dark purple whereas, B157-4 displayed a light green halo which did not change colour. The appearance of dark purple colour, in the case of B157 suggested that laccase was being secreted into the medium resulting in the oxidation of ABTS (Figure 26). Correspondingly, oxidation of ABTS was not detected in the B157-4 plates, suggesting the loss of lacasse activity by its disability to reduce molecular oxygen to water. Laccase production in the putative disruptant B157-4, was studied by activity staining of total protein electrophoresed on a polyacrylamide gel.

iii) Immunoblot Analysis.

Total extracellular protein from \( mg/l \) mutant at different time intervals after conidia germination, was collected, filtered, concentrated, and electrophoresed under
Figure 26. Growth of *mgII* mutant on 1% ABTS Yeast Extract Glucose Agar plates were inoculated with a 0.6 (diameter) cm agar block from a 14 day-old culture of the wild type B157 and mutant B157-4. Addition of ABTS to the 24 h old culture showed a light green halo which gradually turned to dark purple in the case of wild type B157. However, mutants B157-4 did not show any change in color.
B157 (wild type)  B157-4 (disruptant)
Figure 27. Western blot analysis of extracellular proteins from wild type B157 and mutant B157-4 using anti-laccase antibodies. Lane 1. Positive control - purified laccase. Lane 2. 24 h total extracellular protein from wild type B157. Lane 3-8. 24, 48, 72, 96, 120, 144 h of mutant B157-4 total protein. The amount of total protein per lane was 100 μg.
denaturing conditions on SDS-PAGE gels. Total protein from B157 was used as positive control. Subsequently, after transfer, they were hybridised to rabbit anti-laccase polyclonal antibodies. Anti-laccase antibodies cross reacted with a single band in the 24 h sample from B157, whereas total protein from various growth stages of \textit{mgl1} mutant did not show any hybridisation (Figure 27).

\textbf{\textit{MGL1} Disruption Affects Melanin Production}

The effect of disrupting \textit{MGL1}, also manifested as absence in melanin production. It was observed on a variety of culture media (oat meal, yeast-extract Glucose, potato dextrose, complete medium agar plates). Melanin production is known to be an essential factor for pathogenicity in \textit{M. grisea} (Woloshuk et al, 1989; Chumley and Valent, 1990). The conversion of 1,8-DHN to DHN melanin has been suggested to be mediated by a phenoloxidase of the laccase type (Bell and Wheeler, 1986). Studies with inhibitors of melanin synthesis have shown that DHN melanin is required for penetration of rice, cucumber, and bean by appresoria of \textit{Magnaporthe grisea}, \textit{Colletotrichum lagenarum}, and \textit{C. lindemuthianum} respectively. The oxidation of 1-napthol to dimers, and the subsequent addition of 1-napthol units to form trimers, tetramers and polymers as shown in the \textit{in vitro} assay in chapter 1, suggested that laccase is involved in the oxidation of 1,8-DHN to DHN-melanin. Phenotypic observation of mutant B157-4 and wild type B157 grown on oat meal agar plates for 9 days, shows the inability of the mutant B157-4 to produce melanin (Figure 26). In the wild type strain, melanin begins to appear at 96 h after spore germination and progressively increases up to 144 h. We examined the relationship between expression of \textit{MGL1} gene product, laccase, and appearance of melanin production in culture at 24 h intervals. Laccase activity was found to be highest at 24h (21.0 U) after spore germination and was totally absent after 96h after spore germination (Chapter 1). Total
Figure 28. Colony morphology of the MGL1 knockout transformant. Wild type isolate B157 (left) and MGL1 knockout transformant B157-4 (right) were cultured on oatmeal agar medium for 9 days. The wild type shows sporulating areas interspersed with aerial hyphae at the periphery, and melanin production, whereas the knockout B157-4, shows reduced growth, absence of melanin production and reduced sporulation.
protein was extracted from wild type isolate B157 and mutant B157-4 at 24 h intervals upto 110 h after spore germination and protein blots were prepared and probed with anti-laccase antibodies. \textit{MGL1} gene product was detected upto 96 h in the case of wild type only, and was clearly absent in the mutant B157-4 (Figure 27). The major mode of oxidative polymerization of DHN to melanin in the melanin biosynthetic pathway in \textit{M. grisea} most likely involves the action of polyphenol oxidase of the laccase group (Bell and Wheeler, 1986) This was consistent with the observations that laccase from \textit{M. grisea} was able to oxidise several substrates such as 1-napthol, DOPA, syringaldizine to form colored pigments (Chapter 1). It is possible that lack of melanin production in \textit{MGL1} mutant is due to the absence of the enzyme laccase.

\textbf{MGL1 Mutant Phenotype shows Reduced Growth}.

A 6 0 (diameter) cm inoculum from a 2 day old culture of both the wild type strain B157 and the \textit{mgl1} mutant was used for radial growth measurement studies. It was essential to use 2 day old plates as \textit{M. grisea} begins to sporulate after 60 h. Hence, the growing mycelial tip was employed as inoculum. Radial growth measurements on yeast-extract glucose agar plates of the \textit{mgl1} (B157-4) mutant exhibited considerable reduced growth as compared to the wild type strain B157. Radial growth measurements (mm) at 24 h intervals of the \textit{mgl1} mutant had 65% reduced aerial hyphal growth and conidiation as compared to wild type B157. Wild type B157 produced on an average of $10^7$ conidia per plate, whereas \textit{mgl1} mutant produced $10^2 - 10^3$ conidia per ml (Figure 28). The reduction in conidiation may be caused by the limited ability of \textit{mgl1} mutant to produce aerial hyphae.

\textbf{Leaf Detachment Assay to Evaluate Conidia}

The effect of disrupting \textit{MGL1} on conidia formation was carefully evaluated. A 6 0 (diameter) cm inoculum from a 9-day old YEG, oatmeal agar and potato dextrose agar
**Figure 29.** Colony morphology of the *mgll* mutant and wild type *MGL1* isolate. Both the strains were cultured on Yeast Extract Glucose Agar medium. The plate indicated reduced growth relative to wild type after 7 days, also indicated the absence of melanin production in the *mgll* as compared to the wild type *MGL1* isolate.
plates of B157-4 was inoculated on yeast-extract glucose agar plates, oat meal agar plates and potato dextrose agar plates respectively, and incubated for 9 days at 26\(^\circ\)C. The role of host factors, in the induction of conidiation of the pathogen was investigated. Detached leaves from a compatible (HR-12) host cultivar were inoculated with B157 and B157-4 and incubated under high humidity conditions. Microscopic examination of the inoculated leaf surface revealed, that the wild type strain B157 was able to grow on the leaf surface and conidiate, whereas, the \(mgl1\) (B157-4) mutant failed to penetrate the leaf surface. Thus, the lack of penetration could be attributed to its inability to form appresoria and its defective spore morphology.

**Pathogenicity Tests:**

**Inactivation of \(MGL1\) Leads to a Reduction in Pathogenicity towards Rice.**

Pathogenicity assays were carried out using the wild type isolate B157, the \(mgl1\) mutant B157-4, and another independently obtained transformant, B157-34 in which the \(Mgl1::Hph\) construct had integrated at a non-homologous site in the genome. The results are shown in Figure 4. The wild type isolate B157 caused large spreading lesions on compatible rice cultivars HR-12 as did the transformant B157-34 (Figure 30A, H). We were interested to test whether \(mgl1\) mutant B157-4 could still infect rice plant as it was impaired in its ability to form appresoria. Wounding experiments were carried out by abrading rice plant leaves and inoculating the plants with equal amount of biomass (fungal mycelia) directly on the abraded sites (see Methods). Inoculations with 0.2% gelatin solution resulted in predominantly white wound sites with little or no plant necrosis (Figure 30C). Inoculations with wild type B157 mycelia caused lesions in and around wound sites (Figure 30D). Inoculations with B157-4 mycelia resulted in necrotic patches with small hypersensitive lesions away from wound sites (Figure 30F). A more quantitative analysis of pathogenicity was carried out by measuring
Figure 30. Disruption of MGL1 Results in Reduced Pathogenicity on Rice

Rice seedlings of cultivars HR-12 (compatible) and Tadukan (incompatible) were spray inoculated with conidial suspension from wild type isolate B157 in control experiments. Wounding experiments were performed due to the inability of mgl1 mutant B157-4 to form appresoria as indicated below. Typical leaves were collected 7 days after inoculation and photographed.

(A) Conidial suspension of wild type isolate B157 inoculated on compatible cultivar HR-12.

(B) Conidial suspension of wild type isolate B157 inoculated on incompatible cultivar Tadukan.

(C) Control leaves of compatible cultivar, HR-12, wounded with an emery board.

(D) Mycelial suspension of wild type B157 applied on wounded leaves of compatible cultivar HR-12.

(E) Mycelial suspension of wild type B157 applied on wounded leaves of incompatible cultivar Tadukan.

(F) Mycelial suspension of Mgl1 mutant B157-4 applied on intact leaves of compatible cultivar HR-12.

(G) Mycelial suspension of Mgl1 mutant B157-4 applied on intact leaves of incompatible cultivar Tadukan.

(H) Conidial suspension of ectopic transformant B157-34 inoculated on compatible cultivar HR-12.
lesion densities and disease leaf area on infected rice leaves (see Methods). The percentage disease leaf area (DLA) was reduced 6.6 fold in plants inoculated with B157-4 mycelia as compared to wild type isolate B157 mycelia (Figure 31). The degree of disease development in wounding experiments, measured as the percentage ratio of the mean leaf area showing lesions, to the total leaf area inoculated, was 8.81% in plants inoculated with the *mg/l* mutant B157-4 mycelia, which was dramatically lower than that observed in plants inoculated with the wild type isolate B157 (58%). However, compatible cultivar HR-12 and incompatible cultivar Tadukan had values of 50.58 and 9.88% DLA respectively, when inoculated with B157 conidia. These values were as expected, indicating hypersensitive reaction in incompatible cultivar Tadukan. The % DLA obtained from wounding experiments from mutant B157-4 mycelia (8.81%) with compatible cultivar HR-12 were nearly unchanged to that of incompatible cultivar Tadukan (8.12%), infected with wild type B157 conidia (Figure 31). To test whether mycelia from B157-4 were still alive in these wound sites, we excised leaves from susceptible HR-12 plants inoculated with either wild type or mutant mycelia. Excised leaf sections were placed on water agar plates and incubated in direct light at high humidity for 3-days. Under these conditions, wound sites inoculated with wild type isolate B157 mycelia produced abundant normal conidia. Conidia from wild type isolate B157, which can penetrate healthy leaf surfaces because of normal conidial phenotype, served as a positive control. However, no conidia were obtained from wounds inoculated with B157-4 mycelia. These findings suggest that B157-4 is able to penetrate rice leaves to a limited extent through wound sites, although the disease symptoms produced were equivalent to an hypersensitive response, in sharp contrast to the lesions formed in the compatible interaction with mycelia from wild type.
Figure 31. Quantitative Analysis of Pathogenicity Assays of *MGL1* Mutant with Compatible and Incompatible Cultivars

Bar graph of percentage disease leaf area vs host/pathogen combination as indicated on the X-axis. Photographs of leaves from plants inoculated correspond to the bars in the same order. Quantification of the % disease leaf area from Mgl1 mutant B157-4 mycelial suspension inoculated on wounded leaves of susceptible cultivar HR-12, produces an hypersensitive response equivalent to unwounded leaves of resistant cultivar Tadukan, spray inoculated with conidial suspension from wild type isolate B157. Error bar indicates standard error of the mean.
**mgll Mutants Show a Defective Conidial Phenotype**

*mgll* mutants produced elongated conidia (Figure 32B) as compared to pyriform conidia of the wild type (Figure 32A). The transformant B157-34 was unchanged in its ability to form conidia relative to B157. In order to investigate the endogenous mechanisms regulating appresorium formation and conidia germination, an inducive surface such as GelBond film was used. *mgll* mutant conidia collected from several oatmeal agar plates were allowed to germinate on the hydrophobic GelBond surface. Surprisingly, the conidia from the mutant failed to form appresoria even after 48 h of incubation. In contrast, the wild type conidia exhibited normal appresoria formation. The longer incubation periods *in vitro* was essential to overcome variations such as surface responsiveness and to synchronize the fate of germ tube towards appresoria formation. The hardness or the degree of hydrophobicity of the contact surface had no effect on conidium germination in the case of wild type and mutant. However, appresoria formation was definitely impaired in the *mgll* mutant. The ability of the *mgll*.Hph transformants to form appresoria was assayed on GelBond (FMC) film.

Figure 32C shows differentiated appresoria from the wild type B157 in which 91% of the pyriformed three-celled conidia examined (*n = 100*) germinated and formed melanised appresoria by 24 hrs. The pyriformed three-celled conidia germinate and form a round melanized appresoria. Figure 32D shows the absence of appresorial formation in strain B157-4 at the corresponding time. The subapical swollen structures in the *mgll* mutant was elongated and lacked thickened melanized cell walls. Even after 48 hr, none of the germinated B157-4 conidia examined formed any appresoria. This phenotypic features indicate that these structures were unable to form new cell wall layers or generate high level of turgor pressure which is essential for penetration during infection. The inset (Figure 32E) shows a close-up of a typical conidium from...
Figure 32. Inability of Mgll Mutant B157-4 to Undergo Appresoria Formation Essential for Infection

Conidial suspension were prepared from Mgll mutant, B157-4 and the wild type isolate B157 and tested for their ability to make appresoria on GelBond film.

(A) Three-celled pyriformed conidia of B157

(B) The Mgll mutant B157-4 has a abnormal shaped morphology. It has elongated conidia, with tapered ends at both ends, in contrast to the wild type conidia.

(C) Conidia of B157 that germinated and differentiated into a melanized appresorium.

(D) Conidia of B157-4 that germinated and continued indeterminate hyphal elongation without the elaboration of an appresorium after a 48-hr time period.

(E) The conidia of mutant B157-4 (Inset), appears to undergo swelling at 24-hr time interval, which further elaborates into hypha as shown in (D).

Assays for appresorial development were observed and photographed at x 400 magnification.
strain B157-4 that failed to produce an appresorium but is able to undergo normal germination. A long germ tube has formed that appears to have undergone multiple hooking and swelling events. Swelling and hooking of the germ tube is a characteristic event prior to appresorium formation (Bourett and Howard, 1990). Hence, it appears that mgll mutants undergo primary differentiation events that precede appresorial formation but are unable to complete differentiation into mature appresoria. Ectopic transformant B157-34 was also noted for its phenotype along with the mgll mutants. It was unaffected in conidia formation, germination and appresoria formation. We conclude that MGL1 may play a role in morphogenesis of conidia and appresorial development and is required for normal vegetative growth.

**MGL1 Gene Product Inhibitor, Tropolone, Inhibits Appresorium Formation in Wild Type B157**

Disruption of the MGL1 gene appears to disturb the normal conidial morphogenesis of the wild type isolate B157 conidia. *in vitro* inhibition assays of laccase enzyme demonstrated that 80 μM was sufficient to cause 50% inhibition of the enzyme activity (Chapter 1). In order to ascertain the role of the MGL1 gene product in spore development, we decided to test the role of tropolone, a laccase enzyme inhibitor on wild type B157 conidia. The addition of the inhibitor would be expected to act at the enzymatic level, and exhibit certain, if not all characteristics obtained by disrupting MGL1 *in vivo*.

Tropolone (2-hydroxy-2,4,6-cycloheptatrienone), a laccase inhibitor was tested for its efficacy to inhibit appresorium formation in the wild type. The inhibition of germination on wild type conidia was inversely proportional to inhibitor concentration of 10, 80, 150 μM and 1 mM. 10 μM concentration of the inhibitor did not have any effect on appresorium formation. 36% of conidia had germinated with remaining...
Figure 33. Inhibition of Appresorium Formation by Laccase Inhibitor, Tropolone in Wild-Type Isolate B157 on Hydrophobic Surface.

The effects of 80 μM tropolone on appresorium formation resulted in germination at 24 h with apparent lack of appresoria. Extended incubation time up to 48 h resulted in hyphal elongation as indicated in panel C and D. Higher concentration of 150 μM tropolone completely inhibited germination and appresoria formation as shown in panel E and F. Control appresoria assays in the absence of inhibitor pronounced the formation of appresoria and normal development indicated in panel A and B. Assays for appresorium formation was monitored using Nikon Optiphot-2 light microscope under a magnification of X 400.
conidia ungerminated (Figure 33C) at 80μM concentration, while there was total inhibition of germination at 150μM by 24 h (Figure 33E). At a comparable time interval of 48 h, hyphal extension from existing germinated conidia produced vegetative hyphae that failed to undergo appresorium formation at 80 μM concentration (Figure 33D). As expected, increasing concentration at 150 μM concentration completely inhibited conidia germination (Figure 33D). Laccase inhibitor at a concentration of 1mM, completely inhibited conidia germination. Corresponding control in the absence of inhibitor showed normal germ tube formation and appresoria development (Figure 33A,B). In contrast, at the inhibitor concentrations tested for the wild type, there was no pronounced effect on the mgll mutant as expected due to the absence of laccase enzyme. This suggested that MGL1 gene product may have a role to play in the morphogenesis of conidia development and it may affect its role downstream of conidia development viz germ tube formation and appresoria formation.
DISCUSSION

The production of asexual spores is a primary determinant of disease severity in many plant diseases caused by phytopathogenic fungi. Fungal conidia are the infectious propagules responsible for initiating infection as well as for disease dissemination. For the rice blast fungus to complete a disease cycle, three distinct phases are essential: infection, colonisation and sporulation. In mature appresoria, cell walls are melanized, presumably to provide a differential permeability barrier for turgor generation (Bourett and Howard, 1990). Melanin-deficient mutants that cannot generate this turgor pressure are defective in penetrating plant cell surfaces, but they can cause lesions if inoculated through wound sites (Chumley and Valent, 1990).

Molecular dissection of MGL1 in Pathogenesis

Gene disruption has become the method of choice to elucidate the functions of genes in fungal-plant interactions (Sweigard et al, 1992, Apel et al, 1993, Talbot et al, 1993, Hwang et al, 1995, Mitchell and Dean, 1995, Apel-Birkhold and Walton, 1996, Froeliger and Carpenter, 1996, Lau and Hamer, 1996) Two transformants, B157-7 and B157-10 were obtained with the altered mgl1 DNA containing the hygromycin resistance gene. The pattern of disruption in MGL1 gene indicated that there were no apparent changes in restriction fragments or acquisition of plasmid sequences in the genome. Thus, transformants of M. grisea have been produced in which the MGL1 has been replaced by a non-functional copy constructed in vitro. The mgl1 disruptants produced no detectable mRNA, and also failed to produce laccase protein, typical of the wild-type strain, as detected by ABTS staining for laccase activity. The results obtained from the ABTS plate-agar showed that the enzyme laccase in the wild type, retained its ability to utilise oxygen and breakdown ABTS into its corresponding oxidative products. The oxidising ability of the disruptant on the other hand, was
debilitated due to the absence of laccase, for which the primary requirement is molecular oxygen. Furthermore, laccase activity was found only at/beyond the advancing edge of the mycelial tip in the case of the wild type. However, older mycelia beyond 96 h did not show any activity with syringaldizine as substrate. The presence of laccase activity at the advancing mycelial tip suggested that it may play a role in the very early stages of pathogenesis, corresponding to the time of host penetration. This observation substantiated the result previously described in chapter 1, where laccase enzyme has been shown to be produced early in the growth cycle of \textit{M. grisea}.

Mycelial tips from disruptant B157-4, on the other hand, did not show any laccase activity with syringaldizine, which suggested that its activity was abolished due to the disruption in the \textit{MGL1} gene. To establish that the knock out had indeed taken place in the entire coding region of the \textit{MGL1} gene, northern analysis of total RNA was used to confirm the absence of the \textit{MGL1} transcript in B157-4. The lack of transcript indicated that the \textit{MGL1} gene disruption had occurred in a manner which would not give rise to truncated \textit{MGL1} gene products. Further confirmation by western analysis of total protein from B157-4 indicated clearly that the entire coding region of the \textit{MGL1} sequence was replaced by the \textit{gpd-hph-trpC} cassette resulting in the loss of ability to produce any laccase protein. The results obtained clearly demonstrated that the gene coding for laccase has been disabled by the insertion of the \textit{HPH} gene, resulting in the absence of the transcript and protein respectively.

\textit{MGL1} was selected as a gene likely to be important in the pathogenesis of rice blast because of its high level of expression at 24 h after spore inoculation and its varied role in cellular morphogenesis in \textit{Lentinus edodes} (Leatham and Stahmann, 1981), \textit{Schizophyllum commune} (De Vries et al., 1986), \textit{Agaricus bisporus} (Smith et al., 1989), pigmentation in \textit{Aspergillus nidulans} (Hermann et al., 1983, Clutterbuck, 1972).
and pathogenesis in *Cryphonectria parasitica* (Rigling and Van Alfen, 1991), *Botrytis cinerea* (Bar-Nun and Mayer, 1990, Viterbo et al, 1993) Gene disruption mutants of *MGL1* are severely reduced in their ability to cause disease symptoms on susceptible rice cultivars due to an inability to form appresoria

Several lines of evidence indicate that mutant *mgll* is defective in penetration and therefore is incapable of invasive growth to establish complete blast symptoms. First, *mgll* mutant cannot penetrate and infect healthy host leaves in mycelia inoculation assays. Second, the mutant is able to infect through wounds but the percentage disease leaf area obtained for the mutant are comparable to that of an incompatible interaction. A surprising observation from the wounding experiments with the susceptible host indicated that *mgll* mutant elicited a defense response in the form of a hypersensitive reaction in contrast to the large spreading lesions produced in a compatible interaction. The diminished ability of the infectious hyphae of *mgll* mutants to successfully invade rice leaf cells, as evident by the reduction in lesion size, could be attributed to its poor growth rate, which in turn would allow more time for the host plant to limit lesion formation. It remains to be determined whether this hypersensitive response in the wounded plants caused by *mgll* mutant is triggered by hyphae which have the ability to cause infection during the infection cycle (Talbot, 1995), or by other soluble factors released from the invading fungus.

Appresorium formation is a complicated process that involves surface attachment and recognition, spore germination, germ tube growth, cytoskeletal reorganization (Kwon et al, 1991), melanin biosynthesis and localization (Howard and Ferrari, 1990), turgor generation (Howard et al, 1991) and appresorium specific gene expression (Talbot et al, 1993). Tropolone, an inhibitor of phenoloxidase in *Vicia faba* (Takahama, 1992) and weak inhibitor of melanin formation in the larval hemolymph of *Bombyx mori*
(Hashimoto et al., 1994) also inhibited laccase activity in vitro. These observations were consistent with our data that the tropolone caused inhibition of germination of conidia in *M. grisea* (Figure 8) and its enzyme activity in vitro (data communicated). The failure of the *mgll* mutants to penetrate plant cells is directly related to its inability to form appresoria and its lack of melanin production. In *M. grisea*, the apparent ability to infect wounded host plants by the *buf*, *rsy* and *alb* (melanin-deficient) class of mutants indicate that the branch point metabolites as well as the primary metabolites are not required for pathogenicity after the host’s outer barriers have been penetrated (Chumley and Valent, 1990). In *Cochliobolus lagenarum*, laccase deficient strains are unable to penetrate host tissues due to the absence of melanin (Kubo and Furasawa, 1991). By analogy, the absence of melanin formation in the *mgll* mutants suggests that the *MGL1* gene product may be involved in the oxidative polymerisation of 1,8-DHN to melanin by *MGL1* coding laccase. Melanin-deficient *mgll* mutants of *M. grisea* fail to infect intact host plants and cause very limited infection of wounded plants. Hence, the results obtained from studies mutant indicate that the *MGL1* gene product is very essential for melanin production, an essential requirement for blast infection.

**MGL1 Role in Conidiation**

In the *MGL1* mutant *B157-4*, an abnormal morphology in the conidial shape was observed instead of the pyriform structure of wild type *B157*. Previous studies on the *SMO* locus from *M. grisea* revealed its abnormal conidia shape, although no alterations in growth rate or conidial germination rate was detected (Hamer and Givan, 1990). In contrast, our findings indicate that a mutation in a single gene *MGL1*, confers several interesting debilitating phenotypes. Six genes have been identified in the sporulation pathway, *con1*, *con2*, *con5*, *con6*, and *con7* - that control various steps in the sporulation pathway (Shi et al., 1998). The most pronounced defect in *mgll* mutants is...
in conidia formation. However, microscopic examination revealed that *mgl1* mutants are similar to the *con5* mutant (Shi et al, 1998), in that a certain percentage of conidia produces swollen hyphal tips which failed to develop conidia. It is however not known whether *con5* are defective in melanin production. Interestingly, the absence of *MGL1* gene product not only leads to deformed conidia, but also reduced growth rate, absence of melanin formation and most importantly, reduced pathogenicity. Our results indicate that spore morphology can be modified by laccase enzyme, which may act independent of the sporulation pathway proposed by Shi et al., (1998). It is also possible that the enzyme, laccase, may interact with the *CON* genes to produce several pleiotrophic effects such as reduced growth rate, lack of melanin production as is evident from mutation studies in several *CON* genes (Shi et al., 1998). Hence, the defective morphology of the conidia most likely suggests a role for *MGL1*, coding for laccase enzyme, in a pathway independent of a model previously proposed in *M. grisea* (Shi et al, 1998). Experiments to localize and study the accumulation of the *MGL1* gene product during the infection process are currently underway. In conclusion, the results obtained demonstrate that disruption of the *MGL1* gene leads to several pleiotrophic effects and exerts its influence at the morphogenetic level unique to spore development.

We speculate that although the infection processes in fungi may be distinct, the function of laccase is conserved and its activity may be utilized widely by pathogenic fungi to induce differentiation events necessary for pathogenesis. It will be interesting to see whether developmental expression of *MPG1* (Talbot et al., 1993), *CPKA* (Xu et al., 1997), *PMK1* (Xu and Hamer, 1996) in the *mgl1* mutants are affected by the lack of its expression, as these mutants are also impaired in sporulation, appresorium formation and pathogenicity.
The accumulated evidence suggests that mutations affecting genes regulating conidial development in general have strong pleiotrophic effects on pathogenesis such as reduced growth, absence of sporulation and a total loss of, or a significant reduction in pathogenicity. Most of the conidial mutants are deficient in other attributes important in pathogenesis (Shi et al., 1998). In a broader context, the morphogenetic genes such as the *MGL1* gene and several *CON* genes (Shi et al., 1998) can be described as pathogenicity factors as they are essential for normal fungal development and subsequent propagation in the plant. The morphogenetic defect as outlined by the *MGL1* mutation could represent an intervention point, as the various morphological/physiological effects observed in the *mgl1* mutant encompass most of the defects observed in the various *con* mutants put together. Anti-laccase compounds against laccase may allow the fungus to infect the plant but may prevent further infection cycles by preventing sporulation. A practical objective from the above obtained results in *M. grisea* would be to further the understanding of autonomy of a developmental process such as conidiation and identify check-points in order to reduce the blast epidemic.