ABSTRACT

OH, YEON YEE. Genome-Wide Transcription Studies on Infection Structure Formation and Function in *Magnaporthe grisea.* (Under the direction of Dr. Ralph A. Dean.)

Rice blast caused by the filamentous ascomycete fungi, *Magnaporthe grisea* (anamorph *Pyricularia oryzae*) is the most destructive disease of rice through out the world. To gain access to its host, *M. grisea* develops a specialized infection structure called an appressorium. To understand the mechanisms regulating formation and function of this structure, we performed microarray experiments using the *M. grisea* whole genome oligonucleotide array containing *M. grisea* and rice elements. The most dramatic change in gene expression occurred during spore germination where 21% showed differential expression with the vast majority being up-regulated. Approximately 3 % of the predicted genes were differentially expressed during appressorium formation in response to both a hydrophobic surface signal and exogenous cyclic AMP. Our data shows that germination stimulates a major transcriptional response characterized by a dramatic transcription of genes involved in metabolism and biosynthesis. In contrast, induction of appressorium formation triggered a significant decrease in this suite of genes, including the translational apparatus, with a coordinate increase in the expression of genes involved in protein and amino acid degradation, lipid metabolism, secondary metabolism and cellular transportation. Significantly, the set of up-regulated genes was enriched for those encoding predicted secreted proteins. We identified 42 transcriptionally regulated transcription factors during appressorium formation, the majority of whose putative functions are regulation of secondary metabolism, nutrient assimilation and cell development.

Functional characterization of differentially expressed genes using targeted gene disruption revealed novel pathogenicity factors, a subtilisin protease SPM1 and a NAD specific glutamate dehydrogenase Mgd1 in *M. grisea*. Our finding shows that protein turnover and amino acid metabolism are essential for proper appressorium formation and the infection process. Further, we found many differentially expressed genes, which included highly conserved transcription factors, were not required for appressorium formation and function. This may suggest that *M. grisea* employs a number of failsafe and backup systems, such as functional redundancy and compensatory processes in order to protect appressorium formation and to ensure the fungus can successfully invade its host.

Genome wide transcriptional profiles followed by comprehensive functional studies provided broad and in depth insight into infection structure development in *M. grisea*. Our data will directly benefit efforts to find novel fungal pathogenecity factors and further to develop disease management systems.

Genome-Wide Transcription Studies on Infection Structure Formation and Function in *Magnaporthe grisea*

by

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BIOGRAPHY

Yeon Yee Oh was born on March 13th, 1970 in Inchon, Korea. She received her Bachelor of Science and Master of Science degrees from the Department of Agricultural Biology, Seoul National University in 1993 and 1995, respectively. Her master's thesis entitled "Temporal Dynamics of Triflumizole in Tomato Foliage and Effect of Temperature on Residue Level" was directed by Dr. Eun Woo Park. From 1995 to 2000, she started at Bayer CropScience in Korea as a research scientist, where her work was focused on fungicide development and disease control.

In December, 2002, she began studies on the biology of rice blast disease under the direction of Dr. Ralph A. Dean, Department of Plant Pathology, North Carolina State University.

She married Sang-Hoon Sin on October 21st, 1995 and has two daughters, Soo Min and Young Min.

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Literature Review

Rice and rice blast

Rice, *Oryza sativa,* is the primary food source for more than half of the world's population. It is most widely grown in Asia, covering more than 10% of cultivated land. Thanks to the adoption of green revolution technology, which included nitrogen fertilization, increased irrigation systems, breeding for high yield with disease resistance, and pest control, the annual world wide yield of rice has increased significantly from 257 million tons in 1966 to 600 million tons in 2000. The continuing growth of the world's population will demand 40% more rice production by 2030. Satisfying the demand for increased in rice production is challenged by shrinking cropland, less water, and labor shortages [1-3].

Rice blast disease caused by the filamentous ascomycete fungi, *Magnaporthe grisea* (anamorph *Pyricularia oryzae*), has historically been the most destructive disease of the world's rice crop. High pathogen variability combined with monoculture growth of rice has resulted in devastating rice blast epidemics with a dramatic loss of yield in many parts of the world. In Egypt, fields growing the Giza 173 cultivar were badly infected by rice blast in 1984. This accounted for an average 50% reduction in yield. The following year, in India, about 140,000 tons of rice were lost, which was about 0.7% of the total yield [4]. Recently, almost 865,000 hectares of rice fields were devastated with rice blast in Japan [5].

Blast occurs in a wide range of climates, from temperate to tropics. The pathogen is mainly spread by wind and it overseasons in the infected plant debris or seeds left in the fields. The disease is favored by high humidity (> 90%), cool night time temperature (around 20℃) and high nitrogen levels. Release and germination of spore depends on leaf

wetness [6].

Breeding of blast resistant cultivars has been widely adopted for rice blast control, but results have not been satisfactory due to the rapid occurrence of new races which overcome the cultivar's resistance. The population of *M. grisea* is highly dynamic depending on the prevailing host plant genotypes. Breakdown of resistance can be explained in terms of typical gene for gene concepts [7, 8]. Monogenic fungal resistance involves recognition between the resistance gene from the host plant and the avirulence gene from the pathogen. Identification of resistance and avirulence genes has made it possible to find more diverse effective genes and to understand the host pathogen interaction at the molecular level.

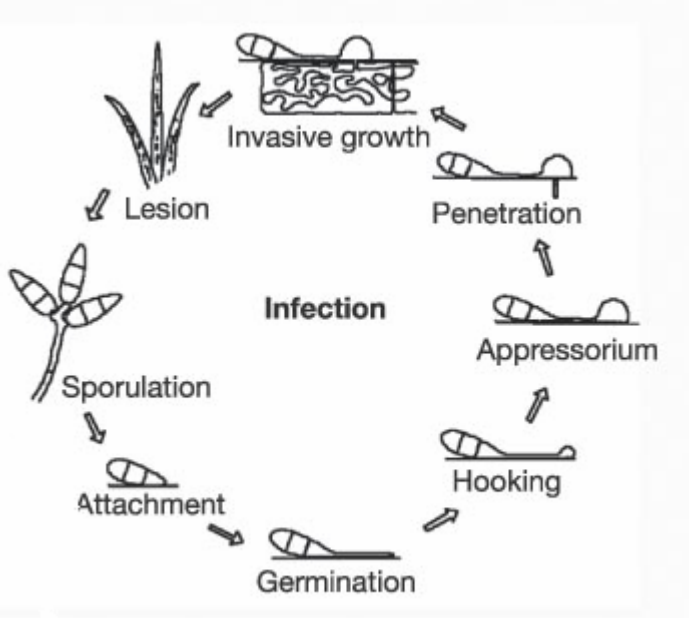
Chemical application is another common way for the control of rice blast. Characterizing the biosynthesis pathway of 1.8-dihydroxynaphtalene (DHN) melanin using UV mediated mutagenesis of *M. grisea* [1, 9], enabled the development of effective and widely used fungicides affecting this pathway [10, 11].

Magnaporthe grisea, as an model organism for the study of fungal disease

In addition to its economic importance, rice blast provides a reliable model system to study host pathogen interactions. Advantages include (i) diversity of fungal populations, enabling comprehensive analysis of host specificity and pathogenecity; (ii) a well defined infection process which has been characterized at the cellular and molecular level; (iii) whole genome sequences of both rice and *Magnaporthe grisea* facilitating gene identification and characterization and (vi) *Magnaporthe grisea* is phylogenetically closely related to other model filamentous fungi enabling studies into the evolution of

fungal pathogenesis [12].

Based on its developmental stages, the infection process of *M. grisea* can be divided to attachment, germination, appressorium development, penetration, *in planta* growth and sporulation.



Life cycle of *Magnaporthe grisea* [13]

Attachment of spores on the surface of the host plant is the first critical step of fungal infection. Once the spore is hydrated, an adhesive mucilage is released from the spore tip [14, 15]. During germination, mucilaginous substances continue to be extruded at the tips of the germ tube, which are essential for germ tube attachment and appressorium formation [16]. Spore adhesion and appressorium formation is inhibited by hydrolytic enzymes such as α-mannosidase, α-glucosidase, and protease. This suggests the adhesive materials are composed of glycoproteins [16, 17]. Germination is also inhibited at high spore concentrations, which might be due to a lipophilic self inhibitor. Self inhibition can be overcome by hydrophobic wax from rice leaf [18].

In response to surface signals, the germ tube tip undergoes a cell differentiation process to form a specialized infection structure, the appressorium. Frank first named "appressorium" as the adhesion body which was selectively formed by the bean pathogen *Gloeosporium lindemuthianum* only on the host surface [19]. Appressorium development involves a number of steps: nuclear division, first septum formation, germling emergence, tip swelling and second septum formation. The mitosis first occurs soon after surface attachment and a nucleus from the second round of mitosis during tip swelling migrates into the hooked cell before septum formation. A mature appressoria normally contains a single nucleus [20-22]. The outside plasma membrane of the mature appressorium is covered by a melanin layer except for the region in contact with the surface of the substratum, where the penetration peg, a specialized hyphae that penetrates the tissue surface develops [22-24].

Cellular glycerol concentration sharply increases during spore germination, but it rapidly decreases at the point of appressorium initiation, and then gradually increases again during appressorium maturation. This glycerol accumulation generates high turgor pressure in the appressorium, and melanin is necessary for maintaining the glycerol gradient across the appressorium cell wall [25].

Appressoria are induced in response to physical cues including surface hardness and hydrophobicity, as well as chemical signals of exogenous cAMP, ethylene, the host's ripening hormone and the plant cutin monomer, hexadecanoic acid [26-28] whereas long chain fatty acids and tripeptide sequence Arg-Gly-Asp (RGD) inhibits appressorium induction [29, 30].

Signal cascades during appressorium development

Highly conserved among fungi, the cAMP signaling pathway is one of the most well characterized signal pathways regulating fungal nutrient sensing, morphogenesis and virulence [31-33]. In *M. grisea*, exogenous cyclic AMP and its analogs induce appressorium on non-inductive surfaces [28]. The components of cAMP signaling pathway have been intensively studied using direct gene deletion. G protein α subunit genes, MagA. MagB and MagC are required for sexual development. MagB disrupted mutants showed a significant reduction in appressorium formation, sporulation, and virulence [34]. Constitutive activation of MagB bypassed the requirement of surface hydrophobicity for appressorium development but resulted in the reduced virulence [35]. Similar to MagB, the adenylate cyclase, Mac1, which is activated upon binding to GTP-G protein α to produce cyclic AMP, is essential for appressorium formation, pathogenecity, and mating. Exogenous cyclic AMP functionally compensated the $Mac1^-$ mutant [35, 36].

Protein Kinase A (PKA) in the inactive state consists of two catalytic subunits and two regulatory subunits. Cyclic AMP selectively binds to the regulatory subunits and alters their conformation, causing them to dissociate from the complex producing functional active catalytic subunits. A gene replaced mutant of the catalytic (CpkA) subunit of PKA in *M. grisea* was unable to develop appressoria, did not respond to exogenous cAMP, and lost pathogenecity on rice [37]. Further analysis of CpkA mutants provided controversial results of delayed appressorium formation and appressorium induction by exogenous cAMP, indicating CpkA is involved in appressorial penetration [37, 38].

The mitogen-activated protein (MAP) kinase signaling pathway is one of the major mechanisms for sensing external signals and transducing them into the cell to invoke the appropriate biological response. In budding yeast, *Saccharomyces cerevisiae*, sharing the common components, distinct MAP kinase pathways are required for mating, morphological changes, osmoregulation and cell wall integrity [39, 40]. In fungal pathogens, MAP kinase signal pathways are involved in fungal pathogenecity [41]. *M. grisea* contains 3 MAP kinases, PMK1, Mps1, and OSM1, which are homologous to *S. cerevisiae* MAP kinase FUS3/KSS1, Slt2 and HOG1 respectively, and all of which have been characterized by targeted gene deletion [42-44]. The GFP-PMK1 fusion protein was highly expressed in appressoria and developing spore rather than in vegetative hyphae and mature spore [45]. Different from the fus3/kiss double deleted mutant in *S. cerevisiae*, which was defective in mating, the *M. grisea* pmk1 null mutant showed normal growth and sexual development in laboratory conditions but failed to develop appressoria and infection hyphae [43]. Mobilization of the stored glycogen into the developing appressorium was impaired in the pmk1 null mutant [24], but the mutant responded to the exogenous cyclic AMP resulting in the initial tip differentiation. This suggests cross talking between cAMP and MAP kinase signaling pathways during infection structure development [43].

The importance of the PMK1 MAP kinase signal pathway in fungal pathogenecity was confirmed by the characterization of the other components of that pathway. MST1 and MST11 are the upstream components of the PMK1 pathway and are homologous to Ste7 and Ste11 in budding yeast respectively. Similar to PMK1, they are necessary for appressorium formation [46]. MST12, a counterpart of STE12 which is a

transcription factor downstream of FUS3/KSS1 MAP kinase signal pathway, is dispensable for melanized appressorium formation but is necessary to develop the infection hyphae with complete loss of pathogenecity [47]. Thus, other transcription factors regulate appressorium formation. Direct comparison of gene expression between wild type and pmk1 mutant by subtractive library screening led to the identification of two homologous genes, GAS1 and GAS2. Localized in the cytoplasm of the cell, GAS1 and GAS2 are especially abundant in mature appressoria. Even though appressorium formation was unaffected, significant reduction in fungal infection was found in GAS1 and GAS2 deletion mutants [48]. As expected from the function of a homologous gene Slt2 in yeast, Mps1 is responsible for cell wall integrity against cell wall degrading enzymes. Although mps1 null mutants develop melanized appressoria, Mps1 is required for the appressorium penetration into host cells [44].

Under hyperosmotic pressure, mycelia of *M. grisea* accumulate the compatible solute arabinitol and it is controlled by OSM1 MAP kinase, a homolog of HOG1 which is responsible for the regulation of cellular turgor in yeast [42]. In contrast to the PMK1 and Mps1, OSM1 is not required for appressorium development and fungal infection. Since the high osmotic turgor pressure is required for appressorium function, it is expected that the stage specific osmoregulatory mechanisms may also exist in *M. grisea* [42].

The calcium/calmodulin-dependent signaling pathway is involved in the growth and pathogenesis in the fungal pathogens of humans, *Crytococcus neoformans, Candida albicans* and *Aspergillus fumigatus* [49]. Compared to other signal pathways, the calcium/calmodulin signaling pathway is not yet well characterized in *M. grisea*. However the importance of this pathway on appressorium formation was first indicated

by the complete inhibition of appressorium formation in the presence of a calcium chelator (EGTA) and calmodulin antagonists [50]. Expression of calmodulin was significantly reduced by self inhibitors that inhibit spore germination and appressorium formation [51]. Direct evidence of this pathway on appressorium formation was provided by the characterization of CYP1, a cyclophilin-encoding gene [52]. Gene expression of CYP1 was highly induced during plant infection [53]. Cyclophilin forms a complex with cyclosporin A, which inhibits the enzyme activity of calmodulin dependent protein phosphatase, calcineurin. CPY1 deleted mutants were less sensitive to cyclosporin A and had a large central vacuole and fewer lipid bodies in spores. In mutants, melanized appressoria which contained few lipid droplets, failed to generate full turgor and showed reduced plant penetration [52]. The phenotypes exhibited by mutants indicates a role of calcium/calmodulin signaling pathway in lipid metabolism in *M. grisea*, which might act by interacting with other signaling pathways such as cAMP signaling and MAP kinase signaling. Further investigation on this pathway remains to be performed.

Melanin biosynthesis in *M. grisea*

Melanins, a large group of diverse substances, are macromolecules formed by oxidative polymerization of phenolic or indolic compounds. In fungi, melanin results in black or brown pigmentation and plays an important role in fungal pathogenesis. The two most well known melanins are 1,8-dihydroxynaphthalene (DHN) melanins and L-3,4-dihydroxyphenylalanine (DOPA) melanins, which are named after the pathway intermediates. Among plant fungal pathogen, DHN melanin biosynthesis is well characterized. In this pathway, melanin is synthesized via the polyketide pathway.

Malonyl-CoA is first converted to 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-THN) by a polyketide synthase (PKS) and then 1,3,6,8-THN is reduced to scytalone by tetrahydroxynaphthalene reductase (4HNR). Dehydration of scytalone by scytalone dehydratase (SCD) forms 1,3,8-trihydroxynaphthalene (1,3,8-THN). 1,3,8-THN is then reduced to vermelon by 1,3,8-THN reductase (3HNR) and vermelon is dehydrated to 1, 8-DHN possibly by SCD. Dimerization of 1,8-DHN following polymerization to melanin might be catalyzed by a polyphenol oxidase. [54-57].

Ultraviolet-light mutagenesis created many non-pathogenic melanin defective mutants in *M. grisea* and they provided three distinctive phenotypes of albino (Alb^-), rosy (Rsy^-), and buff (Buf^-). Three unlinked genes corresponding to these phenotypes, ALB1, RSY1, and BUF1 genes were cloned [22, 58] and, according to the whole genome sequence, they correspond to the genes MG07219 (PKS), MG05059 (SCD) and MG02252 (3HNR) in the DHN-melanin biosynthesis. This showed the importance of melanin biosynthesis for fungal pathogenesis. In addition, 4HNR, MGG_07126 in this pathway was also identified in the *M. grisea* [54], but the oxidizing enzymes which catalyze in the last step of the pathway remain to be characterized.

PKS, SCD and 3HNR are highly conserved in pathogenic filamentous fungi and are often clustered together. Targeted gene deletion mutants have demonstrated their role in pigmentation and pathogenecity [59-62]. SCD and 3HNR appears to be regulated by orthologous Zn2Cys6 binuclear transcription factor Pig1 in *M. grisea*, Cmr1 in *Colletotrichum lagenarium*, and Cmr1 in *Cochliobolus heterostrophus*. Cmr1 deletion mutants did not express SCD and 3HNR during vegetative growth and exhibited significant defects in mycerial pigmentation. But interestingly enough, mutants exhibited

gene expression during appressorium development and produced fully melanized appressorium [60, 63]. Detailed studies on stage specific gene expression of melanin biosynthetic genes and their regulation have not been examined.

Recycling processes in appressoria

Since appressoria are directly linked to pathogenecity, enormous effort had been devoted to identify genes which are involved in appressorium formation and function in *M. grisea*. Cytological studies, differential gene expression, random mutagenesis and homology based gene characterization have resulted in the identification of considerable numbers of pathogenecity factors in *M. grisea*.

Spores of *M. grisea* contain high level of energy storage materials such as carbohydrates and lipids. At the onset of germination, spore glycosome and lipid droplets quickly localize into the appressorium. Lipid bodies are degraded by triacylglycerol lipase during turgor generation. As recycling organelles, enlarged vacuoles with scattered peroxisome complexes are frequently seen in mature appressoria [24]. Glycogen and lipid mobilization were blocked in a PMK1 deletion mutant and were significantly delayed in a cPKA mutant.

Several genes involved in lipid metabolism and fatty acid degradation have been recognized as novel pathogenecity factors. They include genes for peroxisomal fatty acid β-oxidation, glyoxylate cycle such as the multifunctional β-oxidation protein MFP1, carnitine-dependent transport of peroxisomal acetyl-CoA MgPex6, peroxisomal carnitine acetyl transferase PTH2, isocitrate lyase and malate synthase [64-67].

Acetyl-CoA which is produced in fatty acid β-oxidation can be used for melanin biosynthesis and glycerol accumulation [24]. Peroxisomes are organelles that perform a wide range of metabolic functions, especially those closely related to pathogenecity such as the glyoxylate cycle, lipid metabolism, and stress response. Currently, in eukaryotic cells, more than 30 peroxisome biogenesis related genes have been characterized [68], but many of them are still unknown in *M. grisea*.

Using light and transmission electron microscope studies coupled with lipid and vacuole staining dyes, Weber et al. revealed that the lipid droplets in appressoria enter the vacuole in similar manner to autophagocytosis for further degradation [69]. Further evidence for the role of autophagic cell death in appressorium formation has come from functional characterization of autophagy genes, MgATG1 and MgATG8 in *M. grisea*. MgATG1 and MgATG8 are homologous to autophagy related genes in yeast, Serine/Threonine Kinase (ATG1) and Ubiquitin-like protein (ATG8) respectively and were found to be essential for functional appressorium formation [70, 71]. A large group of genes are involved in autophagy [72], but most have yet to be characterized in *M. grisea*.

Genomic and proteomic approaches toward *M. grisea*

Differential gene expression analysis is a powerful approach to reveal new insight into functional biology. Moreover, recent advances in high-throughput sequencing and microarray technologies now enable investigations of gene expression patterns at the whole genome level. Fungal development processes are accompanied by significant transcriptional regulation. During spore germination, the filamentous fungi *Neurospora*

crassa and *Ustilago maydis*, and the soil amoeba *Dictyostelium discoideum*, undergo global transcriptional changes. Using microarray techniques, novel and previously known germination regulated genes were identified. Cross comparison of gene expression profiles revealed the orthologous genes with similar expression patterns, which suggests evolutionary conserved mechanisms for spore germination [73-75]. Microarray analysis also revealed a strong gene expression shift from germination to haustorial development in the rust fungi *Uromyces fabae* and many genes that were specially induced by the host plant in haustoria were identfied [76]. Examination of the dynamics of gene expression during sexual development identified a group of transporter genes that were associated with perithecium formation in Fusarium head blight fungus *Gibberella zea* [77].

At the time of fungal infection, both the pathogen and the host plant exhibit changes in gene expression patterns, which are likely essential for fungal survival and proliferation as well as host resistance. Increased gene expression for the production of phytoalexin and pathogenesis related (PR) proteins were identified in the soybean plant after infected with *Phytophthora sojae* and a number of fungal genes such as cutinase and endoglucanase, also were induced during this infection process [78]. More detailed fungal gene expression during interaction with the host has been reported for the powdery mildew pathogen *Blumeria graminis*. Transcript profiles from pre and post plant infection showed dynamic changes in primary metabolism during infection and revealed a group of genes which appeared to be co-regulated with previously known virulence factors [79]. Studies on *in planta* gene expression led to the identification of a novel virulence factor, FKBP12 protein in *Botrytis cinerea* [80].

In *M. grisea*, studies on the stage specific gene expression have identified important pathogenecity factors such as a hydrophobin MPG1 and GAS homologs [48, 53]. A number of experimental and analytical tools have been applied to study differential gene expression. Analysis of expressed sequence tags (ESTs) from *M. grisea* infected rice cDNA library revealed 72 fungal genes and 221 plant genes that were suggested to be involved in the plant fungal interaction [81]. Serial analysis of gene expression (SAGE) in appressoria induced by cAMP and germinating spores led to the identification of a 110 unique sequence tags that were under the control of exogenous cAMP. Several of these tags corresponded to previously characterized pathogenecity related genes in *M. grisea*, including MPG1, MAS1 and MAC1. Microarray analysis of *M. grisea* array containing 3500 cDNA clones resulted in the identification of 85 up regulated and 38 down regulated genes in spores and/or appressoria, compared to mycelia [82]. One hundred forty two genes were found to be unique in an appressorium cDNA library which was subtracted with cDNA from spore and mycelia [83].

The recent availability of the whole genome sequence for *M. grisea* has dramatically increased the scope of data acquisition and analysis [13]. A total of 8,177 ESTs were identified in cDNA libraries from diverse growth conditions and tissue types including appressorium and nitrogen starvation. Ninety five percent of the ESTs matched the *M. grisea* genome while 59% were found only in a specific library showing stage specific gene expression [84]. In whole genome microarray experiments, gene expression of 520 genes were regulated during nitrogen starvation [85]. Combined analysis of massively parallel signature sequencing (MPSS), robust-long serial analysis of gene expression (RL-SAGE) and microarray studies revealed a total of 2,430 mycelial genes

and 1,886 appressorial genes [86]. On the other hand, compared to transcription studies, proteomic approaches are in their infancy. Two dimensional gel based protein profiles identified five differentially induced proteins during appressorium formation. They included scytalone dehydratase (SCD) in melanin biosynthesis and another four whose putative function is protein mobilization [87]. Protein identification using 2-D electrophoresis and gene expression studies with suppression subtractive hybridization (SSH) indicated increased amino acid synthesis during appressorium formation by *Phytophthora infestans* [88]. Even though a number of advanced techniques have been applied to study gene expression in *M. grisea*, the disparate experimental design, scales and analytical tools used, and the lack of supporting biological evidence has made it difficult to find overarching patterns of gene expression during appressorium development and plant infection by *M. grisea*.

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Transcriptome analysis reveals new insight into appressorium formation and function in the rice blast fungus, *Magnaporthe grisea*

Abstract

Rice blast disease is caused by the filamentous Ascomycete *Magnaporthe grisea*. This disease causes significant annual losses to rice production world-wide. Infection by *M. grisea* requires the development of an appressorium, a specialized infection structure. High quality genome sequence is available for both *M. grisea* and rice.

To understand the mechanisms of appressorium development, we performed whole genome microarray experiments. The most dramatic change in gene expression occurred during spore germination where 21% of the genes represented on the array showed differential expression, with most genes being up-regulated. Approximately three percent of the predicted genes were differentially expressed during appressorium formation in response to both a hydrophobic surface signal and exogenous cyclic AMP. Functional characterization of 16 differentially expressed genes including a subtilisin protease (*SPM*1) and NAD specific glutamate dehydrogenase (*Mgd*1) by targeted gene disruption revealed a hitherto unknown finding that protein degradation and amino acid metabolism are essential for the appressorium formation and infection process.

Genome wide transcriptional profiles followed by comprehensive functional studies provided novel insight into infection structure development in *M. grisea*. Our data will directly benefit efforts to identify fungal pathogenecity factors and the development of new disease management strategies.

Introduction

Discovery of how parasites recognize an appropriate host and deploy the suites of effector molecules necessary to insure successful infection is central to understanding parasite biology and requisite for the development of effective control measures. Host recognition may involve a combination of physical, chemical, or other environmental cues, which initiate an elegant but complex series of reactions that culminate in the transfer of the cue from the cell surface to the nucleus where transcription of virulence associated genes is initiated. *Magnaporthe grisea* is typical of many fungal pathogens of plants in that it elaborates a specialized infection cell called an appressorium to infect its host. *M. grisea* is the causal agent of rice blast, the most destructive fungal disease of rice world wide and a seminal model for the study of the molecular basis of fungal-plant interactions. Following spore attachment and germination on the host surface, the emerging germ tube perceives physical cues such as surface hardness and hydrophobicity, as well as chemical signals including wax monomers that trigger appressorium formation [1-3].

Appressorium formation begins when the tip of the germ tube ceases polar growth, hooks, and begins to swell. The contents of the spore are then mobilized into the developing appressorium, a septum develops at the neck of the appressorium, and the germ tube and spore collapse and die. As the appressorium matures, it becomes firmly attached to the plant surface and a dense layer of melanin is laid down in the appressorium wall, except across a pore at the plant interface. Turgor pressure increases inside the appressorium and a penetration hyphae emerges at the pore, which is driven through the plant cuticle into the underlying epidermal cells [4-9]. Melanin deposition in

the cell wall of the appressorium is essential for maintaining turgor pressure. Genetic mutations or chemical treatments that inhibit appressorium formation and function effectively block penetration and subsequent disease development [6, 10].

Highly conserved signaling networks that transfer cues from the environment to the nucleus play a crucial role in regulating pathogen-host interactions. For *M. grisea,* the MAPK, cAMP and to a lesser extent Ca^{++} signaling pathways have been shown to be essential for appressorium formation and function [11-15]. In addition, the cAMP signaling pathway regulates several other aspects of fungal growth and development including nutrient sensing and cell morphogenesis [16-18]. In *M. grisea*, exogenous cyclic AMP and analogs induce appressorium formation in non-inductive environments [19]. Subsequent functional characterization of proteins in the cAMP signaling pathway, including MagB, a subunit of G protein, Mac1, adenylyl cyclase, and cPKA, the catalytic subunit of protein kinase A, provided clear evidence for the essential role of cAMP in regulating appressorium morphogenesis [12, 20-22]. However, while the core pathways are highly conserved, we are only beginning to reveal the downstream genes and pathways that direct appressorium formation.

Appressorium function is dependent on generating high levels of turgor, which in *Magnaporthe* results from high concentrations of glycerol. How glycerol is generated in the appressoria remains to be clearly defined, but because appressoria develop in the absence of nutrients, it has been suggested that glycerol must be derived from storage products. Carbohydrate catabolism in yeast is regulated by the cAMP response pathway, however, there is no genetic evidence that metabolism of storage glycogen or trehalose is required for appressorium turgor generation [23]. *TRE*1, which encodes the main intracellular trehalase activity in conidia, is not required for appressorium function [24].

On the other hand, targeted mutagenesis of genes involved in degradation of storage lipids or beta oxidation of fatty acids such as *MFP*1 or genes involved in peroxisome function such as *PEX*6, prevent appressorium function but do not appear to affect the accumulation of glycerol [25]. Thus, although spores and developing appressoria contain substantial amounts of lipids and carbohydrates, it appears that glycerol may be derived from other cellular materials. Appressorium formation is accompanied by collapse of the spore, a process involving autophagy whereby cellular contents of the spore are re-cycled into the developing appressorium [26]. This opens up the possibility that glycerol may be derived from other cellular components in addition to storage lipids and carbohydrates.

Studies of appressorium formation and early stages of host invasion suggest that *M. grisea* is not only capable of perceiving its host but is able to evade host detection during pre-penetration and tissue colonization [27]. Bacteria have evolved a specialized type III secretion system to deliver proteins into plant cells to help evade host recognition and promote invasive growth [28]. *M. grisea* mutants defective in secretion, delta Mgapt2 strains for example, are unable to cause disease [29]. Thus, secreted proteins likely play a significant role in fungal pathogenesis. The *M. grisea* genome contains a large and diverse complement of secreted proteins, however, their function remains largely unknown. Other effector molecules, including secondary metabolites, may be delivered by transporters. It is known that ABC type transporters such as *ABC*3 are required for appressorium function [30]. *M. grisea* contains at least 23 polyketide synthases, several non-ribosomal peptide synthases and more than 120 highly diverged cytochrome P450 monooxygenases suggesting a significant capacity to produce a diverse array of secondary metabolites [31]. The nature of these metabolites and the role they play in the infection process is not well defined.

Although evidence collected to date provides important clues as to processes involved in appressorium formation and function, a complete understanding of the metabolic changes and genes contributing to infection related morphogenesis is far from complete. One powerful method for refining and extending knowledge of the infection process is to identify alterations in transcription as *M. grisea* undergoes appressorium formation. To date, only limited gene expression studies have been performed to identify genes associated with appressorium formation and function in fungal pathogens [32-34]. Published studies have only examined subset of the total gene complement and have been far from exhaustive. The recent completion of the *M. grisea* genome sequence greatly enables genomic analyses [31].

In this study, we made use of a whole genome oligo microarray chip containing over 13,000 *M. grisea* elements representing 10,176 predicted genes, and conducted global gene expression profiles during spore germination and appressorium formation on both an inductive hydrophobic surface and in response to cAMP (Figure 1). From this data, we distilled a consensus set of genes differentially expressed in response to both physical and chemical cues, and constructed putative biological pathways that participate in appressorium formation. Our data shows that germination stimulates a major transcriptional response characterized by a dramatic transcription of genes involved in metabolism and biosynthesis. On the other hand, induction of appressorium formation triggered a significant decrease in genes associated with the translational apparatus, with a coordinate increase in the expression of genes involved in protein and amino acid degradation, lipid metabolism, secondary metabolism and cellular transportation. Significantly, the set of up-regulated genes is enriched for those encoding predicted secreted proteins. To directly assay the role of these gene sets in appressorium formation

and function, we performed targeted gene deletion studies on many of the most highly up-regulated genes. Our findings reveal that protein degradation and amino acid metabolism are essential for the infection process. Further, we find many differentially expressed genes are not required for appressorium formation and function. This may suggest that *M. grisea* employs a number of backup systems, such as functional redundancy and compensatory processes in order to protect appressorium formation from being de-regulated.

Results

Genes involved in core biological processes undergo dramatic transcriptional changes during spore germination.

Microarray analysis revealed that about 29 % of the 10,176 *M. grisea* genes present on the array underwent significant changes (≥ 2 fold, $p < 0.05$) in expression during at least one of the developmental processes tested including spore germination, germ tube elongation and appressorium development (Table 1). The most dramatic change in gene expression occurred during spore germination (Phil7 vs. Spore) where 21% showed differential expression with the vast majority being up-regulated. Seventy three percent of the genes differentially expressed during spore germination exhibited no further change in expression during germ tube elongation or appressorium formation. Very few further changes (< 1%) in gene expression were observed during germ tube elongation (Phil12 vs. Phil7).

To explore the cellular processes active during spore germination, differentially expressed genes were first grouped according to GO terms. Examination of gene expression with GO categories revealed that during spore germination genes involved in major biological processes such as metabolism (GO:0008152) and biosynthesis (GO:0009058) were significantly over represented ($p < 0.01$) in the up-regulated gene set (Table 2). In particular, genes associated with carbohydrate metabolism (GO:0005975), amino acid and derivative metabolism (GO:0006519) and protein metabolism (GO:0019538) were over represented. In contrast, genes associated with the GO category for transcription (GO:0006350) was under represented in the up-regulated gene set. The GO category for transcription contains mainly transcription factors and other proteins

involved in DNA binding. Typically, transcription factors are post transcriptionally regulated and thus their expression would not necessarily be expected to be over represented during spore germination.

Thigmotrophic and chemical induction of appressorium formation trigger similar patterns of gene expression.

Approximately 3 - 4 % of the entire set of *M. grisea* ORFs were differentially expressed during appressorium initiation (Pho7 vs. Phil7) and maturation (Pho12 vs. Phil12) on the inductive surface. In response to exogenous cAMP, about 10% of expressed genes were differentially expressed (cAMP9 vs. Phil9; Table 1). Considerably more genes were found to be induced rather than repressed by both physical and chemical (cAMP) stimulation. Overall, good correlations (Pearson's correlation coefficient $r>0.5$) were observed between appressorium related expression profiles induced by physical cues (appressorium initiation and maturation) and by cAMP (Figure 2). In contrast, gene expression profiles during spore germination and germ tube elongation correlated poorly with those observed for appressorium formation. The highest correlation ($r=0.66$) was found between appressorium maturation and cAMP induced appressoria where 66% of differentially expressed genes showed a similar expression pattern. Approximately 54% of genes differentially expressed during appressorium initiation exhibited a similar expression pattern in response to cAMP (Table 1, Figure 2).

Microarray based gene expression pattern is consistent with expression analysis from reverse transcriptase PCR and quantitative RT-PCR

To confirm gene expression patterns derived from our microarray experiments, we performed reverse transcriptase PCR with 5 selected up-regulated genes, 3 down-regulated, and 2 showing no expression change (Figure 3). Genes were selected based on their overall expression levels, i.e. represented high to medium to low expressed genes. If genes contain an intron, primers were designed to bridge the intron to distinguish amplification of transcript from any possible genomic DNA contamination (Table 3). Reverse transcriptase PCR results were consistent with the microarray data, albeit the absolute levels of expression fold change showed slight variation (Figure 3). Two genes *MPG*1 and *PTH*11 [35, 36] were also subjected to analysis by quantitative RT-PCR. Both genes are required for pathogenesis. *MPG*1 has been shown to be highly expressed during appressorium formation [37]. However, our microarray and RT-PCR results indicated that both genes were more strongly up-regulated during germ tube elongation than appressorium formation (Figure 3).

Appressorium consensus gene sets reveal key biological processes for appressorium formation

To identify genes that participate in appressorium formation, we compared gene expression profiles of appressorium initiation, maturation and cAMP induced appressoria. A total of 240 genes were up-regulated and 117 were down-regulated during appressorium initiation or maturation and in response to cAMP (Figure 4). These genes, referred to as appressorium consensus genes, were functionally grouped into GO categories based on manual curation as described in materials and methods (Figure 4,

Table 4). Overall, we noted a significant decrease in expression of genes involved in protein synthesis during appressorium induction. On the other hand, expression of genes associated with protein and amino acid degradation, lipid degradation, secondary metabolism including melanin biosynthesis and cellular transportation exhibited a dramatic upshift. Moreover, this set of genes exhibited nearly a 4-fold enrichment for genes encoding secreted proteins. A detailed discussion of the functional groups exhibiting differential expression is presented below.

1. Major changes in amino acid and protein metabolism

Two of the major functional categories of genes in the up-regulated appressorium consensus gene set were those with predicted roles in protein and amino acid degradation. Protein sequence analysis of putative proteases recognized subgroups according to the active site and substrate specificity such as acid proteases (MGG_03056.5, MGG_09032.5), aspartyl proteases (MGG_09351.5, MGG_00981.5), subtilisin-like proteases (MGG_03670.5, MGG_09246.5), calpain (calcium-dependent cytoplasmic cysteine proteinase)-like proteases (MGG_08526.5, MGG_03260.5), a cysteine protease required for autophagy (MGG_03580.5), a carboxylpeptidase (MGG_09716.5), and a tripeptidyl peptidase (MGG_07404.5).

MGG_03670.5 (named *SPM1*) and MGG_09246.5 are putative proteases bearing the signature for subtilisin peptidase. A BLASTp search revealed that *SPM1* and MGG_09246.5 have 39% amino acid identity and 55% similarity to each other and both match serine proteases from various microorganisms. The possibility of *SPM1* as a pathogenicity candidate in *M. grisea* was first proposed based on its prevalence in a cDNA library of mature appressoria [38]. *SPM1* was also found to be abundant in SAGE

tags derived from cAMP induced mature appressoria [33]. Although *SPM1* contains a predicted signal peptide, the protein appears to be targeted to the vacuole [38]. As previously reported [39], *SPM1* targeted deletion mutants produced melanized appressoria but exhibited severely reduced pathogenicity on rice and barley plants. Disease lesions failed to expand and sporulation was severely reduced [39]. In addition, further characterization revealed vegetative growth of deletion mutants was decreased on the various media such as oatmeal, V8 and minimal media but little difference was observed on complete media (Figure 5). On the other hand, targeted deletion mutants of the putative protease encoded by MGG_09246.5 appeared normal and formed typical pigmented appressoria and developed disease symptoms on barley and rice plants indistinguishable from wild type (Figure 6).

Protein degradation is highly regulated in many instances. Many short-lived proteins destined for degradation are selectively tagged by ubiquitin. It is noteworthy that several proteins involved in this process including polyubiquitin (MGG_01282.5) and ubiquitin activating enzyme E1 like protein (MGG_07297.5) were up-regulated. Additionally, gene expression of putative ubiquitin protein ligases (MGG_11888.5, MGG_01115.5) exhibited increased expression in response to cAMP. Following selective tagging proteins are degraded by the proteasome. Several probable 26s proteasome regulatory protein subunits (MGG_05477.5, MGG_05991.5, MGG_01581.5, MGG_07031.5) were up-regulated by cAMP. Currently, it is unknown which proteins are selectively tagged or how the proteasome regulatory proteins influence appressorium formation.

In addition to evidence for elevated protein degradation during appressorium formation, expression of genes involved in amino acid metabolism was also up-regulated.

A putative aminotransferase (MGG_09919.5), a cystathionine γ-lyase (MG10380.5) that catalyses the transition of cystathionine to cysteine, 2-oxobutanoate and ammonia, a cysteine dioxygenase (MG6095.5) in cysteine degradation pathway, and a threonine deaminase (MGG_07224.5) required for threonine degradation were up-regulated. 2-oxobutanoate (α-ketobutyrate), produced by cystathionine γ-lyase and threonine deaminase, can then be further metabolized through the TCA cycle. Turnover of the cellular storage amino acids, arginine and proline, to glutamate depends on the nutrient status of the cell. Genes involved in arginine and proline degradation to glutamate, such as arginase (MGG_10533.5), ornithine aminotransferase (MGG_06392.5), delta-1-pyrroline-5-carboxylate dehydragenase (MGG_00189.5), and proline oxidase (MGG_04244.5) were up-regulated during appressorium formation.

NAD(+) dependent glutamate dehydrogenase (NAD-GDH) provides a major conduit for feeding carbon from amino acids back into the TCA cycle. The enzyme catalyzes the oxidative deamination of glutamate to produce α-ketoglutarate and ammonia (glutamate + NAD^+ → α-ketoglutarate + NH_4 + NADH). Our gene expression data showed that the *M. grisea* NAD-GDH homolog MGG_05247.5, which we have named *Mgd1*, was present in the up-regulated appressorium consensus gene set. Previous work using SAGE had shown that transcripts of *Mgd1* were abundant in mature appressoria of *M. grisea* induced by cAMP [33]. In yeast, NAD-GDH is repressed by ammonium and derepressed by glutamate and carbon starvation [40-42]. Increased enzyme activity was observed during yeast to mycelium morphogenesis in *Mucor racemousus* and *Benjamineilla poitrasii* and hyphal adhesion and arial hyphae development in *N. crassa* [43-45]. NAD_GDH gene deletion mutants showed poor growth on glutamate as a sole nitrogen source in yeast and *Aspergillus nidulans* [40, 46].

To evaluate the function of *Mgd1* in *M. grisea*, we generated 4 independent targeted deletion mutants. Mutants lacked aerial hyphae when grown on complete media (Figure 7). In addition, growth was severely reduced on poor carbon sources such as Tween 20 and PEG compared to ectopic and wild type strains. The mutants also grow more poorly than ectopic and wild type strains on glucose limiting conditions in the presence of glutamate and glutamine. To determine the role of *Mgd1* in virulence we evaluated mutants for appressorium formation and the ability to cause disease. Mutants had a reduced ability to form mature appressoria (45%) on an inductive surface, others produced appressorium that appeared immature (41%) or were abnormal and highly swollen (4%) (Figure 6). When inoculated onto susceptible barley and rice plants, the mutants exhibited highly reduced virulence and produced many fewer and smaller lesions (Figure 6). Thus, *Mgd1* appears to be required for efficient metabolism of carbon and/or nitrogen from the break down of proteins under nutrient limiting conditions as experienced when cells are attempting to form appressoria.

In contrast to the activated expression of genes involved in protein and amino acid degradation, a major portion of the down-regulated genes encode components of the ribosome; 16 constitute the large ribosomal large subunit and 6 the small subunit (Figure 4, Table 4). Expression of all of these genes was up-regulated during spore germination and remained unchanged during germ tube elongation. However, upon appressorium induction the average level of expression fell 30% during appressorium initiation, and by more than 2-fold during appressorium maturation. Similar changes in gene expression patterns were observed in appressoria induction by cAMP. This pattern of down-regulation of genes associated with ribosome biogenesis has been observed commonly in a number of organisms subjected to nitrogen starvation or upon treatment with rapamycin,

a potent inhibitor of TOR kinase. In *M. grisea*, this set of genes, with the exception of MGG_05716.5, was down-regulated significantly (>2 fold) when cells were shifted to media lacking nitrogen for 12 hr [39]. In the experiments reported here, spores were germinated in water and thus would expect to be starved for both nitrogen and carbon, however, it was only upon appressorium formation that expression of genes associated with ribosome biogenesis fell. The role of TOR is complex, it is not only associated with starvation but with development. In yeast, TOR interacts with a number of other proteins including STE20 and regulates sexual development [47]. In addition, rapamycin is a potent inhibitor of filamentous growth in fungi, including *Aspergillus fumigatus* [48]. Thus, during appressorium formation, the TOR pathway may be involved in redirecting resources away from polar growth to breaking down cellular components in order to generate materials necessary for the penetration process to be effective.

2. Increased gene expression for lipid metabolism

Lipids are essential components of living cells as well as major sources of energy reserves. Several genes involved in the synthesis of the cell membrane components were induced during appressorium formation and they included a 7-dehydrocholesterol reductase (MGG_03765) that catalyzes the last step of cholesterol biosynthesis pathway, oxysterol binding protein (OSBP) (MGG_00853.5) involved in cholesterol biosynthesis, a probable sterol carrier protein (MGG_02409.5) involved in cholesterol trafficking and metabolism, glycerol-3-phosphate acyltransferase (MGG_11040.5) required for phospholipid biosynthesis, diacylglycerol cholinephosphotransferase (MGG_03690.5) involved in phosphatidylcholine biosynthesis pathway, and delta 8 sphingolipid desaturase (MGG_03567.5) involved in sphingolipid metabolism. Conversely, gene

expression of a fatty acid omega hydroxylases (MGG_10879.5, MGG_01925.5) and a cholinesterase (MGG_02610.5) involved in lipid degradation was found to be decreased.

β-oxidation of fatty acids in fungi occurs mainly in the peroxisome. Peroxisomes are membrane bound subcellular organelles where diverse anabolic and catabolic metabolisms, including peroxide metabolism, glyoxylate metabolism, and phospholipid biosynthesis, are conducted [49]. During fatty acid metabolism, the very long chain fatty acids (VLCFA; C22 or longer) are first transferred to coenzyme A by a very long chain fatty acyl-CoA synthetase. In *Saccharomyces cerevisiae*, a very long chain fatty acyl-CoA synthetase, FAT1, disruption mutants showed reduced growth on media containing dextrose and oleic acid and VLCFA accumulated in cells [50]. Our data showed that a very long chain fatty acyl-CoA synthetase (MGG_08257.5) was up-regulated, suggesting fatty acid catabolism is involved in appressorium formation and function.

Recently, several genes for peroxisome structure, translocation of peroxisomal target proteins and metabolism in the peroxisome have been shown to be involved in pathogenicity, cellular differentiation and nutrient assimilation in fungi [51-54]. In *M. grisea*, isocitrate lyase (*ICL1*) of the glyoxylate cycle, *HEX1* from the woronin body, *PTH2* peroxisomal acetyl carnitine transferase, *MFP1* the multifunctional β-oxidation protein and *MgPex6* required for peroxisome biogenesis were found to be necessary for functional appressorium development and fungal infection [25, 55-57]. A putative fatty acid binding persoximal protein MGG_07337.5 was identified in the up-regulated set of appressorium consensus genes. MGG_07337.5 encodes a protein with 40% identity and 59% similarity to the peroxisomal nonspecific lipid transfer protein, PXP-18, which is encoded by *POX18* from *Candida tropicalis* and is highly conserved in filamentous fungi. *POX18* mRNA was shown to be enriched by oleic acid and n-alkane rather than by

glucose. PXP-18 appears to function in peroxisomal production of acetyl-coA either by guiding lipids through the oxidation processes or by protecting the acyl coA oxidase enzyme [58-61]. To address the function of the PXP-18 homolog in *M. grisea*, targeted deletion mutants were created. However, despite other evidence for the role of the peroxisome in appressorium formation and plant infection [25, 51, 62], the putative peroxisomal protein MGG_07337.5 was found to be dispensable for the development of a mature pigmented appressorium and disease symptom development on barley and rice plants in this study. Mutants were indistinguishable from wild type for other aspects of growth and development examined (Figure 6).

3. **Carbohydrate metabolism - cell wall degradation, remodelling and carbon scavenging during appressorium development**

Carbohydrates represent a major component of fungal biomass. Glycogen and various polyols are significant storage carbohydrates, whereas chitin, glucans and other polymers are primary constituents of the fungal cell wall. Inspection of our microarray gene expression analysis revealed a group of enzymes for cell wall degradation, glucan mobilization and cell wall glycoprotein processing in the set of appressorium consensus genes. Several chitinases such as MGG_00086.5 and MGG_01876.5, a beta-1,3 exoglucanase (MGG_00659.5), beta-glucosidase (MG10038.5), and polysaccharide dehydrogenase (MGG_01922.5) were up-regulated. However, other glucan degrading enzymes showed opposite expression profiles. For example, glucan 1,4-alpha-glucosidase (MGG_01096.5), endo-1,4-beta-glucanase (MGG_05364.5), beta-1,3-glucosidase (MGG_10400.5) and alpha-L-fucosidase (MGG_00316.5) were down-regulated. The gene expression of a putative cell wall degrading protein (MGG_03307.5) containing a

LysM associated with general peptidoglycan binding function and a glucosamine-6-phosphate deaminase (MG10038.5) in the glucosamine degradation pathway was increased but a UDP-N-acetylglucosamine-pyrophosphorylase (MGG_01320.5) that catalyzes the formation of UDP-N-acetyl-D- glucosamine (UDP-GlcNAc), which is an essential precursor of cell wall peptidoglycan lipopolysaccharide, was repressed. These results suggest dynamic changes in glucan metabolism occur during appressorium formation.

Fungal cell walls contain high level of mannoproteins (30-50% in yeast cell walls). These proteins are first glycosylated (glycosylphosphatidylinositol (GPI) anchored) by mannosyltransferase adding mannose to their serine or threonine amino acid residues and then further processed by mannosidases. During appressorium development, several genes encoding these enzymes, alpha-1, 2-mannosyltransferase (MGG_00695.5), beta-1,4- mannosyltransferase (MGG_10494.5), alpha 1,6 mannosyltransferase (MGG_03361.5) and alpha-mannosidase (MGG_00994.5) were up-regulated. In addition, gene expression of other homologs of an alpha-1, 6-mannosyltransferase (MGG_00163.5), and a mannosidase (MGG_00084.5) were increased by cAMP treatment, implying that the active production of mannoproteins might aid to stabilize the cell wall during the rapid expansion of the appressorium. This hypothesis is supported by the finding that expression of two genes (MGG_03436.5 and MGG_02778.5), which encode putative mannosylated proteins, was strikingly increased. Expression of MGG_03436.5 was the most highly up-regulated in the appressorium consensus gene set (56.6, 59.6, 76.9 fold changes for AI, AM and CI, respectively). MGG_03436.5 is a small hypothetical protein composed of 169 amino acids in 3 exons with no known functional domains. The deduced amino acid sequence showed 23% and 21% identity to that of *A.*

nidulans cell wall mannoprotein MnpA (7.E-04) and *Aspergillus flavus* antigenic cell wall protein MP1 (1.E-04). Interestingly, a putative paralog of MGG_03436.5 in *M. grisea*, MGG_02778.5, was also highly up-regulated in appressorium maturation and response to cAMP (15.5 and 15.1 fold changes for AM and CI respectively). MGG_02778.5 was previously reported as the most abundant EST in a subtracted mature appressorium cDNA library [32]. The function of MGG_03436.5 was investigated by targeted mutagenesis. Surprisingly, no noticeable phenotypic differences including appressorium formation and pathogenicity in MGG_03436.5 gene deleted mutants were observed (Figure 6). This may suggest functional redundancy among related cell wall proteins. The *Mnp*A- null mutant in *A. nidulans* showed an irregular outer cell wall layer, however, no phenotypic differences in spore germination, growth and cellular development were observed [63, 64].

The expression of other genes involved in the utilization of non preferable carbon sources was also up-regulated during appressorium formation. For example, coupled with increased expression of MGG_02129, a homolog of central regulator AlcR of alcohol metabolism in *A. nidulans*, an alcohol dehydrogenase I (MGG_03880.5) and a NAD^+ - aldehyde dehydrogenase (MGG_03263.5), which is involved in ethanol degradation for the production of acetyl-CoA, were up-regulated. Likewise, another gene involved in sugar alcohol degradation, L-arabinitol 4-dehydrogenase (MGG_01231.5), which hydrolyzes L-arabinitol to L-xylulose, was up-regulated. Gene expression of rhamnosidase A (MGG_05246.5), galactose oxidase (MG10878.5), a putative cytochrome P450 for alkane assimilation (MGG_05908.5) and lactate dehydrogenase (MGG_05735.5) were increased but expression of the dTDP-D-glucose 4, 6-dehydratase

in rhamnose biosynthesis pathway (MGG_09238.5) and 2-isopropylmalate synthase in leucine biosynthesis pathway (MGG_13485.5) were down-regulated.

4. **Secondary metabolism during appressorium formation**

Fungi produce an extensive array of secondary metabolites derived from a number of different biochemical pathways including the polyketide, isoprenoid and shikimate acid pathways as well as through modification of amino acids. Polyketides constitute a large class of secondary metabolites produced by filamentous fungi. They are synthesized from large multi domain enzymes, polyketide synthases (PKS) that share significant similarities to fatty acid synthases. Polyketide synthesis requires the coupling of malonyl-CoA to the elongating chain. It is note worthy that the expression of MGG_07613.5, a putative acetyl-CoA carboxylase, the enzyme that catalyzes carboxylation of acetyl-CoA to produce malonyl-CoA was up-regulated in the appressorium consensus gene set.

Melanin is one of the most thoroughly studied polyketides in *M. grisea* and other pathogenic fungi. The three genes involved in the synthesis of dihydroxynaphthalene (DHN)-melanin, a polyketide synthase (PKS), a synthalone dehydratase (SCD), and a hydroxynaphthalene reductase (THR), are clustered in the plant pathogenic fungus, *Alternaria alternata* and the opportunistic human pathogen, *A. fumigatus*. Targeted gene disruption experiments showed that these genes are essential for spore pigmentation and fungal pathogenicity [65-67]. In *M. grisea*, the melanin biosynthesis genes, *ALB*1, *RSY*1 and *BUF*1 are required for appressorium function but are not clustered [6, 10]. *ALB*1, *RSY*1 and *BUF*1 correspond to MGG_07219.5 (a PKS), MGG_05059.5 (a SCD) and MGG_02252.5 (a THR), respectively. In our study, *ALB*1, *RSY*1 genes were present in the set of appressorium consensus genes and were highly up-regulated (Table 4). BUF1

was also induced in appressorium initiation (3.9 fold change, p = 0.056) and significantly up-regulation in cAMP induced appressoria (15.6 fold change, p = 0.000). The genes were most highly induced during appressorium initiation which is consistent with observations reported previously for their putative orthologs, *PKS*1, *SCD*1 and *THR*1 in *Colletotrichum lagenarium* [68, 69].

It is noteworthy that closer inspection of the genomic region on chromosome I containing the PKS *ALB*1 revealed the presence of a BUF1 homolog, MGG_07216.5. Positioned between these two genes is MGG_07218.5, a putative transcription factor. All 3 genes exhibited similar expression patterns during appressorium formation (Figure 8). MGG_07218.5 has an open reading frame of 1926 potentially encoding a protein of 487 amino acids with GAL4-like Zn2Cys6 binuclear cluster DNA-binding domain. A similar domain is also found in the *Pig1* transcription factor (MGG_07215.5), previously reported to regulate mycelial melanin biosynthesis in *M. grisea* [70]. *Pig1* is located next to the *BUF*1 homolog (MGG_07216.5). However, *Pig1* is not required for pathogenicity [70] and was not found to be differentially expressed in our experiments.

Although widely regarded that the PKS gene MGG_07219.5 encodes ALB1, published evidence appears to be absent. Thus to confirm MGG_07219.5 corresponds to ALB1, targeted gene knock out mutants were created. Mutants exhibited the expected albino phenotype and were non pathogenic on rice and barley. However, deletion of the putative transcription factor MGG_07218.5 resulted in no detectable phenotypic changes. Mutants exhibited no change in pigmentation, growth on various carbon and nitrogen sources or sporulation. In addition, mutants produced appressoria of normal appearance on a hydrophobic surface and produced disease symptoms on both rice and barley indistinguishable from wild type (Figure 6). Thus, this transcription factor does not

appear to regulate melanin production, at least on its own. Examination of the promoter regions of *ALB*1 (MGG_07219.5) and the *BUF*1 homolog (MGG_07216.5) revealed putative GAL4 type transcription factor binding sites. GAL4 type transcription factors commonly form both homo and hetero dimers. Whether it is no more than a coincidence that these two transcription factors are physically associated with this genomic region or perhaps together both regulate melanin biosynthesis during appressorium formation awaits further investigation.

This genomic region on chromosome I also contains other genes associated with pigmentation. Next to the PKS *ALB*1 is a putative multicopper oxidase (MGG_07220.5), which is potentially orthologous to a spore pigmentation related gene in *A. fumigatus*, *abr1*. Signal intensity of MGG_07220.5 was low, but showed a significant increase during appressorium initiation. This region also includes a putative threonine deaminase (MGG_07224.5) and a putative peptide transporter (MGG_07228.5), both of which exhibited up-regulated expression in the appressorium consensus gene set. The melanin biosynthesis gene cluster in *A. fumigatus* also contains the genes for yellowish-green 1 (*ayg1*) and a laccase (*abr2*) in addition to *alb1* (PKS), *arp1* (SCD) and *arp2* (THR) [67]. MGG_12564.5 is a hypothetical protein with 55% identity and 70% similarity to *ayg1* and MGG_08523.5 has 36% identity and 55% similarity to *abr2*. Both MGG_12564.5 and MGG_08523.5 were significantly up-regulated during appressorium formation although the signal intensity was low. However, in contrast to reduced spore pigmentation in *ayg1* and *abr2* deletion mutants in *A. fumigatus*, targeted gene disruption mutants of MGG_12564.5 or MGG_08523.5 in *M. grisea* resulted in no obvious phenotypic changes including appressorium pigmentation and pathogenicity on barley plants compared to the wild type (Figure 6).

In addition to the PKS *ALB*1 required for melanin biosynthesis, several other PKS genes involved in possible toxin biosynthesis were induced during appressorium formation. Increased gene expression was found for the putative PKS MGG_04775.5, which appears to be the ortholog of PKS1 and PKS2 required for T-toxin production in *Cochliobolus heterostrophus*. The genomic neighborhood around MGG_04775.5 contains a serine hydrolase (MGG_04774.5), an ABC transporter (MGG_13762.5), and a polyphenol oxidase (MGG_13764.5). These clustered genes were all up-regulated in the appressorium consensus gene set except MGG_13762.5 which was only up-regulated on the hydrophobic surface. Similar to the Tox1 locus in *C. heterostrophus* [71] which contains two PKS genes, MGG_04775.5 was found to be closely located with another PKS1 homolog, MGG_13767.5. However, this gene exhibited no significant changes in gene expression. Expression of MGG_07803.5, another PKS gene, also did not change significantly, however, several neighboring genes, MGG_07784.5, MGG_07785.5, a manganese peroxidase (MGG_07790.5), an oxidoreductase (MGG_07793.5) and a major facilitator superfamily transporter (MGG_07808.5) were induced during appressorium formation. It is possible this cluster of genes directs the synthesis of an unknown secondary metabolite.

Transcript levels of MGG_10072.5, which has 69% identity with PKSN required for alternapyrone biosynthesis in *Alternaria solani*, was dramatically increased during appressorium maturation and cAMP treatment (52 and 100 fold change, respectively). Interestingly, the genomic region containing MGG_10072.5 also contains a putative FAD-dependent oxygenase (MGG_13597.5) and 2 cytochrome P450s (MGG_10070.5, MGG_10071.5), which is very similar to genomic organization containing the PKSN locus in *A. solani* [72].

HMG-CoA synthase (3-hydroxy-3-methylglutaryl-coenzyme A synthase) and HMG-CoA reductase (3-hydroxy-3-methylglutaryl-coenzyme A reductase) catalyze the conversion of acetoacetyl-CoA to HMG-CoA and HMG-CoA to mevalonate, the limiting steps for the synthesis of isoprenoids, such as cholesterol and egosterol as well as numerous secondary metabolites, via the mevalonate pathway. Both HMG-CoA synthase (MGG_01026.5) and HMG-CoA reductase (MGG_08975.5) were up-regulated in the appressorium consensus gene set. Intriguingly, a PKS (MGG_08969.5), a regulatory enzyme (MGG_08974.5) and a secondary metabolite transporter (MGG_08970.5) flank MGG_08975.5. The gene expression levels of these genes were not significantly changed. However, in other fungi which contain similar arrangements of the apparently orthologous genes, these genes confer important regulation and biological properties. In *Penicillium citrinum,* the orthologous genomic region contains a cluster of genes that synthesize ML-236B (compactin), a lovestatin-like inhibitor of HMG-CoA reductase [73]. Furthermore, MGG_08969.5 appears orthologus to NPS6, a gene required for fungal virulence and resistance against oxidative stress in plant pathogenic ascomycetes fungi [74, 75], suggesting that this gene cluster may play an important role in the pathogenicity of *M. grisea*.

Several other genes involved in secondary metabolism were found in the up-regulated appressorium consensus gene set. For example, MGG_00385.5 and MGG_00573.5 encode proteins homologus to an ochratoxin-A non-ribosomal peptide synthetase in *Penicillium tetracenomycin* and an O-methyltransferase involved in polyketide synthesis in *Streptomyces glaucescens*, respectively. Other examples include MGG_06585.5 and MGG_04911.5, which are respectively similar to a putative short-chain dehydrogenase/reductase Fum 13p and a putative cytochrome P450

monooxygenase Fum15p in fumonisin biosynthesis gene cluster of *Gibberella moniliformis*. Genes encoding other key enzymes catalyzing critical steps in secondary metabolism were also up-regulated. For example, phenylalanine ammonia lyase (PAL; MGG_10036.5), which catalyzes the non–oxidatative deamination of phenylalanine to cinnamic acid and ammonia, the opening reaction in phenylpropanoid pathway that generates various precursors of secondary metabolites such as toxins, antibiotics and pigments was in the set of up-regulated appressorium consensus genes. Other important genes for secondary metabolism such as squalene hopene cyclase (MGG_00792.5); a key enzyme for hopanoid (triterpenoid) biosynthesis and isoflavone reductase (MGG_06539.5); a key enzyme in phenylpropanoid biosynthesis were up-regulated.

The identification of several genes central to secondary metabolism with elevated expression strongly implies the existence of appressorium specific fungal metabolites that may play a role in establishing the pathogenic interaction. For example, it has been shown that HC-toxin synthesis is highly induced during appressorium development in *Cochliobolus carbonum* [76, 77]. To investigate the role of secondary metabolites in appressorium formation and function, we selected two key genes for functional analysis, the PKS ortholog of PKS1 (MGG_04775.5) required for T-toxin in *C. heterostrophus* and the PAL gene (MGG_10036.5). However, unlike PKS1 and PKS2, which are required for T-toxin production and high virulence of *C. heterostrophus* [71], targeted knock out mutants of MGG_04775.5 were indistinguishable from the wild type, were able to form appressoria and were pathogenic towards barley and rice. Likewise, deletion mutants of MGG_10036.5 appeared to have a normal phenotype in growth, development and pathogenicity compared with the wild type (Figure 6). PAL was also found to be

dispensable for sexual development and virulence in the fungal pathogen *Ustilago maydis* [78].

5. Transporters

Fungal transporters play an essential role in pathogenicity by exporting host-specific and non-host specific secondary metabolites including toxins into the host plant tissue or provide a protective role by removing plant defense compounds or disease control agents from the fungal cell [79]. During appressorium development, the expression of 35 transporters with various substrate specificities were differentially up-regulated with the most striking being transporters related to toxin export and ion transport.

During appressorium induction, gene expression of 11 transporters was increased whereas in mature appressoria, 25 transporters were up-regulated. Up-regulated transporters included several from the major facilitator super family (MFS) such as MGG_02167.5, MGG_01778.5, MGG_10869.5, MGG_06794.5, MGG_03640.5 and MGG_03843.5 which share close homology with toxin efflux pumps and multidrug transporters as well as MGG_07062.5, MGG_09827.5 and MGG_04511.5 and MGG_00275.5, which appear to be involved in exporting monocarboxylate, lactate and nicotinamide mononucleotide, respectively. Other up-regulated transporters included the ABC transporter (MG10410.5) which is homologus to mdrA2 in *Dictyostelium discoideum* and MGG_06604.5, a homolog of human CLN3, which is involved in the ATP-dependent transport of arginine into the vacuole, and putative peptide trasporters, MG10200.5, MGG_07228.5 and MGG_08258.5. In contrast, gene expression of several putative carbohydrate transporters (MGG_04927.5, MGG_09193.5, MGG_03298.5, MGG_07843.5, and MGG_08968.5) was down-regulated, as might be expected due to the lack of nutrients in the surrounding environment.

Enhanced gene expression was also detected in a group of putative ion transporters, such as a K+ potassium transporter (MGG_02124.5), a cation efflux pump (MGG_07494.5), a CorA-like Mg2+ transporter (MGG_02763.5), P-type ATPases (MGG_00930.6 and MGG_04852.5) and other ion transporters (MGG_05085.5 and MGG_04105.5). A putative large conductance mechanosensitive channel (MGG_01489.5) was down-regulated during appressorium formation.

Several fungal transporters have been recognized to play a role in cellular development and are regarded as virulence factors. In *M. grisea*, an ATP driven efflux pump, *ABC*1 (MGG_13624.5) was strongly induced by azole fungicides and the rice phytoalexin sakuranetin. Mutants lacking *ABC*1 were unable to colonize host tissue [80]. Likewise, deletion of the multidrug resistance transporter, *ABC*3 (MGG_13762.5) led to complete loss of pathogenicity although appressorium formation was unaffected [30]. Deletion mutants of *Pde*1, a P-type ATPase were impaired in appressorium development and pathogenicity [81]. In our experiments, no significant changes in gene expression of *ABC*1 and *Pde*1 were detected during appressorium development. However, MGG_04852.5, the closest homolog of Pde1 (50% identity) was present in the up-regulated appressorium consensus set. *ABC*3 was highly induced during early stages of appressorium development on the hydrophobic surface.

6. Elevated vesicle transport and secreted proteins.

The vesicular secretory pathways have not been well studied in plant pathogenic fungi, however, increased expression of genes involved in membrane trafficking and signal transduction was apparent during appressorium formation. Up-regulated genes included a homolog of yeast phosphatidylinositol transfer protein, Sec14p, for vesicle budding from Golgi complex (MGG_00871.5), a putative phospholiphase-D for coated vesicle

formation (MGG_05804.5), a putative dynamin GTPase for vesicle detachment from membrane (MGG_08732.5), and putative lipid binding proteins with C2 domain, a Ca2+-dependent membrane-targeting module for signal transduction or membrane trafficking (MGG_09947.5 and MGG_01150.5). Another putative transmembrane protein with C2 domain, MGG_01094.5 was down-regulated. The up-regulation of a number of genes associated with vesicle transport and secretion is consistent with our observation that expression of secreted proteins was enriched during appressorium formation.

Secreted proteins are likely to be key determinants of host fungal pathogen interactions. Gene expression of the putative secreted proteins was found to be highly enriched in flax rust haustoria suggesting they play a significant role in host parasite interaction during fungal infection [82]. The overall percentage of putative secreted proteins in the *M. grisea* proteome is 7 %. However, about 26 % of appressorium consensus genes contains proteins with translocation signals and includes several previously characterized pathogenicity related genes such as *GAS* (gEgh16 homologs expressed in appressorium stage) homologs and hydrophobin proteins. *GAS*3 (MGG_04202.5) and *GAS*1 (MGG_12337.5) were previously shown by differential hybridization analysis to be highly abundant in an appressorium specific cDNA library [83]. Deletion mutants developed appressoria but showed reduced pathogenicity on rice and barley leaves. In our study, we found that both genes were highly up-regulated throughout appressorium formation. In addition, other *GAS* homologs (MGG_02253.5 and MGG_09875.5) were also differentially expressed. During appressorium morphogenesis, MGG_09875.5, the closest paralog to *GAS*1 (62% identity), was strongly down-regulated (0.1, 0.2, 0.2 fold change in AI, AM and CI) while MGG_02253.5, the closest paralog to *GAS*3 (45% identity) was down-regulated during appressorium

initiation but up-regulated in mature and cAMP induced appressoria (0.3, 2.5, 2.7 in AI, AM and CI). Stage dependent gene expression of GAS homologs was previously reported in *Blumeria graminis*. *gEgh*16H was strongly induced during plant penetration and haustorium formation whereas gEgh16 was induced during spore germination [84]. In our experiments, MGG_09875.5 showed a similar expression pattern to *gEgh*16 and appears to be a germination specific gene.

The hydrophobin *MPG1* (MGG_010315.5) was previously shown to be highly expressed in the infected leaves compared to growth in complete media. Further, deletion mutants produced less appressoria and exhibited reduced pathogenicity [36]. In this study, similar to *PTH11*, gene expression of *MPG1* was strongly down-regulated on the hydrophobic surface and by exogenous cAMP treatment compared to germ tubes that continued to develop vegetatively. This result suggests *MPG1* may be involved in surface recognition and hyphal attachment to the surface rather than directing appressoria development after surface attachment. We also observed that the expression profile of another extracelluar protein SnodProt1 homolog (MGG_05344.5), which has some physical properties in common with hydrophobins, was also down-regulated during appressorium formation. SnodProt1 is a member of cerato-platanin family, which in *Phaeosphaeria nodorum* was shown to be involved in pathogenicity and plant defense response in wheat [85]. Recently, the SnodProt1 homolog in *M. grisea* has been functionally characterized [86]. Targeted deletion mutants exhibited reduced pathogenicity and impaired in planta growth, but there was no evidence that purified SnodProt1 protein had phytotoxic properties as suggested for other fungal homologs [87].

Other secreted proteins have hydrolytic enzyme activity potentially involved in degradation of the host cell wall and may play some role in appressorium development

and function. For example, putative exogenous cutinases (MGG_02393.5, MGG_11966.5) and a fungal lignin peroxidase (MGG_07790.5) were highly up-regulated. Until recently the role of cutinase was largely discounted based on the finding that the cutinase *CUT*1 (MGG_01943.5) is not essential for pathogenicity [88]. However, expression of this gene was not up-regulated in our experiments. Skamnioti and Gurr reported that *CUT*2 (MGG_09100.5) is required for full virulence. As reported by Skamnioti and Gurr, we also found this gene was highly up-regulated during late stages of appressorium formation on the hydrophobic surface [89]. To further investigate the role of cutinases, we knocked out the putative cutinase MGG_02393.5 because this gene was also up-regulated by both hydrophobic and cAMP. However, gene deletion mutants exhibited no observable changes in virulence or other phenotypic differences.

7. Cell signalling pathways

In addition to cAMP, several cell signalling pathways have been shown to be involved in regulating appressorium formation. Calcineurin is a Ca^{2+}/calmodulin-dependent serine/threonine phosphatase, which is involved in many signal pathways for cation homeostasis, cell differentiation, morphogenesis, cell wall integrity and pathogenicity [90-93]. Phosphorylation activity of calcineurin is inactivated when it is coupled with cyclophilin and other FK506 binding proteins (FKBPs) in the presence of inhibitors such as cyclosporine A (CsA) and tacrolimus (FK506). In *M. grisea*, cyclophilin (*CYP*1; MGG_10447.5) has been shown to be involved in fungal virulence and appressorium turgor generation [94]. CsA inhibits hyphal growth and appressorium formation in a *CYP*1-dependent manner, supporting a role for calcineurin in regulating appressorium development. Transcriptional activity of calcineurin (MGG_07456.5) remained unchanged, however, expression of MGG_06035.5, a putative ortholog of FKBP

decreased. In addition, MGG_01150.5, a putative ortholog of CTS1 (calcineurin temperature suppressor), which is required for the full virulence of *Crytococcus neoformans* was up-regulated during appressorium formation. The differential gene expression of the components of calcineurin dependant signalling pathway in the appressorium consensus gene set provide further evidence for a role of multiple signal pathways in regulating appressorium morphogenesis and function in *M. grisea.*

Many external signals are transmitted to the inner workings of the cell through receptors, which are embedded in the plasma membrane. During appressorium induction, several genes encoding putative G-protein coupled receptor-like proteins with 7 membrane spanning domains (*Pth*11 MGG_05871.5, MGG_02692.5, MGG_05214.5, MGG_10571.5) were significantly down-regulated although the expression of a CFEM containing protein (MGG_09570.5) was up-regulated. Also, expression of another CFEM protein ACII (MGG_05531.5), which interacts with MAC1 adenylate cyclase in cAMP signalling pathways was very intensive and was up-regulated in young appressoria but was dramatically reduced in mature appressoria and was significantly down regulated by cAMP. We also observed increased gene expression of MGG_00438.5, which encodes a putative transmembrane protein, PAT 531. Previous studies reported that deletion of this gene resulted in reduced pathogenicity of *M. grisea* towards weeping lovegrass [95].

Discussion

In this study, we performed microarray studies to identify genome wide fluctuations in gene expression during germination, appressorium induction and maturation. We then used these data as a guide to identify and subsequently characterize core biological processes, in some cases previously unrecognized processes, required for infection related development and pathogenesis. Key to this study was the experimental design. First, we performed a direct comparison of gene expression during spore germination on an inductive surface verses a non-inductive surface. Secondly, we compared the expression patterns with those obtained from a direct comparison of conidia germinating in the presence of cAMP verses its absence. The integration of these data sets revealed a core set of appressorium-induced genes common to the different stimuli. Subsequent bioinformatics and functional analyses of these so called consensus genes shed new light on the biochemical processes required for appressorium formation and function.

Prior to this study, little was known regarding the role of protein degradation and amino acid metabolism in appressorium formation and function. We found that during appressorium formation a number of genes required for protein metabolism were differently expressed at significant levels. In particular, several proteases, including the putative vacuolar subtilisin-like protease *SPM1*, which is required for penetration and *in planta* growth, were up-regulated specifically during appressorium formation. Our data also revealed that genes involved in targeting proteins for degradation as well as the machinery involved in protein degradation were also up-regulated. Several proteases contained an export signal, suggesting they may be secreted and act as virulence factors as has been shown for homologs in related fungi [96]. Coupled with elevated expression

of genes for protein degradation, we found genes involved in amino acid metabolism to be stimulated during appressorium formation. For example, a NAD-dependent glutamate dehydrogenase (*Mgd1*) was significantly up-regulated in a manner very similar to *SPM1*. *Mgd1* deletion mutants were unable to make normal appressoria and showed significantly reduced virulence. In sum, the combination of global gene expression analysis and subsequent functional interrogation revealed a hitherto unknown connection between protein catabolism, carbon and nitrogen scavenging from amino acids and appressorium formation and function.

Our data also revealed that many genes previously implicated in appressorium formation and function were not up-regulated. Lipid metabolism has been strongly linked to generating the high levels of glycerol found in appressoria [8]. Triacylglycerol lipase activity has been reported to be induced during appressorium formation and to remain high [8]. In our microarray study, only one (MGG_00528.5) of 7 genes encoding this activity showed any evidence of induction and even then expression levels were low. Furthermore, deletion of any of the seven genes had no effect on infection, although this observation may be attributed to functional redundancy [8, 97]. In addition, we observed that *Mfp*1, the gene that encodes the second step in lipid degradation, was more highly expressed in spores than during germination or appressorium formation. Deletion mutants of *Mfp*1 exhibited reduced ability to penetrate, but no decrease in turgor pressure, suggesting lipids are not the primary source of glycerol in appressoria [97]. Beta-oxidation of fatty acids occurs in peroxisomes, and enlarged vesicles with scattered peroxisomes are found in appressoria. Disruption of genes involved in peroxisome biosynthesis (such as *MgPex*6) does not block appressorium formation, but does affect appressorium function. However, in our experiments, homologs of genes involved in

peroxisome biosynthesis exhibited little evidence of being up-regulated during appressorium formation.

Autophagy appears to be important for appressorium function. *M. grisea* mutants lacking either the autophagy genes *MgATG*1 or *MgATG*8 form appressoria, but they are non-functional. Spores of mutants lacking *MgATG*8 do not collapse suggesting that the recycling of storage materials is essential to generate a functional appressorium. Again, we found no evidence for these genes being up-regulated during appressorium function. Both genes were induced slightly during germination, but showed little or no further induction during appressorium formation. This pattern of expression was observed for many autophagy orthologs identified. A few exceptions were noted such as MGG_03580.5, a ATG4 homolog which was up-regulated during appressorium formation. Also, MGG_07297.5 and MGG_09559.5, putative orthologs of ATG7 and ATG9 respectively, were induced significantly during germination and in response to cAMP.

In recent years, attention has focused on effector molecules, many of which are secreted proteins or secondary metabolites. These molecules may serve as virulence determinants or help shield the fungus from being detected by its host. In some cases, the host may recognize these molecules and activate its defence mechanisms. For example, the *M. grisea* avirulence gene *ACE*1 encodes a PKS [98, 99]. In this case, it is presumed that the metabolite synthesized by this gene product confers biological function. Other avirulence genes such as avr-pita encode a small-secreted protein that has been shown to directly interact with its cognate resistance gene product, Pita, in rice [100]. It is reasonable to expect that during pre-penetration phases of the host-pathogen interaction the pathogen would begin to mobilize effector molecules. Indeed during appressorium

formation, we detected a nearly 4-fold enrichment of genes encoding products with a putative signal peptide in the up-regulated consensus gene set as well as increased expression of numerous genes associated with secondary metabolism and secretion. We found several examples of genes clustered around PKS genes, suggesting they maybe co-regulated. Several phytotoxic secondary metabolites have been isolated from *M. grisea* such as pyricularin and picolinic acid, but little is known regarding the genes that are required for their synthesis [101]. This is a research topic that would appear to warrant further investigation.

We found that some genes known to be essential for appressorium development appeared to be down-regulated in our expression studies. The importance of MPG1 (MGG_010315.5), a hydrophobin and PTH11 (MGG_05871.5), a membrane spanning protein in appressorium development are well document, but our data showed they were more highly expressed in spores germinating on a hydrophilic surface than in incipient appressoria. This suggests the gene products are required initially and perhaps transiently for subsequent morphological changes and is consistent with a role in surface sensing and attachment. Once the environmental cue is detected and the intracellular signal pathways activated, these proteins may no longer be needed for appressorium formation to proceed.

While disruption of anyone signal cascade is often sufficient to affect appressorium formation, considerable evidence suggests that there is cross-talk between the signal pathways. Previous studies have shown that expression of *GAS*1 and *GAS*2, which are highly expressed during appressorium formation, requires *Pmk*1, suggesting they are regulated by the MAPK pathway [83]. The presence of these genes in the appressorium consensus gene set is consistent with cross-talk between the cAMP and MAPK pathways. In addition, our microarray data suggests the involvement of the

calcineurin dependent and TOR signal pathways in regulating appressorium formation. Clarifying the role and interconnection of these different signal pathways may be served by examining gene expression in different mutant backgrounds, particularly within the first few hours of germination.

While gene expression analysis is a useful tool to identify genes associated with a particular process, it is by no means definitive. In a recent report, 250 *M. grisea* mutants defective in appressorium formation and/or pathogenesis were created by agrobacterium mediated random insertion mutagenesis [102]. The majority of the mutation sites were located in intergenic regions. Interestingly, most of the genes flanking the insertion sites showed no differential expression changes in our microarray experiments. Moreover, of 90 genes with well-characterized roles in pathogenicity in *M grisea*, only 9 exhibited changes in transcript levels during appressorium formation. Conversely, the majority of appressorium consensus gene mutants generated in this study retained their ability to differentiate infection structures and cause disease. We postulate that the lack of an observable phenotype variant in gene knock-out mutants may be due to functional redundancy among possible (unidentified) paralogs or the activation of alternative compensatory pathways. For example, *M. grisea* appears capable of compensating for the loss of MGG_02393.5, but appears to be unable to fully compensate for the loss of *CUT*2, even though both homologs exhibited similar expression patterns. Deleting *SPM*1 had a dramatic effect on sporulation and virulence, but deleting its paralog MGG_09246.5, showed no detectable phenotypic changes despite both genes have very similar expression patterns. Thus, *M. grisea* is unable to fully compensate for some genes but not their closely related homologs. This suggests some level of functional specificity may exist. To address issues related to functional redundancy employing RNA interference to

silence all members of a gene family may be effective. In other situations, it may be valuable to explore the use of over-expression to evaluate the role of particular genes.

Genome wide expression profiling during spore germination and appressorium formation revealed that the blast pathogen *M. grisea* undergoes significant changes in gene expression during infection related cellular differentiation. Functional analysis and characterization of the differentially expressed genes provides new insight into appressorium morphogenesis, in particular regarding the role of protein degradation for appressorium function. We provide a comprehensive list of genes that might be involved in appressorium formation and function solely or in combination with other genes. Our data will be beneficial for further studies on fungal pathogenesis including gene expression studies in other fungal pathogens. We expect the emergence of additional functional information regarding *M. grisea* genes with no known biological function will help broaden the scope of future analyses.

Materials and Methods

Appressorium induction by physical cue and exogenous cyclic AMP

M. grisea strain 70-15 spores were collected from 10 day old V8 agar plates and adjusted to 10^5 conidia/ ml. Spore suspensions were placed on the appressorium inducing (hydrophobic) and non-inducing (hydrophilic) surface of GelBond (Cambrex Bio Science Rockland, Inc. Rockland, ME USA) at 6.25 x 10^4 spores/ cm^2 and incubated at 25□ in the dark and for 7 and 12 hr. By 7 hr, on the hydrophobic side of GelBond film, essentially all spores germinated and tips of germ tubes had started to hook and swell forming young appressoria. These young light-colored appressoria developed into dark mature appressoria at 12 hr. On the hydrophilic side of GelBond, germ tubes continuously elongated and branched several times through 12 hr with no other developmental changes. Appressoria were also induced by adding exogenous cAMP (final concentration of 50mM) to the spore suspension placed on the hydrophilic surface of GelBond as described above. In the presence of cAMP, melanized appressoria were evident at 9 hr (Figure 1, A).

RNA sample preparation and microarray data collection.

RNA was purified following standard protocols. Briefly, following incubation, material was flash frozen with liquid nitrogen, scrapped from the support, and ground in liquid nitrogen. Total RNA was extracted using Qiagen RNesay Mini kit (Qiagen, Valencia, CA) according to manufacturer's protocol. RNA was also extracted from spores in water immediately after being collected from agar plates. Quality analysis and quantification

were performed using the Agilent Bioanalyzer (http://www.agilent.com) and the Nano Drop (NanoDrop Technologies, Inc).

RNA from the two biological replicates was pooled in equal amounts and 1ug was labelled by reverse transcriptional incorporation of cyanine 3 (Cy3) and cyanine 5 (Cy5) labelled dCTP, using an oligo(dT) primer. The Agilent Fluorescent Linear Amplification Kit protocol for fluorescent cRNA synthesis (http://www.agilent.com) was used. The concentration and the dye incorporation of labeled cRNA were measured by Nano Drop (NanoDrop Technologies, Inc). According to the microarray hybridization scheme (Figure 1, B), equimolar amounts of cRNA samples, each labelled with Cy3 or Cy5, were co-hybridized to the *M. grisea* Oligo Microarray (Agilent technology, #G4137A) using to the Agilent 60-mer-oligo microarray processing protocol (http://www.agilent.com) as previously described [39]. Following drying, slides were immediately scanned with an Agilent G256BA microarray scanner (http://www.agilent.com). Image files from the scanner were analyzed with the Agilent 2567AA Feature Extraction software (version 1.6.1.1). For signal normalization, the output from Agilent Feature Extraction was first converted into GPR format to conform to the input format requirements for "Bioconductor/Aroma" software (http://www.bioconductor.org/). The data were read into Aroma and "Within Slides LOWESS Normalization" and "Across Slides LOWESS Normalization" methods were applied. After normalization, the data was extracted for each treatment – each having 4 replicates, 2 from Cy3 and 2 from Cy5, from which the mean and other statistics were calculated.

Gene expression profiles and their correlation

Gene expression profiles for appressorium initiation (AI), appressorium maturation (AM) and appressorium induced by cAMP (CI), were determined by comparing the hybridization signals of RNA harvested from the hydrophobic surface after 7 and 12 hr incubation (Pho7) and (Pho12) and the hydrophilic surface after 9 hr incubation with cAMP (cAMP9), directly to those from hydrophilic surface after 7 and 12 hr incubation (Phil7) and (Phil12), and hydrophilic surface after 9 hr incubation without cAMP (Phil9). Expression profiles during appressorium development (AD) and germ tube elongation (GE) were made by comparing the hybridization signals of RNA harvested from hydrophobic surface after 12 hr incubation (Pho12), to that of hydrophobic surface after 7 hr (Pho7), and hydrophilic 12 hr (Phil12) to hydrophilic 7 hr (Pho7). To construct the gene expression profile for spore germination (SG), the hybridization signals of RNA harvested from the hydrophilic surface after 7 hr incubation (Phil7) were compared to those of RNA from ungerminated spores (Spore). For each profile, the ratio of the expression level for the 10,176 *M. grisea* probes was calculated. The $\log_2$–transformed value of the ratio was used for pairwise correlation analysis between expression profiles. Genes were designated as differentially expressed if their average signal intensities were equal or above 200 in at least one condition and their expression ratios were equal or greater than 2 fold with $P<0.05$ (Student's t-test).

Reverse transcriptase PCR and Quantitative RT-PCR

Reverse transcriptase PCR as described previously was conducted on selected genes that were either significantly up-regulated, down-regulated, or showed no difference in their expression profile in the microarray experiments [39]. PCR was performed on

independently generated templates using primers shown in Sup1. Total RNA was prepared from germinating spores on hydrophilic and hydrophobic GelBond surfaces using the RNAeasy Plant Mini Kit (Qiagen) and used for reverse transcription. cDNA was prepared according to procedures for cDNA synthesis from total RNA in Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies) with modification.

For quantitative reverse transcription-PCR (qRT-PCR), total RNA was independently extracted from cAMP induced appressoria on hydrophilic surface as well as germinating spores without cAMP treatment and cDNA was synthesized as described above. qRT-PCR reactions of *MPG*1 and *PTH*11 were carried out using 2 × SYBR Green Master Mix (Applied Biosystems, Foster City, CA) with that of actin gene as an internal control. qRT-PCR was performed using the following profile: 94 ºC for 3 min; 30 cycles at 94 ºC, 45 s; 62 ºC, 45 s; 68 ºC, 2 min; and a final extension at 68 ºC for 7 min. Analysis of the qRT-PCR results was carried out as previously described by Livak and Schmittgen [103].

Gene Ontology (GO) and functional annotation

Orthologs were identified between *M. grisea* predicted proteins and proteins in the GO database (http://www.geneontology.org/GO.downloads.database.shtml) via searching reciprocal best hits with the following cut offs; e-value, 1.0e-3, and identity, 20%. Results from local alignment using BLAST, functional domain comparisons from Interpro and prediction of signal peptides from SignalP 3.0 software and manual literature review were used to make final assignments to GO functional categories.

Targeted gene replacement and mutant screening

Gene replacement cassettes were constructed using adaptamer mediated PCR [104]. Typically 1.3 kb of upstream and downstream sequence of each target gene was amplified with primers that contained adaptamer sequences. A 1.5 kb fragment containing the hygromycin dehydrogenase gene with the *trp*C promoter from *A. nidulans* was amplified from plasmid PCB1003 using the adaptamer sequence attached to the forward HPHF and reverse HPHR primer set (Figure 9).

Using nested primers from inside of the 5' upstream fragment and from inside of the 3' end of the downstream fragment of the target gene, the individual fragments and hygromycin resistance gene fragment were combined and amplified together to construct a hygromycin cassette for gene replacement typically ~ 3.3 kb in length. The hygromycin cassette was transformed into 70-15 protoplasts as previously described [88]. Gene replacement mutants were initially identified by PCR screening with nested primers in target gene region and further confirmed by Southern blot analysis.

Mutant phenotype assays

A series of phenotype analyses were conducted on several knockout mutants and ectopic transformants for each gene functionally characterized. Germination and appressorium assays were conducted using spores collected from 10 day old V8 agar plates and adjusted to 10^5 spores/ml. Spore suspension was spotted on the hydrophobic and hydrophilic surface of GelBond film and rate of germination and appressorium formation was measured after 24 hr incubation at 25 ℃ in the dark. To test for pathogenecity, barley and rice seedlings were spray inoculated with *M. grisea* spore suspension ($3x10^4$

spores/ml, Tween 20 0.025%) and incubated in dark humid conditions at 25 ℃. The number and size of lesions were recorded 7 days post-inoculation. Growth rate assays were conducted by placing 10 ul spore suspension ($3x10^4$ conidia/ ml) on agar plates with complete or minimal media. In other growth rate assays, where minimal media was amended with glutamate and glutamine as a sole nitrogen source, glucose was reduced to 0.125 %. Colony morphology and diameters were recorded periodically for 15 days. The total number of spores on minimal media plates was counted after 15 days incubation. All experiments were conducted in triplicate and performed at least 3 times.

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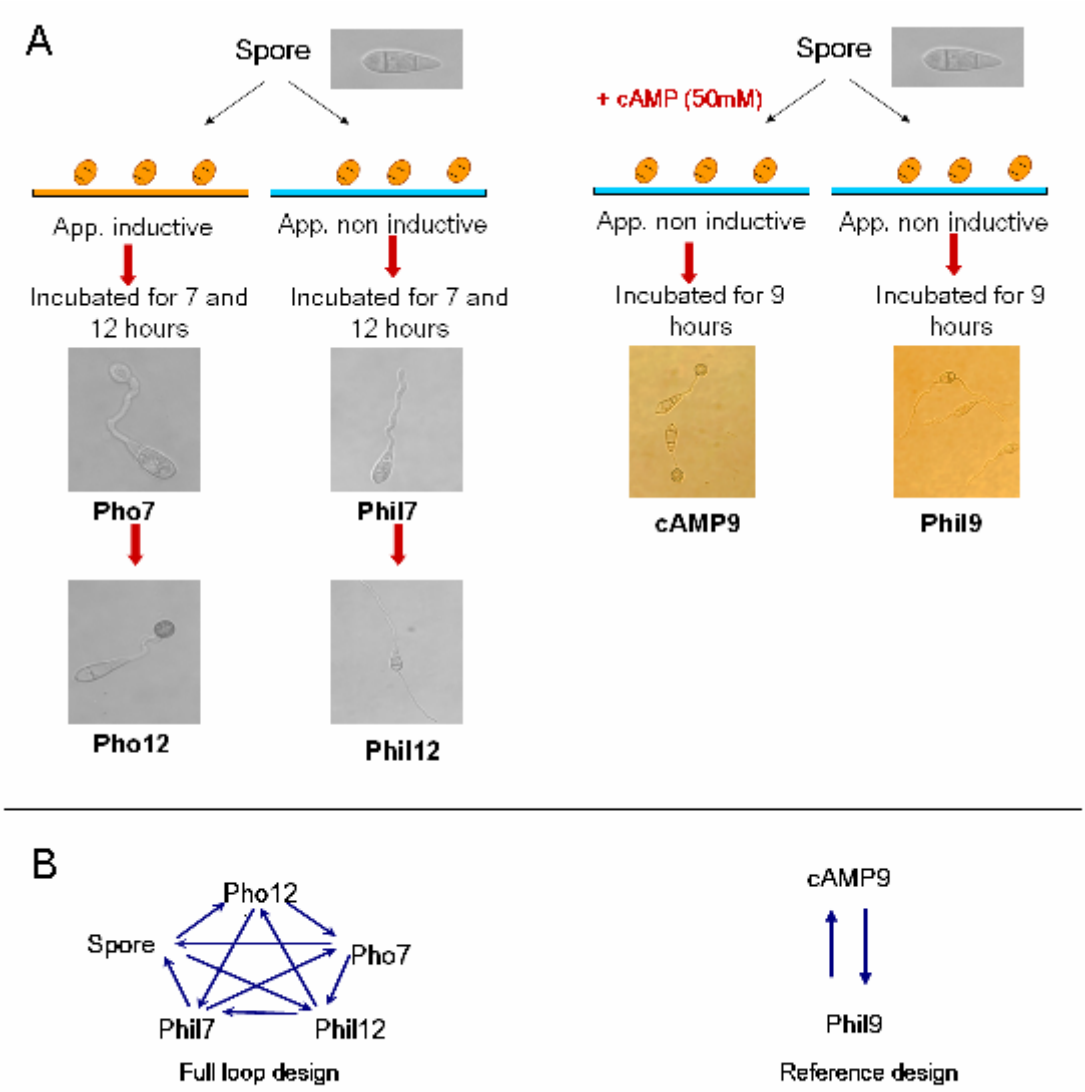
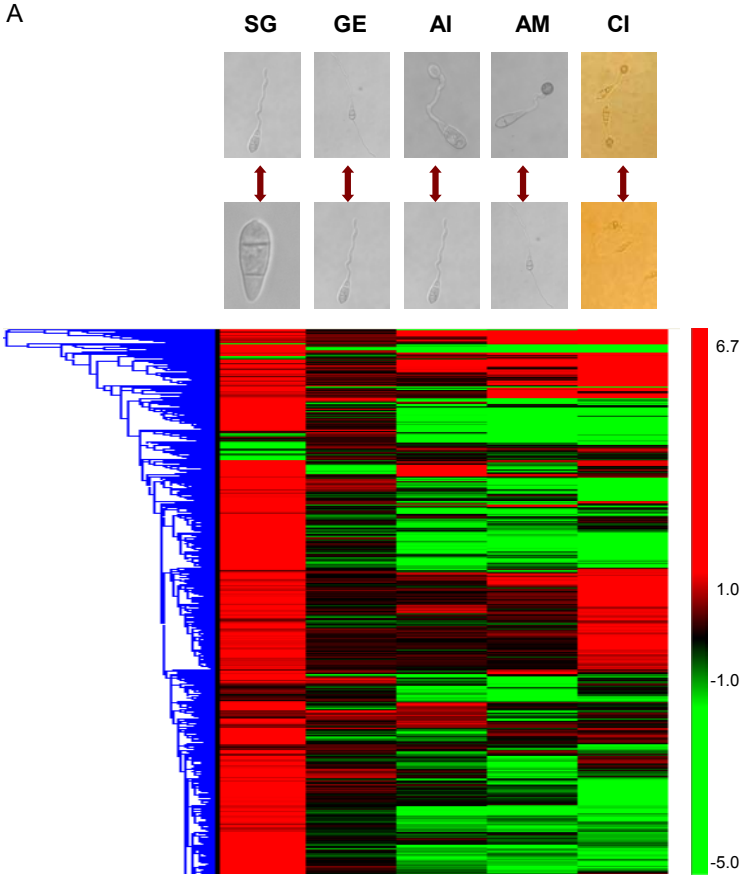


Figure 1. Experimental and microarray design for spore germination and appressorium induction. A. Spores were placed on the hydrophilic (Phil) and hydrophobic (Pho) surfaces of Gelbond and incubated for 7 and 12 h. For induction of appressoria by cAMP, spores were placed on the hydrophilic surface of Gelbond with (cAMP9) and without (Phil9) cAMP and incubated for 9 h. B. Diagrams show microarray design. Arrows connect samples directly compared on 2 channel Agilent *M. oryzae* oligonucleotide microarrays. Arrow heads = Cy5, arrow tails = Cy3.

A

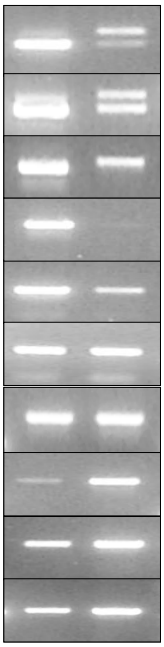


B

	SG	GE	AI	AM	CI
SG	1.00				
GE	-0.28	1.00			
AI	-0.26	0.12	1.00		
AM	-0.22	0.03	0.54	1.00	
CI	-0.19	0.06	0.57	0.66	1.00

Figure 2. Gene expression profile clustering and correlation analysis. A. Hierarchical clustering analysis of gene expression profiles for spore germination (SG), germ tube elongation (GE), appressorium initiation (AI), appressorium maturation (AM) and cAMP induced appressoria (CI). Differential expression of each gene is indicated in color (red shows induced, green showed repressed, number = $\log_2$ (fold change)). B. Correlation coefficient for pairwise gene expression profiles shown in A.

A.



Pho12	Phil12	FC	Gene	Description
		56.6	MGG_03436.5	predicted protein
		3.0	MGG_05247.5	NAD(+)-specific glutamate dehydrogenase
		3.2	MGG_03670.5	subtilisin-like proteinase Spm1 precursor
		5.3	MGG_08523.5	laccase [*Aspergillus nidulans*]
		20.9	MGG_00792.5	squalene-hopene-cyclase [*Thermosynechococcus elongatus*]
		1.0	MGG_06456.5	predicted protein
		1.0	MGG_03982.5	actin
		0.2	MGG_10061.5	catalase-1 [*Neurospora crassa*]
		0.1	MGG_03593.5	predicted protein
		0.2	MGG_09875.5	MAS3 homolog [*Magnaporthe grisea*]

B.

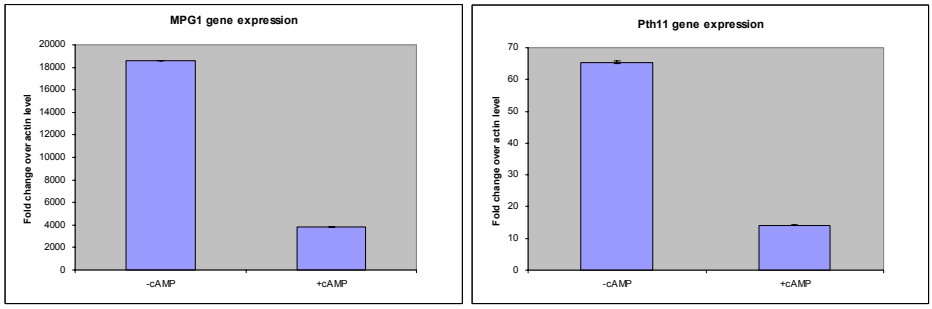
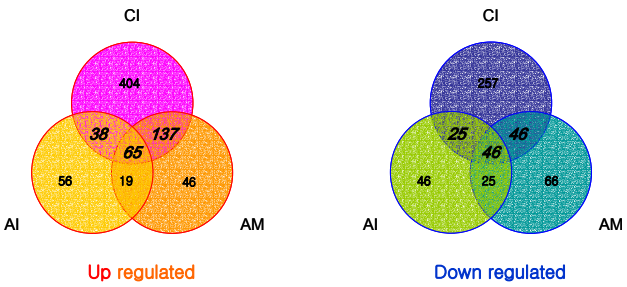


Figure 3. RT-PCR and qRT-PCR of differential of gene expression. A. Reverse transcriptase PCR (RT-PCR) using RNA isolated from spore germinated on the hydrophobic (Pho12) and hydrophilic (Phil12) surfaces of Gelbond after 12 h incubation compared to expression fold change (FC) derived from microarray data from the same time point. B. Real time PCR (qRT-PCR) analysis of MPG1 and Pth11 using RNA from appressoria induced by cyclic AMP (+cAMP) and germinating spores (-cAMP) after 9h incubation on a hydrophilic surface. Gene expression fold change for MPG1 and Pth11 were 0.2 and 0.2, respectively, in our cAMP microarray study.

A



B

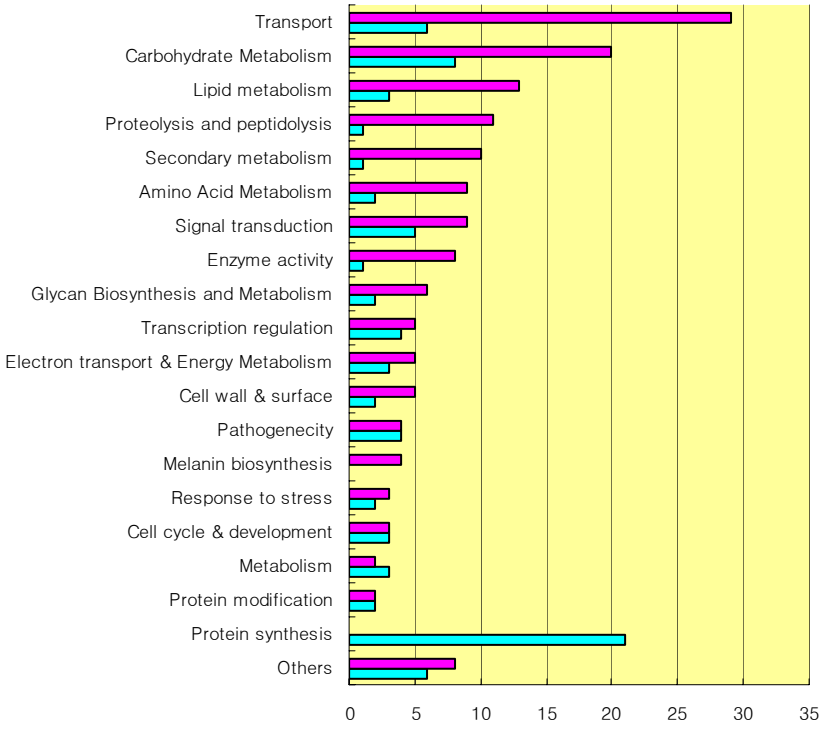


Figure 4. Functional categorization of appressorium consensus genes. A. Appressorium associated expression profiles were combined and 240 up-regulated and 117 down-regulated genes were designated as appressorium consensus genes (in italics). Abbreviations are same as Figure 2. B. Up-regulated (in pink) and the down-regulated (in blue) genes were grouped according to their putative function. X axis indicates number of gene in each functional category.

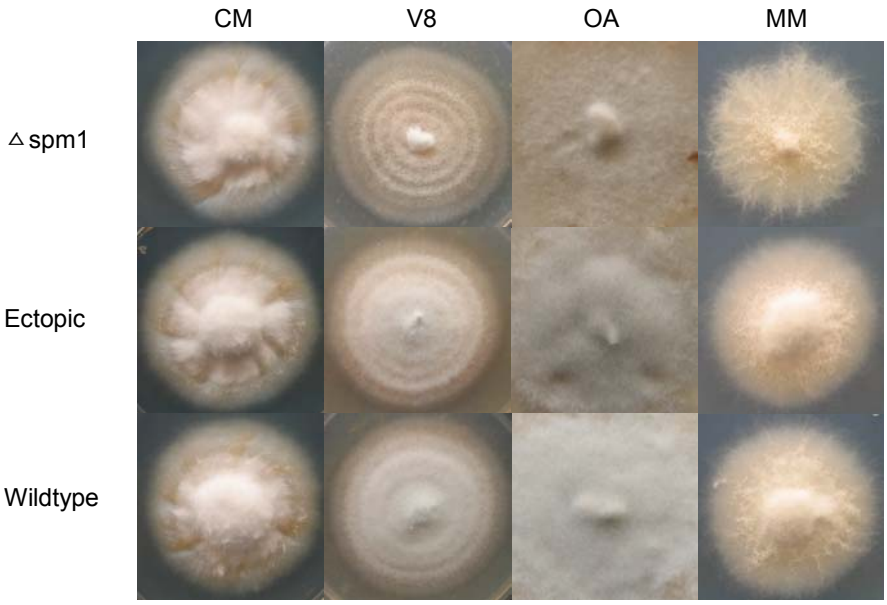
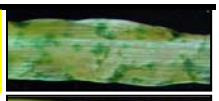
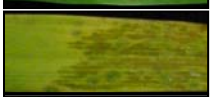
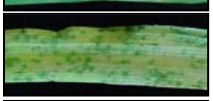


Figure 5. Growth of SPM1 deletion mutant on various nutrient conditions. SPM1 deletion mutant (△ spm1), ectopic strain and wild type 70-15 were incubated on solidified complete media (CM), oatmeal media (OA), V8 media (V8) and minimal media (MM) for 7 days. Results shown are typical for all 4 independent SPM1 deletion mutants.

Target gene	Description	AI	AM	CI	App[a]	Pathogenicity[b]
MGG_03436.5	hypothetical protein	59.6[c]	56.6	76.9		
MGG_00659.5	beta-1,3-exoglucanase [*Trichoderma hamatum*]	15.7	11.6	16.3		
MGG_02393.5	cutinase [*Botryotinia fuckeliana*]	1.6	14.6	29.4		
MGG_07219.5	polyketide synthase [*Colletotrichum lagenarium*]	11.0	5.0	26.0		
MGG_04775.5	polyketide synthase [*Botryotinia fuckeliana*]	7.2	8.4	7.3		
MGG_12564.5	yellowish-green 1 [*Aspergillus fumigatus*]	21.2	3.8	3.0		
MGG_08523.5	laccase [*Emericella nidulans*]	4.3	5.3	2.3		
MGG_03670.5	subtilisin-like proteinase Spm1	1.4	3.2	2.6		
MGG_09246.5	subtilisin-like protease [Verticillium dahliae]	1.1	3.9	7.9		
MGG_08526.4	thiol protease [*Porphyromonas gingivalis*]	1.0	15.0	7.2		
MGG_05247.5	NAD-specific glutamate dehydrogenase, Mgd1	2.6	3.0	4.1		
MGG_10036.5	phenylalanine ammonia-lyase [*Ustilago maydis*]	2.5	19.7	24.1		
MGG_07218.5	transcription factor [*Colletotrichum lagenarium*]	1.1	2.1	39.4		
MGG_09276.5	transcription activator CRG1 [*Cercospora nicotianae*]	12.0	5.5	4.4		
MGG_09031.5	Transcriptional regulator [*Dechloromonas aromatica*]	0.5	0.5	0.3		
MGG_07337.5	Oleate-induced peroxisomal protein POX18 [*Pichia stipitis*]	2.9	4.0	3.0		
70-15. WT						

[a] appressoria development on a hydropholic surface after 20 h incubation.

[b] pathogenicity of target gene deletion mutants on barley seedings 5 days after inoculation.

[c] numbers indicate gene expression fold change for appressorium induction (AI), appressorium maturation (AM) and cAMP induced appressoria (CI).

Figure 6. Appressorium formation and pathogenicity of targeted gene deletion mutants.

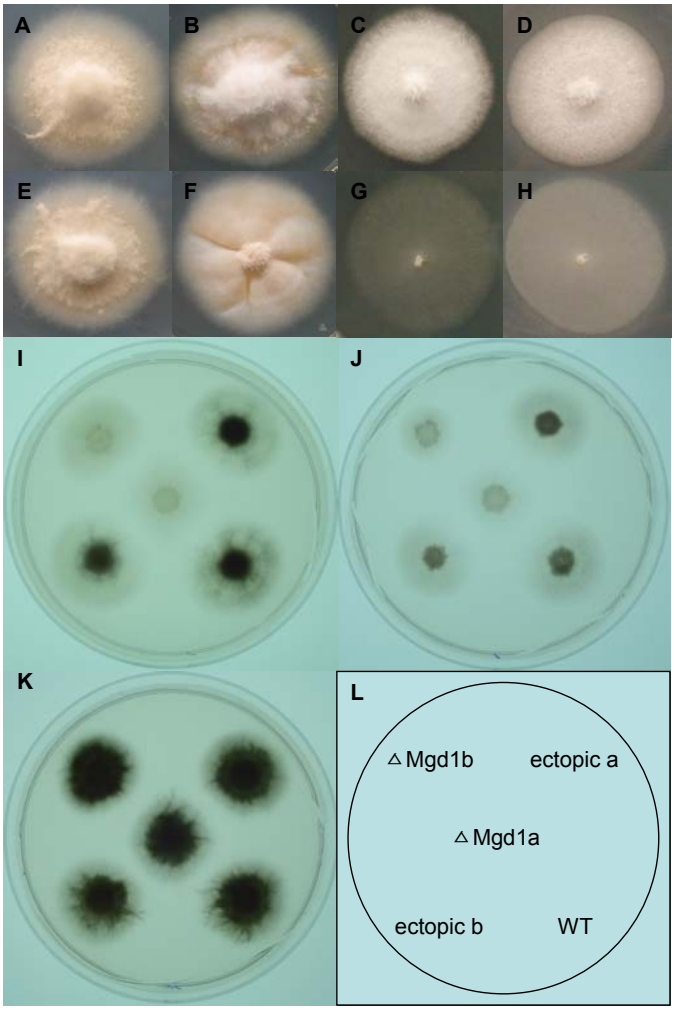


Figure 7. Growth of Mgd1 deletion mutants on various nutrient sources. Wild type 70-15 (A to D) and Mgd1 deletion mutant (E to H) were incubated on minimal media (A, E), complete media (B,F), minimal media with Tween 20 (C, G) or PEG (D,H) as carbon source for 7 days. Results shown are typical of all 4 independent Mgd1 deletion mutants. Ectopics were similar to wild type, data not shown. Mgd1 deleted mutants (△ Mgd1 a,b), ectopics (ectopic a,b) and wild type 70-15 (WT) were incubated for 7 days on minimal media (0.125% glucose) with glutamine (I) and glutamic acid (J) as nitrogen source and minimal media (1% glucose, K) as indicated in panel L. Photographs I to K were taken on a light box to highlight differences in mycelial density.

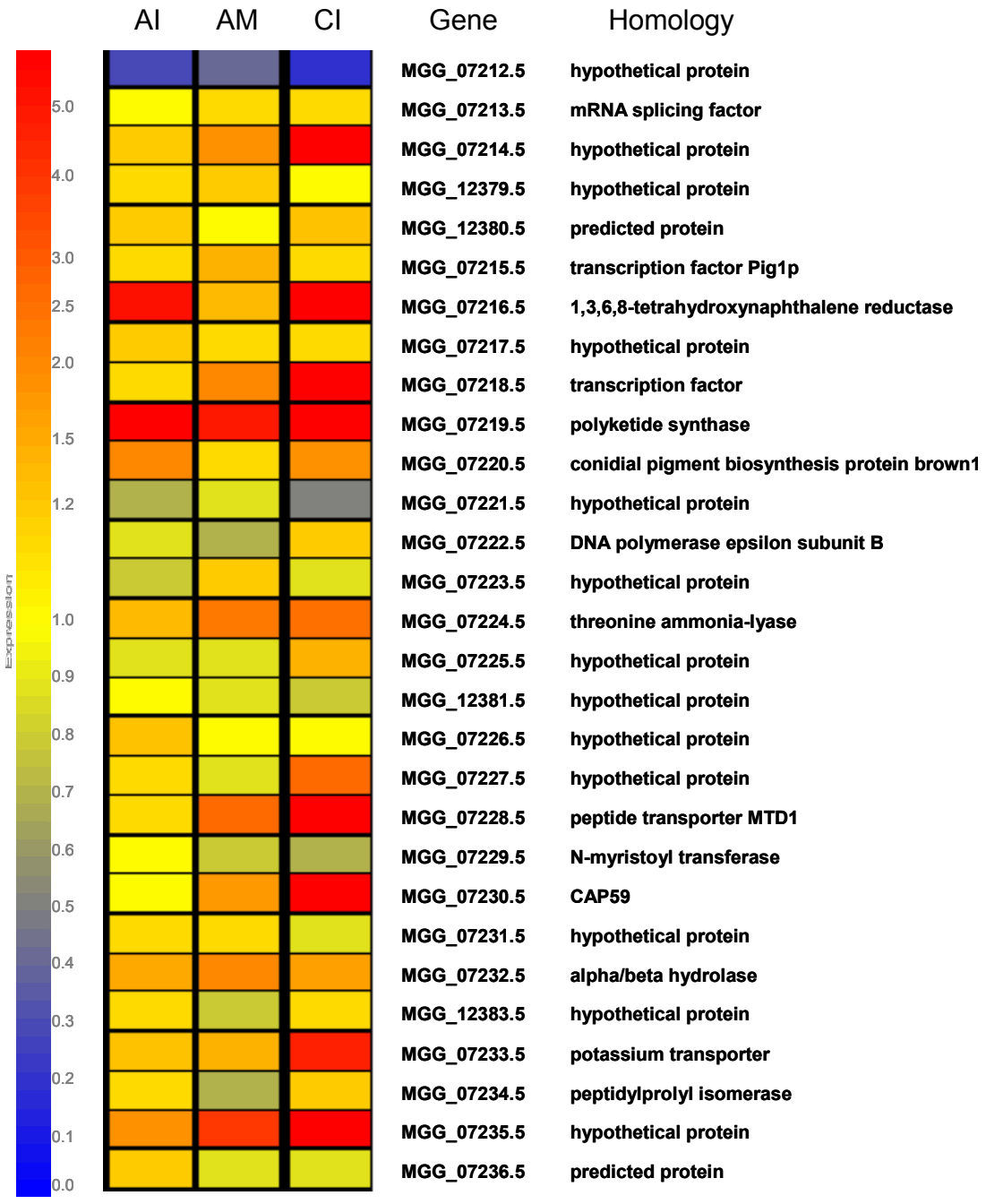


Figure 8. Putative melanin biosynthesis gene cluster in *M. grisea*

Figure 9. Adaptamer mediated PCR strategy for targeted gene deletion

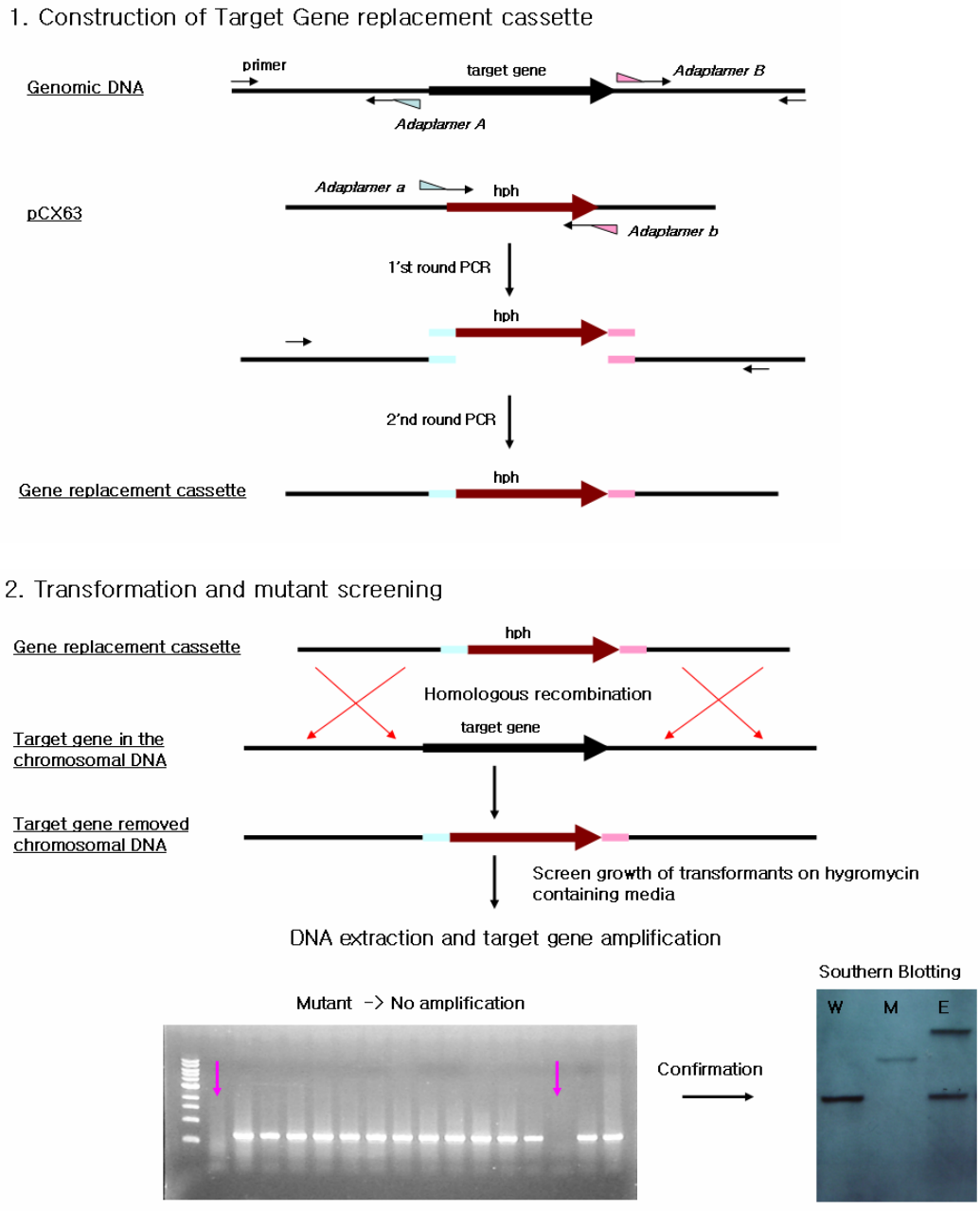


Table 1. Differential expression of 10,176 *M. grisea* genes during spore germination and appressorium formation.

	SG_up[a]	SG_dn	GE_up	GE_dn	AI_up	AI_dn	AM_up	AM_dn	CI_up	CI_dn
SG_up	2,087[b]									
SG_dn		67								
GE_up	17	2	46							
GE_dn	13			17						
AI_up	62	2			178					
AI_dn	107	2	3	11		142				
AM_up	50	7	2		84	2	267			
AM_dn	128	5	19	4	1	71		179		
CI_up	113	8	7	2	103	2	202	1	644	
CI_dn	242	10	13	7		71		92		370

[a] Letters indicate particular expression profiles, SG = spore germination, GE = germ tube elongation, AI = appressorium initiation, AM = appressorium maturation and CI = cAMP induced appressorium. Up = up-regulated and dn = down-regulated.

[b] Each value indicates the number of genes in common between the paired expression profiles.

Table 2. GO Categorization (Biological Process) of differentially expressed genes during spore germination.

Ontology	Description	MG	SG_up		SG_dn	
GO:0007582	physiological process	3,927	1,085		22	
GO:0008152	metabolism	3,094	901	+	21	
GO:0006139	nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	938	200	−	1	−
GO:0019538	protein metabolism		302	+	1	
GO:0006810	transport	775	176	−	1	
GO:0009058	biosynthesis	589	258	+	3	
GO:0006091	generation of precursor metabolites and energy	459	139		13	+
GO:0006350	transcription	427	54	−		
GO:0016043	cell organization and biogenesis	368	92		1	
GO:0006118	electron transport	367	89		12	+
GO:0006464	protein modification	304	67	−	1	
GO:0005975	carbohydrate metabolism	285	99	+	2	
GO:0006996	organelle organization and biogenesis	234	62		1	
GO:0006259	DNA metabolism	232	59		1	
GO:0015031	protein transport	208	48			
GO:0009056	catabolism	198	61		2	
GO:0006519	amino acid and derivative metabolism	182	75	+	2	
GO:0007154	cell communication	177	40			
GO:0007165	signal transduction	160	34			
GO:0007049	cell cycle	152	45			
GO:0006629	lipid metabolism	150	41			
GO:0006950	response to stress	141	28	−		
GO:0006811	ion transport	122	49	+		
GO:0009719	response to endogenous stimulus	102	17	−		
GO:0007028	cytoplasm organization and biogenesis	66	7	−		
GO:0007010	cytoskeleton organization and biogenesis	52	20			
GO:0007275	development	41	10			
GO:0019725	cell homeostasis	27	10			
GO:0000003	reproduction	22	10			
GO:0019748	secondary metabolism	21	8			
GO:0030154	cell differentiation	18	9	+		
GO:0040029	regulation of gene expression, epigenetic	17	4			
GO:0009653	morphogenesis	15	2			
GO:0007005	mitochondrion organization and biogenesis	14	3			
GO:0009607	response to biotic stimulus	13	6			
GO:0016265	death	10	5			
GO:0008219	cell death	10	5			
GO:0040007	growth	9	1			
GO:0016049	cell growth	8	1			
GO:0009628	response to abiotic stimulus	6				
GO:0009605	response to external stimulus	5	1			
GO:0008283	cell proliferation	3	1			
GO:0007610	behavior	1	1			
GO:0016032	viral life cycle	1				
Total		4,189	1,154		23	

Values represent numbers of genes assigned to GO categories in the Magnaporthe genome (MG) and up-regulated (SG_up) and down_regulated (SG_dn) during spore germination. Values followed by + or - indicate the number of genes in a particular GO category that are over or under represented (Chi-square test $p < 0.05$).

Table 3. Primer sequences for RT-PCR and qRT-PCR experiments

Gene name	Forward primer (5'-3')	Reverse primer(5'-3')
MGG_03436.5	ATCCGACATGAAGCTCAGCGATGT	CAAACACCTTGCAGGCCTTCTCAA
MGG_05247.5	AAGCTCAGCAAGGACGGTTA	GGTACACCAGTCTCGGCATT
MGG_03670.5	TTCGACTCTGTGAACGATGC	GAGAAGTAGGCACGGCTGTC
MGG_08523.5	AGATGGCCTTCATCAACGGAAGGT	GACCAAACGTGCCGACAATGTCAA
MGG_00792.5	TGGAGATGAGCAATGAGGACCGAA	AGCTGCAAGTACGGCACCGTATAA
MGG_06456.5	CCAAAGAATCCACGTCCGAGAACT	TGTCGTAATCGTTCTGCTCCAGGT
MGG_03982.5	TTGGTATGGGCCAGAAGGACTCTT	TTCTGCATACGGTCCGAAAGACCA
MGG_10061.5	TGCTTACAGACACATCGCGTGAGA	AGTAGTTGACCTTGCCCTTGGTGA
MGG_03593.5	TTGCGGACAAGATTGTCAACGAGC	ATGGTAGGCTTGCACTTGCCAAAG
MGG_09875.5	TTCGCCAAGGTTACCATTCTCTCC	ACAAGGCAGACGTTCTCTTGACCA
MGG_10315.5 (MPG1)	TAAGAACTCCAAGTCTGGTGCCGA	TGAGCTGGTTGATGGTGAGGATGT
MGG_05871.5 (PTH11)	AGCATCGAAGGCAAATGCATGGAG	TCACCACCAGGACCACATCTTCAA

Table 4. List of functionally annotated appressorium consensus genes

Category	Gene ID	Exp.[a]	SignalP[b]	Blast hit	NCBI_ID	E-value[c]
Melanin biosynthesis						
	MGG_07219.5	UR	N	polyketide synthase [Colletotrichum lagenarium]	BAA18956.1	0.0
	MGG_07216.5	UR	N	1,3,6,8-trihydroxynaphthalene reductase [Magnaporthe grisea]	AAG29497.2	0.0
	MGG_05059.5	UR	N	syctalone dehydratase I [Ceratocystis resinifera]	AAO60167.1	9.E-73
	MGG_07218.5	UR	N	Zn-II 2Cys6 regulatory protein [Leptosphaeria maculans]	AAO49457.1	1.E-10
	MGG_13764.5	UR	N	polyphenol oxidase [Acremonium murorum]	CAB75422.1	0.0
Secondary metabolism						
	MGG_10072.5	UR	N	PKSN polyketide synthase for alternapyrone biosynthesis [Alternaria solani]	BAD83684.1	0.0
	MGG_04775.5	UR	N	polyketide synthase [Botryotinia fuckeliana]	AAR90244.1	0.0
	MGG_00385.5	UR	N	ochratoxin A non-ribosomal peptide synthetase [Penicillium nordicum]	AAS98174.1	6.E-90
	MGG_00573.5	UR	N	tetracenomycin polyketide synthesis O-methyltransferase [Mycobacterium smegmatis str. MC2 155]	ZP_00139515.1	5.E-31
	MGG_00792.5	UR	N	squalene-hopene-cyclase [Thermosynechococcus elongatus BP-1]	NP_683099.1	E-112
	MGG_06539.5	UR	N	CipA protein, putative [Aspergillus fumigatus]	CAE47970.1	5.E-44
	MGG_06585.5	UR	N	Fum13p [Gibberella moniliformis]	AAN74816.1	1.E-24
	MGG_04911.5	UR	N	Fum15p [Gibberella moniliformis]	AAN74818.2	1.E-56
	MGG_04335.5	UR	N	nonribosomal peptide synthetase 12 [Cochliobolus heterostrophus]	AAX09994.1	9.E-25
	MGG_03397.5	UR	N	citrinin biosynthesis oxydoreductase CtnB [Monascus purpureus]	BAE95339.1	3.E-63
	MGG_01391.5	DR	N	cytochrome P450 monooxygenase [Penicillium paxilli]	AAK11528.1	2.E-52
Signal transduction						
	MGG_09947.5	UR	N	transmembrane protein, putative [Cryptococcus neoformans]	AAW42308.1	0.0
	MGG_01150.5	UR	N	calcineurin temperature suppressor Cts1 [Cryptococcus neoformans]	AAN85205.1	7.E-19
	MGG_06035.5	DR	N	probable peptidylprolyl isomerase (FK506-binding protein homolog) [Neurospora crassa]	CAF06078.1	2.E-31
	MGG_00871.5	UR	N	Csr1p [Saccharomyces cerevisiae].	NP_013484.1	6.E-66
	MGG_05804.5	UR	N	phospholipase D [Emericella nidulans]	BAC67175.1	0.0
	MGG_01367.5	UR	Y	secretory phospholipase A2 [Aspergillus oryzae]	BAD01582.1	1.E-27
	MGG_08732.5	UR	N	related to interferon-regulated resistance GTP-binding protein [Neurospora crassa]	CAE81930.1	0.0

Table 4 (continued)

MGG_09570.5	UR	Y	MAC1 interacting protein 1; ACI1 [Magnaporthe grisea]	AAN64312.1	2.E-07
MGG_05353.5	UR	N	putative G protein-coupled receptor alpha [Botryotinia fuckeliana]	CAE55153.1	2.E-16
MGG_00987.5	UR	N	Plasma membrane protein involved in G-protein mediated pheromone [Saccharomyces cerevisiae]	NP_014226.1	6.E-15
MGG_01094.5	DR	N	conserved hypothetical protein [Neurospora crassa]	CAB91433.2	0.0
MGG_05871.5	DR	Y	Pthintegral membrane protein [Magnaporthe grisea]	AAD30436.1	0.0
MGG_05214.5	DR	Y	integral membrane protein [Magnaporthe grisea]	AAD30437.1	2.E-24
MGG_02692.5	DR	N	integral membrane protein [Magnaporthe grisea]	AAD30437.1	1.E-05
MGG_10571.5	DR	N	integral membrane protein [Magnaporthe grisea]	AAD30436.1	2.E-17
MGG_03148.5	DR	N	bacterial signalling protein N terminal repeat family [Aspergillus fumigatus]	XP_753518.1	3.E-86
MGG_13736.5	DR	N	putative G protein-coupled receptor alpha [Botryotinia fuckeliana]	CAE55153.1	2.E-04
Amino Acid Metabolism					
MGG_09919.5	UR	N	amino transferase [Aspergillus fumigatus Af293]	XP_755696.1	E-149
MGG_01906.5	UR	N	putative nicotianamine synthase [Podospora anserina]	AAO25955.1	1.E-30
MGG_03231.5	UR	N	related to pentachlorophenol 4-monooxygenase [Neurospora crassa]	CAD70786.1	7.E-88
MGG_05247.5	UR	N	NAD(+)-specific glutamate dehydrogenase; NAD-GDH [Neurospora crassa]	AAB28355.1	0.0
MGG_06095.5	UR	N	cysteine dioxygenase [Ajellomyces capsulatus]	AAV66535.1	3.E-52
MGG_10036.5	UR	N	phenylalanine ammonia-lyase [Aspergillus fumigatus Af293]	XP_755245.1	2.E-97
MGG_10380.5	UR	N	cystathionine-gamma-lyase [Acremonium chrysogenum]	AAF97598.1	E-153
MGG_10533.5	UR	Y	arginase, putative [Cryptococcus neoformans var. neoformans JEC21]	AAW42854.1	3.E-91
MGG_07224.5	UR	N	threonine deaminase [Arxula adeninivorans]	CAA10977.1	E-162
MGG_00189.5	UR	N	delta-1-pyrroline-5-carboxylate dehydrogenase [Aspergillus fumigatus Af293]	XP_750764.1	0.0
MGG_02378.5	DR	N	glutamate decarboxylase [Chaetomium globosum CBS 148.51]	EAQ84052.1	0.0
MGG_02817.5	DR	N	Glutamate decarboxylase [Saccharomyces cerevisiae]	NP_013976.1	E-122
Proteolysis and peptidolysis					
MGG_03056.5	UR	Y	aorsin [Aspergillus oryzae]	BAB97387.1	E-132
MGG_00981.5	UR	N	aspartyl proteinase [Trichoderma asperellum]	AAU11329.1	1.E-79
MGG_03260.5	UR	N	related to calpain [Neurospora crassa]	CAD37034.1	E-166
MGG_03670.5	UR	Y	subtilisin-like serine protease [Podospora anserina]	AAC03564.2	0.0
MGG_07404.5	UR	Y	tripeptidyl aminopeptidase [Aspergillus oryzae]	AAU10333.1	E-126
MGG_08526.5	UR	N	thiol protease [Porphyromonas gingivalis]	AAA25652.1	2.E-17
MGG_09246.5	UR	Y	subtilisin-like protease [Verticillium dahliae]	AAR10770.1	7.E-82
MGG_09351.5	UR	Y	aspartyl protease [Sclerotinia sclerotiorum]	AAF76202.1	E-126

Table 4 (continued)

MGG_09716.5	UR	Y	carboxypeptidase [Metarhizium anisopliae]	AAB68600.1	E-119
MGG_03580.5	UR	N	Atg4p [Saccharomyces cerevisiae]	NP_014176.2	1.E-49
MGG_09032.5	DR	Y	related to acid proteinase [Neurospora crassa]	CAD36982.1	1.E-18
Carbohydrate Metabolism					
MGG_03361.5	UR	N	glycosyl transferase [Aspergillus fumigatus Af293]	XP_751278.1	E-126
MGG_02393.5	UR	Y	cutinase [Botryotinia fuckeliana]	CAA93255.1	6.E-38
MGG_11966.5	UR	Y	cutinase [Botryotinia fuckeliana]	CAA93255.1	3.E-48
MGG_00086.5	UR	Y	chitinase [Trichoderma viride]	AAG09447.1	E-145
MGG_01876.5	UR	Y	chitinase 3 [Coccidioides posadasii]	AAO88269.1	2.E-60
MGG_03880.5	UR	N	alcohol dehydrogenase [Cochliobolus lunatus]	ABC88428.1	E-138
MGG_03263.5	UR	N	related to aldehyde dehydrogenase (NAD+) [Neurospora crassa]	CAD37029.1	E-152
MGG_10038.5	UR	Y	putative beta-glucosidase [Arthrobacter nicotinovorans]	CAD47965.1	E-126
MGG_00625.5	UR	N	probable glucosamine-6-phosphate deaminase [Neurospora crassa]	CAE85549.1	E-103
MGG_01922.5	UR	N	polysaccharide deacetylase family protein [Pseudomonas syringae]	YP_276070.1	E-110
MGG_00659.5	UR	Y	beta-1,3-exoglucanase [Trichoderma hamatum]	AAP33112.1	0.0
MGG_01231.5	UR	N	L-arabinitol 4-dehydrogenase [Hypocrea jecorina]	AAP57209.1	E-161
MGG_05246.5	UR	Y	alpha-L-rhamnosidase A precursor [Aspergillus aculeatus]	AAK16249.1	3.E-28
MGG_10878.5	UR	N	GAOA_DACDE Galactose oxidase precursor (GAO) [Gibberella zeae PH-1]	XP_391208.1	E-107
MGG_05735.5	UR	N	FMN dependent dehydrogenase [Aspergillus fumigatus Af293]	XP_747805.1	E-109
MGG_05908.5	UR	Y	ALK1 [Yarrowia lipolytica]	BAA31433.1	3.E-86
MGG_00623.5	UR	N	related to hexokinase [Neurospora crassa]	CAE85550.1	E-124
MGG_00450.5	UR	N	phosphoenolpyruvate carboxykinase [Emericella nidulans]	AAL10705.1	0.0
MGG_03497.5	UR	N	pyruvate dehydrogenase [Aedes aegypti]	EAT32992.1	9.E-01
MGG_10051.5	UR	Y	cellobiose dehydrogenase [Aspergillus fumigatus Af293]	XP_747382.1	3.E-13
MGG_01320.5	DR	N	UDP-N-acetylglucosamine pyrophosphorylase [Emericella nidulans]	AAW49004.1	0.0
MGG_09238.5	DR	N	rhamnose biosynthetic enzyme 1, putative, expressed [Oryza sativa	ABF95279.1	2.E-79
MGG_10400.5	DR	Y	glucosidase [Fusarium sporotrichioides]	AAO27749.1	8.E-25
MGG_01096.5	DR	N	glucan 1, 4-alpha-glucosidase [Neurospora crassa]	CAB91426.1	0.0
MGG_05364.5	DR	Y	endoglucanase, putative [Aspergillus fumigatus]	CAF31975.1	1.E-57
MGG_03041.5	DR	N	glucokinase [Aspergillus niger]	CAA67949.1	E-138
MGG_10005.5	DR	N	glycerol kinase [Aspergillus fumigatus Af293]	XP_750736.1	E-167
MGG_13485.5	DR	N	probable 2-isopropylmalalate synthase [Neurospora crassa]	CAE76195.1	0.0
Glycan Biosynthesis and Metabolism					

Table 4 (continued)

MGG_07790.5	UR	Y	manganese peroxidase [Ganoderma applanatum]	BAA88392.1	3.E-24
MGG_03307.5	UR	N	chitinase, class I [Myxococcus xanthus DK 1622]	YP_635494.1	2.E-03
MGG_00994.5	UR	Y	alpha-mannosidase [Aspergillus saitoi]	BAA08634.1	E-149
MGG_10494.5	UR	Y	beta-1,4-mannosyltransferase, putative [Cryptococcus neoformans]	AAW41187.1	3.E-72
MGG_00695.5	UR	N	alpha-1,2-mannosidase subfamily [Aspergillus fumigatus Af293]	XP_751252.1	0.0
MGG_00077.5	UR	N	proteophosphoglycan ppg4 [Leishmania major strain Friedlin]	AAZ14280.1	1.E-05
MGG_00316.5	DR	Y	a-L-fucosidase [Halocynthia roretzi]	BAB85519.1	4.E-53
MGG_05785.5	DR	Y	fructosyltransferase [Aspergillus sydowii]	CAB89083.1	8.E-47
Lipid metabolism					
MGG_01026.5	UR	N	hydroxymethylglutaryl-CoA synthase [Aspergillus fumigatus Af293]	XP_754553.1	0.0
MGG_07613.5	UR	N	cut6 [Schizosaccharomyces pombe]	CAB16395.1	0.0
MGG_03765.5	UR	Y	7-dehydrocholesterol reductase [Homo sapiens]	NP_001351.1	7.E-93
MGG_11040.5	UR	Y	1-acyl-sn-gylcerol-3-phosphate acyltransferase [Saccharomyces cerevisiae]	NP_010231.1	8.E-44
MGG_08257.5	UR	N	fatty acid transporter protein [Cochliobolus heterostrophus]	CAA75802.1	2.E-96
MGG_06704.5	UR	N	Gde1p [Saccharomyces cerevisiae]	NP_015215.1	0.0
MGG_03569.5	UR	N	putative delta 8-sphingolipid desaturase [Kluyveromyces lactis]	BAB93118.1	E-143
MGG_00853.5	UR	N	oxysterol binding protein-like 11 [Mus musculus]	NP_789810.1	8.E-24
MGG_03690.5	UR	N	related to ethanolaminephosphotransferase [Neurospora crassa]	CAE76360.1	4.E-61
MGG_02409.5	UR	N	sterol carrier protein [Aspergillus fumigatus Af293]	XP_752186.1	0.0
MGG_07337.5	UR	N	probable peroxisomal protein POX18 [Neurospora crassa]	CAD21491.1	2.E-41
MGG_02813.5	UR	N	malate synthase [Candida tropicalis]	BAA02681.1	0.0
MGG_11317.5	UR	N	long chain fatty alcohol oxidase [Aspergillus fumigatus Af293]	XP_753079.1	E-145
MGG_02610.5	DR	N	lipase [Bacillus cereus ATCC 10987]	NP_979556.1	1.E-20
MGG_10879.5	DR	N	fatty acid omega-hydroxylase (P450foxy) [Fusarium oxysporum]	BAA82526.1	0.0
MGG_01925.5	DR	N	fatty acid omega-hydroxylase (P450foxy) [Fusarium oxysporum]	BAA82526.1	0.0
Cell cycle & development					
MGG_04891.5	UR	N	GPI-anchored cell surface glycoprotein (flocculin) [Saccharomyces revisiae]	NP_012284.1	1.E-02
MGG_00850.5	UR	N	SesB [Nectria haematococca]	AAS80314.1	4.E-27
MGG_06461.5	UR	N	PREDICTED: similar to G2/mitotic-specific cyclin F [Strongylocentrotus purpuratus]	XP_798496.1	5.E-09
MGG_06538.5	DR	Y	blastomyces yeast phase-specific protein 1 [Ajellomyces dermatitidis]	AAF86474.1	2.E-07
MGG_00513.5	DR	N	con-8 protein [Neurospora crassa]	CAE76281.1	2.E-06
MGG_03336.5	DR	N	late embryogenesis abundant protein [Catharanthus roseus]	AAY84145.1	9.E-05

Table 4 (continued)

Cell wall & surface					
MGG_09477.5	UR	N	related to a-agglutinin core protein AGA1 [Neurospora crassa]	CAC28825.2	2.E-13
MGG_09460.5	UR	Y	cell wall protein [Aspergillus kawachii]	BAD01559.1	2.E-18
MGG_03436.5	UR	N	cell wall mannoprotein MnpA [Aspergillus nidulans]	AAM16156.1	1.E-03
MGG_02778.5	UR	Y	cell wall protein [Aspergillus kawachii]	BAD01559.1	2.E-03
MGG_02796.5	UR	N	cell surface flocculin [Saccharomyces cerevisiae]	CAK18547.1	1.E-03
MGG_04093.5	DR	N	cell wall surface anchor family protein [Streptococcus pneumoniae TIGR4]	AAK75846.1	1.E-38
MGG_04913.5	DR	N	cyanovirin-N-like protein [Tuber borchii]	AAV85993.1	2.E-05
Electron transport & Energy Metabolism					
MGG_09162.5	UR	Y	cytochrome b2, mitochondrial precursor [Cryptococcus neoformans]	AAW45006.1	6.E-50
MGG_09453.5	UR	Y	monooxygenase [Aspergillus flavus]	AAT65719.1	2.E-24
MGG_00790.5	UR	N	oxidoreductase [Agrobacterium tumefaciens str. C58]	NP_533225.1	5.E-07
MGG_02792.5	UR	Y	related to n-alkane-inducible cytochrome P450 [Neurospora crassa]	CAC10088.1	E-116
MGG_02256.5	UR	N	monooxygenase [Penicillium paxilli]	AAK11530.1	4.E-41
MGG_01569.5	DR	N	probable 1, 4-Benzoquinone reductase [Neurospora crassa]	CAE76242.1	2.E-71
MGG_04120.5	DR	N	putative monooxygenase [Bradyrhizobium japonicum USDA 110]	NP_770497.1	E-109
MGG_10800.5	DR	N	sarcosine oxidase [Cylindrocarpon didymum]	BAA96069.1	E-168
Enzyme activity					
MGG_13573.5	UR	N	6-hydroxy-D-nicotine oxidase [Coccidioides immitis RS]	EAS36048.1	9.E-50
MGG_00745.5	UR	N	hydrolase [Aspergillus fumigatus Af293]	XP_753376.1	7.E-47
MGG_07793.5	UR	N	oxidoreductase, short-chain dehydrogenase/reductase family[Aspergillus fumigatus Af293]	XP_749588.1	1.E-40
MGG_04774.5	UR	N	Fsh3p [Saccharomyces cerevisiae]	NP_014923.1	2.E-05
MGG_09942.5	UR	Y	alpha/beta hydrolase [Aspergillus fumigatus Af293]	XP_750599.1	5.E-55
MGG_11608.5	UR	Y	laccase [Gaeumannomyces graminis var. tritici]	CAD10747.1	0.0
MGG_12475.5	UR	N	phloretin hydrolase [Eubacterium ramulus]	AAQ12341.1	6.E-17
MGG_04378.5	DR	N	Alpha/beta hydrolase [Burkholderia sp. 383]	ABB10796.1	4.E-09
Protein modification					
MGG_01282.5	UR	N	Ubi4p [Saccharomyces cerevisiae]	NP_013061.1	E-166
MGG_07297.5	UR	N	ubiquitin-like conjugating enzyme, putative [Cryptococcus neoformans]	AAW45433.1	E-138
Protein synthesis					
MGG_04484.5	DR	N	Protein component of the large (60S) ribosomal subunit [Saccharomyces cerevisiae]	NP_014521.1	4.E-47
MGG_03554.5	DR	N	RL36_TRIHM 60S ribosomal protein L36 (TRP36) [Gibberella zeae PH-1]	XP_381414.1	4.E-34

Table 4 (continued)

MGG_05449.5	DR	N	60S ribosomal protein L16 [Chaetomium globosum CBS 148.51]	EAQ86180.1	E-102
MGG_03727.5	DR	N	putative 60s ribosomal protein [Colletotrichum gloeosporioides].	CAC15500.1	2.E-48
MGG_10680.5	DR	N	probable 40S RIBOSOMAL PROTEIN S24 [Neurospora crassa]	CAD71100.1	1.E-48
MGG_04829.5	DR	N	60S ribosomal protein L9 b [Aspergillus fumigatus Af293]	XP_752277.1	2.E-81
MGG_06269.5	DR	N	ADL127Cp [Ashbya gossypii ATCC 10895]	AAS51793.1	E-118
MGG_04455.5	DR	N	probable ribosomal protein L35 [Neurospora crassa]	CAE76503.1	7.E-51
MGG_06721.5	DR	N	60S ribosomal protein L28 [Chaetomium globosum CBS 148.51]	EAQ86004.1	5.E-61
MGG_07048.5	DR	N	60S RIBOSOMAL PROTEIN L5 [Neurospora crassa]	CAD71058.1	E-133
MGG_07753.5	DR	N	cytosolic large ribosomal subunit L11 [Aspergillus fumigatus Af293]	XP_752052.1	2.E-80
MGG_02953.5	DR	N	ribosomal protein Srp1 [Sclerotinia sclerotiorum]	AAP58401.1	2.E-34
MGG_04612.5	DR	N	probable ribosomal protein L7a.e.B, cytosolic [Neurospora crassa]	CAE85573.1	6.E-97
MGG_06952.5	DR	N	probable ribosomal protein L38 [Neurospora crassa]	CAC28690.1	6.E-19
MGG_06480.5	DR	N	RS12_ERYGR 40S ribosomal protein S12 [Gibberella zeae PH-1]	XP_387468.1	8.E-64
MGG_06837.5	DR	N	RS21_NEUCR 40S ribosomal protein S21 (CRP7) [Gibberella zeae	XP_380974.1	8.E-38
MGG_06479.5	DR	N	40S ribosomal protein S22 [Coccidioides immitis RS]	EAS32089.1	2.E-63
MGG_02659.5	DR	N	ribosomal protein L14 [Aspergillus fumigatus Af293]	XP_747694.1	2.E-38
MGG_04921.5	DR	N	60S ribosomal protein L23 [Chaetomium globosum CBS 148.51]	EAQ93853.1	3.E-73
MGG_05716.5	DR	Y	ribosomal protein S1 [Geobacter metallireducens GS-15]	ABB31109.1	1.E+00
MGG_05296.5	DR	N	ribosomal protein L34-like protein [Ophiostoma novo-ulmi]	AAK58051.1	2.E-53
MGG_03193.5	DR	N	60S ribosomal protein L3 [Chaetomium globosum CBS 148.51]	EAQ83793.1	0.0
Response to stress					
MGG_04358.5	UR	N	hsp16 [Schizosaccharomyces pombe]	CAA19006.1	5.E-06
MGG_03350.5	UR	Y	related to cytosolic Cu/Zn superoxide dismutase [Neurospora crassa]	CAB97297.1	4.E-25
MGG_06747.5	UR	N	glutathione S-transferase [Botryotinia fuckeliana]	AAG43132.1	5.E-96
MGG_10061.5	DR	N	catalase [Cochliobolus heterostrophus]	AAR17473.1	0.0
MGG_03896.5	DR	N	chloride peroxidase [Burkholderia cepacia]	AAL73575.1	4.E-04
Transcription regulation					
MGG_02129.5	UR	N	regulatory protein AlcR [Aspergillus fumigatus Af293]	XP_748311.1	E-110
MGG_00320.5	UR	N	C6 transcription factor [Aspergillus fumigatus Af293]	XP_749091.1	2.E-05
MGG_03288.5	UR	N	CAMP responsive element binding protein 3-like 2 [Mus musculus]	AAH43466.1	1.E-04
MGG_09276.5	UR	N	cercosporin resistance protein [Cercospora nicotianae]	AAD25072.2	4.E-31
MGG_08295.5	UR	N	C6 transcription factor [Aspergillus fumigatus Af293]	XP_750890.1	2.E-20
MGG_09200.5	DR	N	zinc finger protein [Ascobolus immersus]	CAA67549.1	3.E-65

Table 4 (continued)

	MGG_09031.5	DR	N	sporulation negative regulatory protein-like protein [Magnaporthe grisea]	AAX07687.1	E-131
	MGG_06832.5	DR	N	transcription activator-like protein [Nectria haematococca]	AAO72069.1	1.E-03
	MGG_01490.5	DR	N	Transcriptional Regulator, AraC family [Pseudomonas fluorescens PfO-1]	ABA73699.1	7.E-05
Transport						
	MGG_02167.5	UR	N	aflatoxin efflux pump [Aspergillus flavus]	AAM53947.1	E-113
	MGG_01778.5	UR	N	probable aflatoxin efflux pump AFLT [Neurospora crassa]	CAF06057.1	E-163
	MGG_03640.5	UR	N	Mfs1.1 [Coprinus cinereus]	AAF01426.1	4.E-69
	MGG_03843.5	UR	N	multidrug transporter, putative [Cryptococcus neoformans]	AAW45439.1	2.E-72
	MGG_10869.5	UR	N	probable aflatoxin efflux pump AFLT [Neurospora crassa]	CAF06057.1	3.E-21
	MGG_06794.5	UR	N	related to aminotriazole resistance protein [Neurospora crassa]	CAF06028.1	E-173
	MGG_10410.5	UR	N	ABC transporter [Aspergillus fumigatus Af293]	XP_754651.1	0.0
	MGG_07062.5	UR	N	related to monocarboxylate transporter [Neurospora crassa]	CAD70416.1	3.E-25
	MGG_04511.5	UR	N	MFS transporter [Beauveria bassiana]	AAO73599.1	1.E-82
	MGG_00275.5	UR	N	MFS transporter [Beauveria bassiana]	AAO73599.1	2.E-88
	MGG_09827.5	UR	N	Jen1p [Saccharomyces cerevisiae]	NP_012705.1	2.E-90
	MGG_06604.5	UR	Y	Vacuolar membrane protein involved in the ATP-dependent transport [Saccharomyces cerevisiae]	NP_012476.1	4.E-56
	MGG_10200.5	UR	N	small oligopeptide transporter, OPT family [Aspergillus fumigatus]	XP_755859.1	0.0
	MGG_07228.5	UR	N	peptide transporter MTD1 [Schizophyllum commune]	AAF26618.1	5.E-77
	MGG_08258.5	UR	N	probable PEPTIDE TRANSPORTER PTR2 [Neurospora crassa]	CAE75697.1	0.0
	MGG_02124.5	UR	N	potassium transporter hak-1 [Neurospora crassa]	CAE81927.1	0.0
	MGG_07494.5	UR	N	possible cation efflux protein [Aspergillus fumigatus]	CAF32159.1	E-129
	MGG_05085.5	UR	N	putative ion transporter [Candida albicans SC5314]	XP_712877.1	3.E-71
	MGG_04105.5	UR	Y	cation transport-related protein, putative [Cryptococcus neoformans]	AAW42114.1	3.E-22
	MGG_02763.5	UR	N	Lpe10p [Saccharomyces cerevisiae]	NP_015265.1	2.E-58
	MGG_04994.5	UR	N	plasma membrane H+-ATPase [Blumeria graminis]	AAK94188.1	0.0
	MGG_04852.5	UR	N	probable P-type ATPase [Neurospora crassa]	CAE76097.1	0.0
	MGG_00930.5	UR	N	copper resistance-associated P-type ATPase [Candida albicans]	AAF04593.1	E-177
	MGG_03957.5	UR	N	Permease of the drug/metabolite transporter, DMT superfamily [Prochlorococcus marinus str. MIT 9211]	ZP_01005589.1	4.E-12
	MGG_04068.5	UR	N	fructose symporter [Aspergillus fumigatus Af293]	XP_747651.1	3.E-11
	MGG_06336.5	UR	Y	integral membrane protein [Aspergillus fumigatus Af293]	XP_750355.1	E-102
	MGG_11717.5	UR	N	MFS_transporter protein [Streptomyces avermitilis]	BAB69375.1	1.E-14

Table 4 (continued)

	MGG_12650.5	UR	N	MFS transporter [Aspergillus fumigatus Af293]	XP_754894.1	1.E-74
	MGG_13192.5	UR	N	MFS transporter [Aspergillus fumigatus Af293]	XP_747222.1	E-122
	MGG_04927.5	DR	N	transporter-like protein [Magnaporthe grisea]	AAX07640.1	0.0
	MGG_09193.5	DR	N	l-fucose permease [Aspergillus fumigatus Af293]	XP_750566.1	E-148
	MGG_03298.5	DR	N	phthalate transporter [Aspergillus fumigatus Af293]	XP_746562.1	E-132
	MGG_07843.5	DR	N	Tna1 [Paracoccidioides brasiliensis]	AAQ04627.1	E-152
	MGG_08968.5	DR	N	allantoate transporter, putative [Cryptococcus neoformans]	AAW44174.1	1.E-79
	MGG_01480.5	DR	N	related to large-conductance mechanosensitive channel [Neurospora crassa]	CAB91331.2	1.E-36
Pathogenecity						
	MGG_04202.5	UR	Y	putative Egh16H1 precursor isoform B [Blumeria graminis]	AAK25793.1	E-107
	MGG_02253.5	UR	Y	putative Egh16H1 precursor isoform B [Blumeria graminis]	AAK25793.1	3.E-38
	MGG_00438.5	UR	Y	pathogenicity protein [Magnaporthe grisea]	AAD01641.1	E-121
	MGG_12337.5	UR	Y	ASG1 [Magnaporthe grisea]	AAL28112.1	2.E-75
	MGG_09875.5	DR	Y	ASG1 [Magnaporthe grisea]	AAL28112.1	1.E-72
	MGG_10315.5	DR	N	MPG1[Magnaporthe grisea 70-15]	XP_366095.1	9.E-42
	MGG_05344.5	DR	Y	snodprot-FS [Gibberella pulicaris]	AAV83793.1	3.E-44
Metabolism						
	MGG_01473.5	UR	N	2-nitropropane dioxygenase, putative [Cryptococcus neoformans]	AAW45147.1	E-102
	MGG_02497.5	UR	Y	carboxylesterase [Aspergillus fumigatus Af293]	XP_753283.1	1.E-48
	MGG_02987.5	DR	N	Carboxylesterase, type B [Mycobacterium flavescens PYR-GCK]	ZP_01191056.1	4.E-37
	MGG_00357.5	DR	N	Short-chain dehydrogenase/reductase SDR [Mycobacterium sp. JLS]	ZP_01278292.1	1.E-18
	MGG_02798.5	DR	N	Rossman fold oxidoreductase, putative [Cryptococcus neoformans]	AAW42118.1	2.E-31
Others						
	MGG_05053.5	UR	N	UbiE/COQ5 methyltransferase [Aspergillus fumigatus Af293]	XP_748902.1	1.E-26
	MGG_09096.5	UR	N	related to tol protein [Neurospora crassa]	CAD70524.1	9.E-34
	MGG_04110.5	UR	N	queuine tRNA-ribosyltransferase [Aspergillus fumigatus Af293]	XP_746611.1	E-161
	MGG_06823.5	UR	Y	putative purple acid phosphatase [Arabidopsis thaliana]	AAT48877.1	2.E-41
	MGG_05528.5	UR	N	probable cytosceletal binding protein [Neurospora crassa]	CAD21322.1	E-136
	MGG_10892.5	UR	Y	predicted protein [Magnaporthe grisea 70-15]	XP_361209.1	9.E-14
	MGG_06847.5	UR	N	annexin XIV [Neurospora crassa]	CAF06024.1	E-112
	MGG_04726.5	UR	N	DNA repair system specific for alkylated DNA [Xanthomonas oryzae]	YP_199441.1	3.E-08

Table 4 (continued)

MGG_04740.5	UR	N	putative SMK toxin resistance protein [Candida albicans SC5314]	XP_713262.1	7.E-06
MGG_03270.5	DR	N	putative U3 snoRNP component [Candida albicans SC5314]	XP_722611.1	E-113
MGG_01041.5	DR	N	vivid PAS protein VVD [Neurospora crassa]	CAF06140.1	4.E-45
MGG_09461.5	DR	N	UV-endonuclease UVE-1 [Neurospora crassa]	CAD21267.1	E-178
MGG_06911.5	DR	N	probable inosine triphosphate pyrophosphatase [Neurospora crassa]	CAD70978.1	3.E-87
MGG_08008.5	DR	N	formyltetrahydrofolate deformylase [Aspergillus fumigatus Af293]	XP_751045.1	1.E-93
MGG_07749.5	DR	Y	putative alpha subunit of ATP synthase [Cyathea arborea]	CAJ44964.1	2.E-04

[a] Gene expression was marked as upregulated (UR) and down regulated(DR).

[b] Signal peptide cleavage sites were predicted by SignalP 3.0 (www.cbs.dtu.dk/services/SignalP/)

[c] E-values were taken from BLASTX search against NCBI non redundant protein database.

Developmentally regulated transcription factors and insight into their function in *Magnaporthe grisea*

Abstract

Magnaporthe grisea undergoes significant transcriptional changes during appressorium development. Using the functional domain searching tool, Interproscan, we predicted 573 genes in the *M grisea* genome encode protein that regulate gene expression. Microarray analysis revealed several transcription factors were transcriptionally regulated during appressorium development. Many were similar to known transcription factors that are involved in regulating secondary metabolism, nutrient assimilation and cell development. The transcription factor gene MGG_08199.5 which encodes a highly conserved Zn2-Cys6 binuclear protein was upregulated. However, in contrast to their orthologs in *Fusarium solani* and *Aspergillus nidulans*, MGG_08199.5 and its homolog MGG_01836.5 were dispensable for fatty acid assimilation and fungal virulence. The frequent occurrence (>25%) of the putative binding target of MGG_08199 and MGG_01836, CCTCGG in the 5' upstream region of *M. grisea* genes suggests complex transcriptional regulation during appressorium differentiation

Introduction

The rice blast pathogen *Magnaporthe grisea* undergoes unique developmental processes during host infection. After contacting the host surface, fungal spores elaborate a germ tube. The growing germ tube tip senses surface signals and differentiates a specialized infection structure, called an appressorium [1]. In previous whole genome microarray analysis, we revealed dramatic transcriptional changes during spore germination followed by significant changes in expression related to appressorium morphogenesis (Chapter 2). Transcriptional regulation is mainly mediated by transcription factors which selectively bind the promoter region of target genes. In plant pathogenic fungi, transcription factors play key roles in signal transduction, nutrient assimilation, secondary metabolism, morphogenesis and pathogenecity [2-7]. While the activity of many transcription factors is determined by post-translational modification, such as phosphorylation [8], microarray studies revealed that mRNA levels of transcription factors were influenced by environmental stress or during development. For example, in Arabidopsis, analysis of expression profiles of 402 transcription factors revealed 74 transcription factors responded to bacterial infection [9] and 118 responded to the fungal cell wall material chitin [10]. Genome wide expression profiles of rice reveals the differential gene expression of MADS-box transcription factors during rice development as well as in response to abiotic stress [11].

Over the past several years, the number of available genome sequences has risen dramatically. Detailed functional studies and structural characterization of transcription factor domains and target sites have enabled more reliable automated annotation and classification of fungal transcription factors [12, 13].

In this study, using an automated functional domain searching tool, we predict the *M. grisea* genome contains 573 putative transcription factors. We define transcription factors as proteins which have functional domains for transcription regulation. Microarray analysis of spore germination and appressorium formation revealed a number of transcription factors were induced during spore germination. Homologs of transcription factors associated with cell differentiation, nitrogen metabolism, and lipid degradation were differentially expressed during appressorium formation. Functional characterization of a developmentally regulated *M. grisea* transcription factors and distribution of putative binding sequences suggest the existence of functional diversity and redundancy of orthologous transcription factors among filamentous fungi.

Results

***M. grisea* Transcription factors** Among the whole 12,841 protein sequences in *M. grisea*, a total of 573 transcription factors with 78 transcription factor domains were identified (Table 2). The largest transcription factor clan in *M. grisea* presented the fungal transcriptional regulatory protein N-terminal domain (IPR001138) which contains cysteine rich motifs where 6 cysteine residues bind to two Zn atoms forming Cys_6Zn_2 clusters for DNA binding. An example is the Gal4 transcription factor. Proteins with this domain are involved in diverse cellular processes such as carbohydrate metabolism, amino acid metabolism, nitrogen utilization, and peroxisome proliferation, as well as responses to stress [16, 17]. We found that the majority of proteins which have fungal specific transcription factor domain (IPR007219) and all the putative aflatoxin synthesis regulatory proteins (IPR002409) contain the IPR001138 domain. In addition, transcription factors with other Zinc finger, Myb DNA binding, basic leucine zipper and basic helix-loop-helix motifs were found to be abundant in *M. grisea*. These types of transcription factors appear to be common among other filamentous fungi including *Neurospora crassa*, *Fusarium graminearum* and *Ustilago maydis* (http://mips.gsf.de/projects/fungi) (Table 2).

Developmentally regulated transcription factors in *M. grisea*. From the 573 transcription factors, we obtained the expression profiles of 464 genes (Figure 1). Analysis revealed that 146 genes were differentially expressed, with the majority being up regulated during spore germination. Forty-two genes were differentially expressed during appressorium induction, maturation or appressorium induction by cAMP (Figure

2). More transcription factors were differentially expressed in response to cAMP than in response to the hydrophobic surface signal during appressorium induction. Functional annotation of these 42 transcription factors revealed that many of them were homologous to transcription factors involved in secondary metabolism, nutrient scavenging and cell developmental process (Table 3).

In fungi, a number of secondary metabolites, including aflatoxins, fumonisin, and cercosporins are synthesized by genes that are physically clustered [18-21]. Based on expression profiles, we previously suggested that the melanin biosynthesis genes are clustered in *M. grisea* (Chapter 2). One of the genes in this cluster, MGG_07218 contains a Cys_6Zn_2 DNA binding domain and exhibited the most dramatic fold change in response to cAMP signal during appressorium induction.

Recently, Chen identified a gene cluster for cercosporin biosynthesis which consists of 8 genes named CTB1 to CTB8. [21]. Cercosporin is a fungal toxin produced by *Cercospora* species and is required for disease development [22]. A transcription activator CRG1 (cercosporin-resistance gene) was involved in cercosporin production and resistance of the fungus to the toxin [23]. Another transcription factor in the cluster, CTB8 regulated the expression of all clustered genes. Cercosporin biosynthesis was completely blocked in CTB8 deletion mutants [21].

It is noteworthy that MGG_09276.5 and MGG_00417.5, which are the orthologs of CRG1 and CTB8 respectively, were significantly induced during appressorium formation. Interestingly, similar to CTB8, we found that MGG_00417.5 is closely located with MGG_00416.5 and MGG_00428.5 genes which are homologus to CTB1 and CTB4 in CTB cluster.

Appressorium formation requires significant redirecting of metabolism under nutrient limiting conditions. We found that transcription factors which are associated with nutrient scavenging were significantly increased. MGG_02129.5, an ortholog of the positive regulatory gene alcR for ethanol utilization, was upregulated. MGG_05709.5, MGG_01887.5, MGG_01518.5 which are homologous to the regulatory proteins for glycerol utilization (GlcD), the arginine catabolism (ArcA) and nitrate assimilation (Nit-4) respectively were also induced [24-27]. In addition, the transcription factors MGG_08199.5 and MGG_02880.5 which are homologous to regulatory proteins involved in cell wall degradation, exhibited increased gene expression. These transcription factors possibly control the expression of cutinase and xylanase. Among transcription factors involved in fungal morphogenesis, gene expression of MGG_01209.5 and Mstu1 (MGG_00692.5) was up regulated whereas MGG_00494.5 and MGG_01776.5 was down regulated.

Transcription factors for lipid metabolism. Recycling of cellular material is a central process for appressorium development. A genome wide gene expression study revealed the highly activated catabolic process of cell storage material during appressorium morphogenesis (Chapter 2). In particular, a group of cell wall degrading enzymes, cutinases, were significantly induced during appressorium formation. Among 42 differentially expressed transcription factors during appressorium formation, MGG_08199.5 is a putative transcription factor containing domains for fungal transcriptional regulatory protein, N-terminal (IPR001138), and fungal specific transcription factor (IPR007219). The amino acid sequence showed 55% and 43%

identity respectively to *Fusarium solani* cutinase transcription factor 1 beta CTF1β (E value 0.0) and *Aspergillus nidulans* FarB (1e-154). A local blast search of MGG_08199.5 against other protein sequences in *M. grisea* revealed MGG_01836 (27% identity, 5.66E-43) as a close paralog.

MGG_08199.5, CTF1β and FarB share transcription factor domains with high similarity (Figure 3). In *F. solani*, CTF1β and its paralog CTF1α control the constitutive and inductive gene expression of cutinases respectively [28]. The CTF1α appears to be essential for pathogenecity of *Fusarium solani* (Li, unpublished). Similarly, in *A. nidulans*, FarB and its paralog FarA bind to the CCTCGG motif in promoter regions of various fatty acid degradation genes and are involved in fatty acid assimilation [29]. MGG_01836.5 is orthologous to CTF1α (70% identity, E value 0.0) and FarA (60% identity, E value 0.0) and these orthologous proteins have very similar transcription factor domains (Figure 3). In contrast to MGG_08199.5, gene expression of MGG_01836 did not significantly change during appressorium formation.

Functional characterization of MGG_08199.5 and MGG_01836.5. To further investigate the biological function of MGG_08199.5 and MGG_01836.5, we generated gene deletion mutants. Individual null mutants of MGG_08199 and MGG_01836.5 were indistinguishable from ectopics and wild type in regards to vegetative growth and sporulation on complete media, oatmeal media, and V8 media. Moreover, like wild type fungi, the mutants grew vigorously on glucose as a carbon source and showed reduced aerial hyphae formation on oleate and tween80 media (Figure 4). In contrast to the FarA and FarB deleted mutants in *A. nidulans*, there appears to be no clear difference in the

utilization of fatty acids between MGG_08199.5 and MGG_01836.5 mutants and the wild type. When mutant spores were placed on an appressorium inductive hydrophobic surface, they developed typical melanized appressoria after 20 hr. Furthermore, the mutants developed typical disease lesions when spray inoculated on to young barley plants (Figure 5).

The CCTCGG motif in *M. grisea* genes

In a previous study, CTF1α and CTF1β were shown to selectively bind palindromic structure GGCXXGCC in the promoter region of target genes in *Fusarium solani*. Similarly, in vitro, FarA and FarB bind to the CCTCGG motif and this motif is present in the 5' upstream regions of many genes whose predicted function are fatty acid metabolism, peroxisome biogenesis and metabolism [29]. Many of these genes such as isocitrate lyase, carnitine acetyl transferase and cutinase are essential for appressorium function and pathogenecity in *M. grisea* [17, 30-32]. Hynes showed the highly conserved CCTCGG motif is enriched in the upstream region of the cutinase genes in *M. grisea* [29].

To test whether this motif is biased to certain functional groups of genes, we scanned the 1kb of upstream region of all predicted genes in the *M. grisea* sequences for the CCTCGG motif. We identified 2,926 genes that containing the motif in their promoter region. Most of genes have a single copy of the motif but 392 genes have 2 copies, 36 have 3 copies, and 4 have 6 copies. These genes are involved in diverse biological processes not only in fatty acid and carbohydrate metabolism but also transcription, transport, and proteolysis (Table 4).

Discussion

Integrated information of whole genome sequence and genome wide transcription profiles along with public protein domain and annotation databases have made it possible to predict a substantial fraction of the regulatory complement and find transcription factors regulated during appressorium formation in *M. grisea*. Transcription factors were identified based on the existence of functional domains for transcription factor activity. In *M. grisea*, the largest group of transcription factors has the fungal specific TF N- terminal signature (IPR001138) which is also most prevalent in other filamentous fungi (Table 2). The relative occurrence of each type of transcription factors within a species was very similar across the filamentous fungi (Table 2). However, it seems that transcription factors are more highly enriched in *F. solani*. Further studies on the diversity and function of fungal transcription factors remain to be done.

The majority of transcription factors showed no significant change in gene expression during appressorium development. Most changes were seen during spore germination as was observed for non regulatory genes (Chapter 2). Functional analysis of 42 transcription factors which were differentially expressed during appressorium related profiles suggested increased transcriptional regulation for nutrient scavenging, morphogenesis and plant infection. MSTU1 in *M. grisea* and its ortholog GcSTUA in *Glomerella cingulata* were recently reported as pathogenecity factors. Deletion mutants failed to generate sufficient appressorium turgor pressure to penetrate the intact host plant surface [7, 33]. MSTU1 and GcSTUA both contains the APSES DNA-binding domain (IPR003163) which is found in several fungal proteins involved in cell development such as the cell pattern formation-associated protein STUA in *A. nidulans,* the mycelial growth

factor-1 MGF1 in *Yarrowia lipolytica* and the cell-cycle box factor, chain SWI4 in *Saccharomyces cerevisiae*. The significant increase gene expression of MSTU1 and its role in functional appressorium formation supports our original hypothesis that differential gene expression of transcription factors regulates development and pathogenecity in *M. grisea*

Esterases such as cutinase degrade cutin, whereas others are involved in fatty acid degradation. Cutinase production in *F. solani* and fatty acid utilization in *A. nidulans* were regulated by the related transcription factors CTF1α and CTF1β, and FarA and FarB respectively. Thus, we expected their orthologs MGG_08199.5 and MGG_01836.5 to function as central regulators for lipid metabolism and cutinase synthesis in *M. grisea*. However, MGG_08199.5 and MGG_01836.5 deletion mutants did not exhibit phenotypic differences in fatty acid utilization, appressorium formation and pathogenecity on barley seedlings. This may suggest the existence of functional back up system for these transcription factors. In future work, it may be valuable to evaluate the double mutants. Hynes identified a highly enriched CCTCGG motif in the 5' upstream region of genes involved in fatty acid metabolism, especially in fungal species which contains the FarA ortholog. However, in *M. grisea*, the occurrence of this sequence is not restricted to the promoter region of certain functional group of genes. Rather, it seems to be a common motif existing in more than 25% of 5' upstream region. Thus, transcription factors which selectively bind to this motif such as MGG_08199.5 and MGG_01836.5 may be involved in more specific biological metabolism when they work together with other gene specific regulatory proteins.

Currently, with the exception of melanin biosynthesis, little is known regulating

secondary metabolism in *M. grisea*. Functional annotation of differential expressed transcription factors led to identification of a putative gene cluster for toxin biosynthesis in *M. grisea*. Other analyses such as over expression studies of these differentially expressed transcription factors would broaden the understanding of regulatory processes for fungal development. Likewise, an integrated analysis of gene expression profiles and genome wide transcriptional factor binding studies would help reveal the transcriptional regulation network during *M. grisea* appressorium morphogenesis.

Materials and Methods

Prediction of *M. grisea* transcription factors First, we identified Interpro domains mapped to GO:0003700 (Transcription factor activity), GO:0003702 (RNA polymerase II transcription factor activity), GO:0016563 (Transcriptional activator activity), and GO:0016563 (Transcriptional repressor activity). These Interpro domains were combined with the set obtained from a key word search for "transcription factor", "DNA binding" and "regulatory protein" in the Interpro database.

The predicted protein sequences of *M. grisea* were then analyzed with InterProScan program (Version 10.1) [14], where the individual sequences were scanned against the protein signatures of the InterPro member databases including UniProt, PROSITE, Pfam, PRINTS, SUPERFAMILY, ProDom and SMART. The results were then filtered using the set of Interpro domains we identified as being associated with transcription factors. Finally, the results were manually inspected and the domains not suitable for transcriptional regulation were eliminated.

Gene expression profiles of *M. grisea* transcription factors. Gene expression of *M. grisea* transcription factors were extracted from the genome wide expression profiles during spore germination, appressorium initiation and maturation, and appressorium induction by exogenous cyclic AMP (described in chapter 2). Genes were designated as differentially expressed if their expression ratios were equal or greater than 2 fold with $P<0.05$ (Student's t-test).

CCTCGG motif finding. To find the putative transcription factor binding motif CCTCGG in the promoter region of *M. grisea* genes, we extracted 1 kilobase (kb) 5' upstream sequences of *M. grisea* genes (a sequence file is available in from *M. grisea* genome database, http://www.broad.mit.edu/annotation/genome/magnaporthe_grisea). Occurrence and frequencies of the motif were obtained using Perl scripts.

MGG_01836.5 and MGG_08199.5 functional characterization. Genes were functionally characterized by targeted gene deletion. The procedure utilized for targeted gene deletion of MGG_01836.5 and MGG_08911.5 was similar to that previously described in Chapter 2. Briefly, disruption cassettes were constructed by combining and amplifying three individually amplified fragments of upstream and downstream sequence of MGG_01836.5, MGG_08199.5, and hygromycin resistance gene together to construct a 3.3 kb fragment containing the hygromycin cassette for gene replacement. This construct was transformed into 70-15 *M. grisea* protoplasts as described [15]. Mutants were screened by PCR. Primers used for the amplification of each flanking region as well as hygromycin genes are listed in Table 1. Phenotype assays of mutants for vegetative growth, sporulation, appressorium formation and pathogenecity were conducted as described in Chapter 2. To test the utilization of fatty acids, mutants were incubated on minimal medium containing glycerol (1%), oleate (1%), tween 80 (0.1%) and glucose (1%) as a sole carbon source for one week.

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Table 1. Primer sequences for gene specific replacement cassette.

Gene	Primer	Sequence(5' - 3')
MGG_01836.5	F	CCGTCATCAACCGCACACATTTCA
MGG_01836.5	R	TGCGAGCAGTATGGCTTGGAGTAA
MGG_01836.5	FLF	CTGCTCCGTAAACAAGGCATGCAA
MGG_01836.5	FLR	CACGGCGCGCCTAGCAGCGG TTGCTTAGCCGTTTACTTGTGGCG
MGG_01836.5	FRF	GCAGGGATGCGGCCGCTGAC TCTGTCGCTAGGATTGCGAGGATT
MGG_01836.5	FRR	AACGAGGTTCCTGAAGGCGAGTTT
MGG_01836.5	FC	GTCCGGAACTGCAACAAAGCAAGT
MGG_01836.5	RC	AGCGGCAAGTGTTAGTACTGTCGT
MGG_08199.5	F	TTGACTTGAACAACCAGCCAAGCG
MGG_08199.5	R	TGTGCATTTGCAGCTGTCTCTGTG
MGG_08199.5	FLF	AAAGTGGTGAACCAATTCGCACGC
MGG_08199.5	FLR	CACGGCGCGCCTAGCAGCGGATCGCCAAGTATCCGTCTGTGTGT
MGG_08199.5	FRF	GCAGGGATGCGGCCGCTGAC TCTCTTTGCATATACCCACCGCCA
MGG_08199.5	FRR	TGACCAACTAATCTTGCCCGGGAT
MGG_08199.5	FC	TGCACACAAGGAAAGAGGAGGTGA
MGG_08199.5	RC	GTTCCAAACCGTCCAAATCGCAGT

Primer indicate as "F" forward intergenic, "R" reverse intergenic, "FLF" forward left flanking, "FLR" reverse left flanking, "FRF" forward right flanking, "FRR" reverse left flanking", "FC" nested forward and "RC" nested reverse.

Table2. Transcription factor domains in filamentous fungi.

InterPro ID	Description	Mg	Nc	Fg	Um
IPR001138	Fungal transcriptional regulatory protein, N-terminal	117[a](44)[b]	114	319	103
IPR007087	Zinc finger, C2H2-type	95 (14)	96	116	44
IPR001878	Zinc finger, CCHC-type	79	9	13	15
IPR007219	Fungal specific transcription factor	66 (37)	57	164	32
IPR001005	Myb, DNA-binding	33 (18)	39	47	25
IPR012287*	Homeodomain-related	26 (23)			
IPR000330	SNF2-related	24 (3)	25	28	17
IPR004827	Basic-leucine zipper (bZIP) transcription factor	22 (17)	24	32	14
IPR008030	NmrA-like	19	2	3	
IPR007124	Histone-fold/TFIID-TAF/NF-Y	18 (9)	17	19	17
IPR011616*	bZIP transcription factor, bZIP_1	13 (13)			
IPR001789	Response regulator receiver	13 (1)	14	20	11
IPR000910	HMG1/2 (high mobility group) box	11	10	10	8
IPR000679	Zinc finger, GATA-type	10	6	8	11
IPR000571	Zinc finger, CCCH-type	10	10	14	14
IPR001092	Basic helix-loop-helix Dimerization region bHLH	9	15	16	13
IPR001356	Homeobox	8 (7)	9	14	10
IPR002197	Helix-turn-helix, Fis-type	8	14	8	5
IPR002409	Aflatoxin biosynthesis regulatory protein	7 (7)	4	20	1
IPR001594	Zinc finger, DHHC-type	6	5	5	7
IPR003604	Zinc finger, U1-type	6 (6)	7	10	8
IPR003958	Transcription factor CBF/NF-Y/archaeal histone	5 (5)	5	5	6
IPR001025	Bromo adjacent region	5	3	3	2
IPR001222	Transcription factor TFIIS	4 (1)	3	2	4
IPR001606	AT-rich interaction region	4 (1)	4	3	3
IPR011700[c]	Basic leucine zipper	4 (3)			
IPR002078	Sigma-54 factor, interaction region	4	6	4	6
IPR000005	Helix-turn-helix, AraC type	3	6	11	5
IPR000232	Heat shock factor (HSF)-type, DNA-binding	3 (3)	3	3	4
IPR003163	DNA-binding, yeast	3	5	5	5
IPR004181	Zinc finger, MIZ-type	3 (1)	1	1	1
IPR002341	HSF/ETS, DNA-binding	3 (3)	2	3	4
IPR001766	Fork head transcription factor	3	4	4	5

[a] The number of proteins containing the transcription factor domain in *M. grisea* (Mg), *N. crassa* (Nc), *F. graminearum* (Fg) and *U. maydis* (Um).
[b] The number of proteins that match to multiple transcription factor domains.
[c] The domain not included in InterproScan program version 7.1.

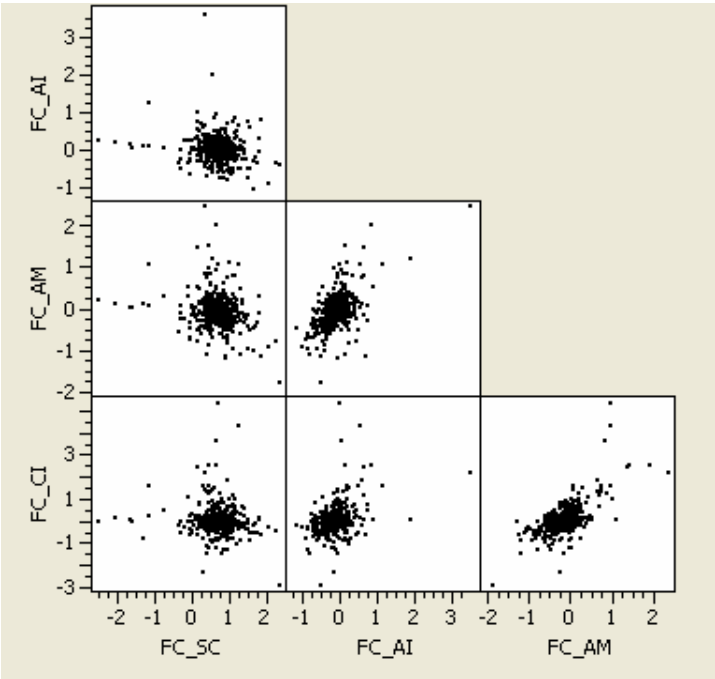


Figure 1. Gene expression of *M. grisea* transcription factors. Expression profiles of spore germination (SC), appressorium induction (AI), appressorium maturation (AM) and cyclic AMP mediated appressorium induction (CI) was compared pairwise and fold change (FC) plotted using a Log2 scale.

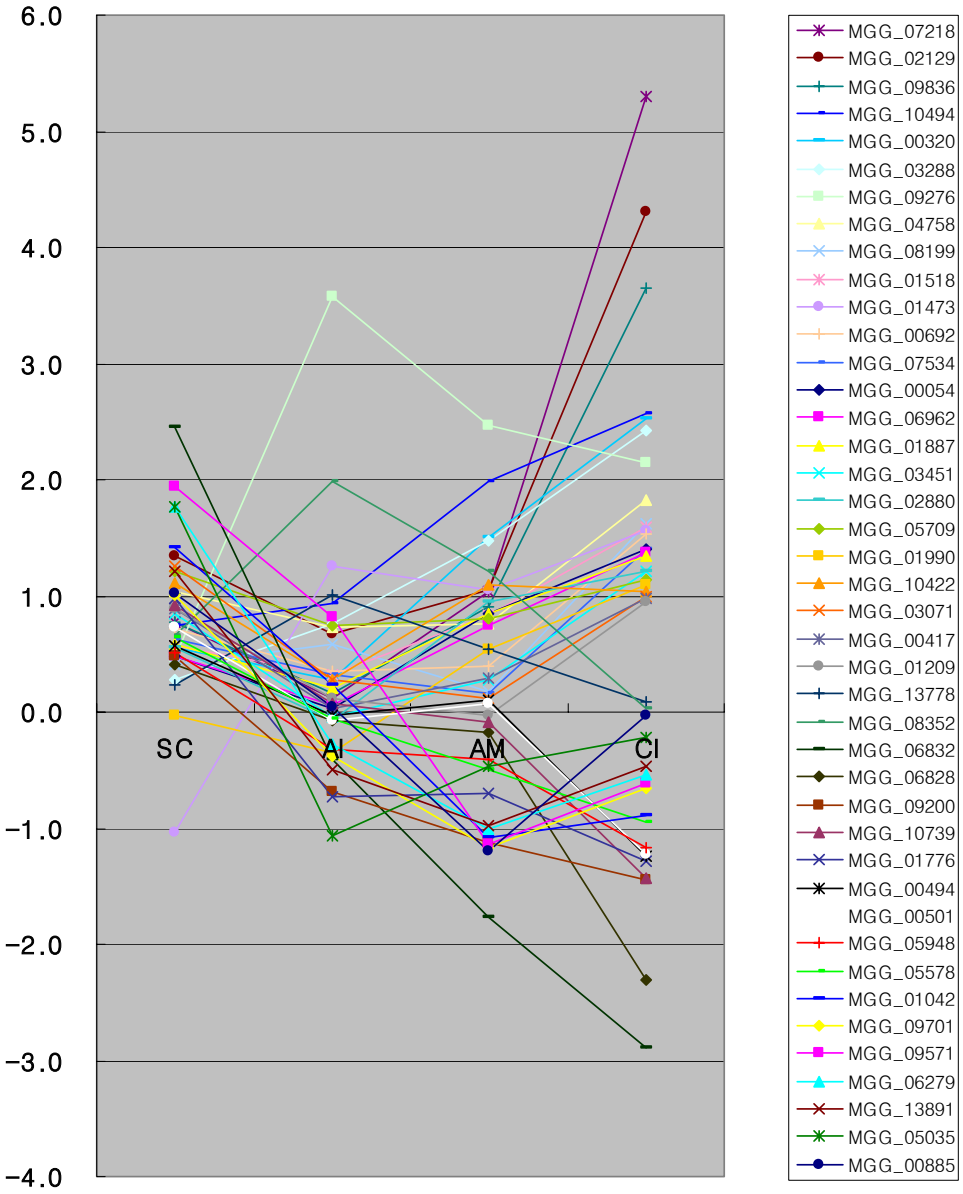


Figure 2. Gene expression of the developmentally regulated transcription factors during appressorium morphogenesis. The X axis indicates expression profiles of spore germination (SC), appressorium induction (AI), appressorium maturation (AM) and cyclic AMP mediated appressorium induction (CI). The Y axis indicates gene expression in Log2 fold change.

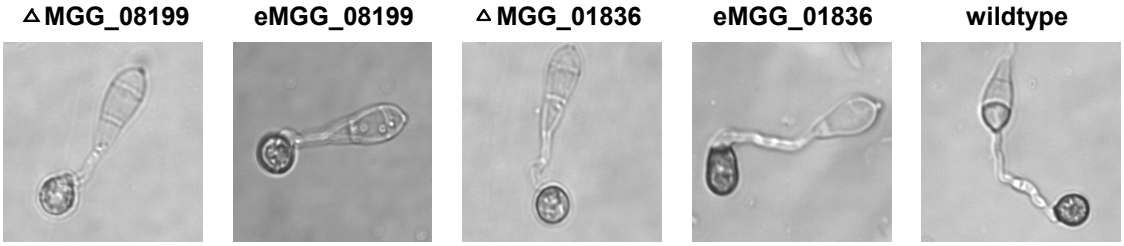
Table. 3 List of the differentially expressed transcription factors during appressorium morphogenesis.

Gene ID	InterPro ID	Putative identity	E-value[a]
MGG_07218	IPR001138, IPR002409	transcription factor [Colletotrichum lagenarium]	1.E-120
MGG_02129	IPR001138	alcR protein [Aspergillus nidulans]	3.E-73
MGG_09836	IPR008030	NAD dependent epimerase, NmrA family [Myxococcus xanthus]	6.E-07
MGG_10494	IPR001005	Beta-1,4-mannosyltransferase Alg1 [Aspergillus fumigatus]	e-115
MGG_00320	IPR001138	C6 transcription factor [Aspergillus fumigatus]	2.E-05
MGG_03288	IPR004827	bZIP transcription factor LziP [Aspergillus fumigatus]	2.E-54
MGG_09276	IPR001138	cercosporin resistance protein [Cercospora nicotianae]	4.E-31
MGG_04758	IPR004827	bZIP transcription factor [Aspergillus fumigatus]	4.E-53
MGG_08199	IPR001138, IPR007219	CTF1B, cutinase transcription factor [Gibberella zeae]	0.E+00
MGG_01518	IPR001138, IPR007219	Nit-4, nitrogen assimilation transscription factor [Neurospora crassa]	0.E+00
MGG_01473	IPR002197	oxidoreductase, 2-nitropropane dioxygenase family [Aspergillus fumigatus]	1.E-142
MGG_00692	IPR003163	Mstu1, pathogenicity related transcriptional factor [Magnaporthe grisea]	0.E+00
MGG_07534	IPR001138	C6 transcription factor [Aspergillus fumigatus]	1.E-03
MGG_00054	IPR001005	MFS transporter [Aspergillus fumigatus]	1.E-92
MGG_06962	IPR002078	small GTP-binding protein YPTI [Hypocrea jecorina]	2.E-98
MGG_01887	IPR001138	ARCA protein [Emericella nidulans]	1.E-67
MGG_03451	IPR007087	C2H2 transcription factor [Aspergillus fumigatus]	1.E-39
MGG_02880	IPR007219	transcription activator [Aspergillus oryzae]	5.E-48
MGG_05709	IPR001092	GLCD beta [Aspergillus nidulans]	6.E-20
MGG_01990	IPR004827, IPR011616	b-ZIP transcription factor IDI-4 [Podospora anserina]	4.E-15
MGG_10422	IPR001138	C6 zinc cluster transcription factor [Aspergillus fumigatus]	2.E-57
MGG_03071	IPR008831	mediator complex subunit SOH1 [Aspergillus fumigatus]	2.E-17
MGG_00417	IPR001138, IPR002409	Zinc finger transcription factor [Cercospora nicotianae]	7.E-23
MGG_01209	IPR007087	Zinc finger protein SFP1 [Saccharomyces cerevisiae]	2.E-33
MGG_13778	IPR001878	Zinc knuckle nucleic acid binding protein [Aspergillus fumigatus]	2.E-54
MGG_08352	IPR008030	NmrA-like [Medicago truncatula]	5.E-15
MGG_06832	IPR001138	C6 transcription factor [Aspergillus fumigatus]	3.E-04
MGG_06828	IPR001005	MFS transporter [Aspergillus fumigatus]	2.E-67
MGG_09200	IPR007087	Zinc finger protein [Ascobolus immersus]	3.E-65
MGG_10739	IPR002197	arsenate reductase Arc2 [Aspergillus fumigatus]	3.E-21
MGG_01776	IPR007087	C2H2 zinc finger protein Zas1A [Schizosaccharomyces pombe]	2.E-27
MGG_00494	IPR001138	PRO1 protein [Neurospora crassa]	0.E+00
MGG_00501	IPR007087	stress response element binding protein [Trichoderma atroviride]	5.E-94
MGG_05948	IPR001878	universal minicircle sequence binding protein (UMSBP) [Leishmania major]	8.E-38
MGG_05578	IPR001138	C6 transcription factor [Aspergillus fumigatus]	2.E-18
MGG_01042	IPR004181	related to DNA repair protein MMS21 [Neurospora crassa]	1.E-31
MGG_09701	IPR007087	Zinc finger transcription factor E2S-VP64 [synthetic construct]	2.E-06
MGG_09571	IPR004826	kinesin K39, putative [Leishmania major]	1.E-14
MGG_06279	IPR001138	citrinin biosynthesis transcriptional activator CtnR [Monascus purpureus]	2.E-15
MGG_13891	IPR001789	putative histidine kinase HHK13p [Gibberella moniliformis]	0.E+00
MGG_05035	IPR004827	hypothetical protein cgd2_3530 [Cryptosporidium parvum Iowa II]	1.E-02
MGG_00885	IPR003228	transcription initiation factor TFIID subunit 12 [Aspergillus fumigatus]	3.E-42

[a] E-values were taken from BLASTX hits to a homolog

Figure 3. Highly conserved fungal specific regulatory domains in MGG_08199.5 and MGG_01836.5. Red box = The fungal transcriptional regulatory protein, N-terminal (IPR001138), Blue box = The the fungal specific transcription factor (IPR007219).

A.



B.

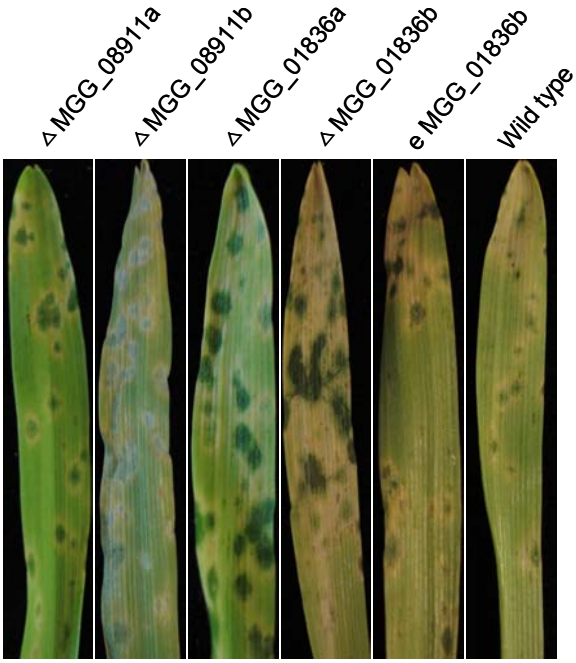


Figure 4. Appressorium formation (A) and pathogenicity assay (B) of MGG_08199.5 and MGG_01836.5 deletion mutants.

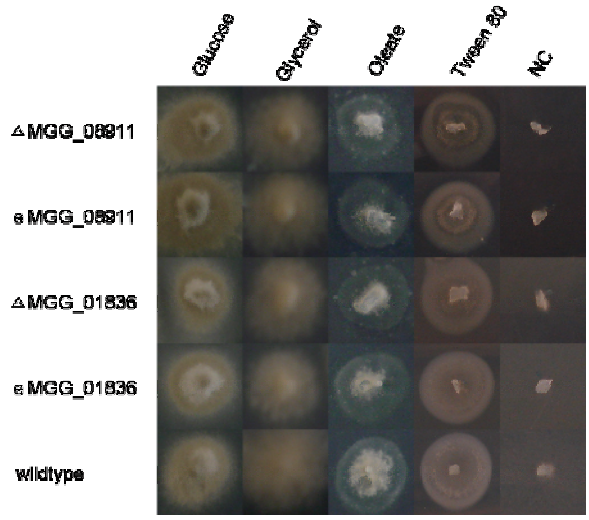


Figure 5. Growth of MGG_08199.5 and MGG_01836.5 deletion mutants on various carbon sources. Fungal cells were grown in minimal medium containing glucose (1%), glycerol (1%), oleate (1%) and tween 80 (0.1%) as a sole carbon source for one week. NC represents media lacking carbon source.

Table 4. GO annotation of *M. grisea* genes that contain CCTCGG motif(s) in the 5′ upstream.

GO	GO Description	MGG[a]
GO:0008152	metabolism	159
GO:0006118	electron transport	113
GO:0006810	transport	112
GO:0006355	regulation of transcription, DNA-dependent	65
GO:0005975	carbohydrate metabolism	54
GO:0006350	transcription	51
GO:0006508	proteolysis	42
GO:0015031	protein transport	31
GO:0006412	protein biosynthesis	29
GO:0006468	protein amino acid phosphorylation	29
GO:0008150	biological_process	28
GO:0006512	ubiquitin cycle	20
GO:0007165	signal transduction	18
GO:0007049	cell cycle	16
GO:0006464	protein modification	15
GO:0006629	lipid metabolism	15
GO:0008643	carbohydrate transport	15
GO:0006511	ubiquitin-dependent protein catabolism	14
GO:0006333	chromatin assembly or disassembly	13
GO:0015074	DNA integration	13
GO:0006278	RNA-dependent DNA replication	12
GO:0009058	biosynthesis	12
GO:0006457	protein folding	11
GO:0006397	mRNA processing	10
GO:0006886	intracellular protein transport	10
GO:0007046	ribosome biogenesis	10
GO:0044237	cellular metabolism	10
GO:0045449	regulation of transcription	10
GO:0051301	cell division	10
GO:0006520	amino acid metabolism	9
GO:0007264	small GTPase mediated signal transduction	9
GO:0006396	RNA processing	8
GO:0006633	Fatty acid biosynthesis	8
GO:0006725	aromatic compound metabolism	8
GO:0006865	amino acid transport	8
GO:0007242	intracellular signaling cascade	8
GO:0015986	ATP synthesis coupled proton transport	8
GO:0016071	mRNA metabolism	8
GO:0043037	translation	8

[a] The number of *M. grisea* genes assigned to the corresponding GO term.