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Global Approaches to Identify Genes Involved during Infection Structure Formation in Rice Blast Fungus, *Magnaporthe grisea*

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The ascomycete *Magnaporthe grisea* is a pathogen of rice blast and is known to form specialized infection structures called appressoria for successful infection into host cells. To understand the molecular mechanism underlying infection process, appressorium-related genes were identified through global approaches including EST sequencing, differential hybridization, and suppression subtractive hybridization. EST database was generated on >2,000 cDNA clones randomly selected from appressorium stage cDNA library. Large number of ESTs showed homology to known proteins possibly involved in infection-related cellular development (attachment, germination, appressorium formation, and colonization) of rice blast fungus. The 1051 ESTs showing significant homology to known genes were assigned to 11 functional categories. Differential hybridization and suppression subtractive hybridization were applied to identify genes showing an appressorium stage specific expression pattern. A number of genes were selected as up-regulated during appressorium formation compared with the vegetative growing stage. Clones from various cDNA libraries constructed in different developmental stages were arrayed on slide glass for further expression profiling study. Functional characterization of genes identified from these global approaches may lead to a better understanding of the infection process of this devastating plant disease, and the development of novel ways to protect host plant.

Keywords **:** appressorium, differential hybridization, EST, rice blast fungus, suppression subtractive hybridization.

Magnaporthe grisea, a filamentous ascomyceta fungus, causes rice blast (Ou 1985). Rice blast is characterized by the appearance of large eye-shaped necrotic lesions on rice leaves. *M. grisea* also infects the panicle and often results in complete yield loss. Rice blast starts when conidia of *M. grisea* land on rice leaf surfaces. After the conidium has attached on the surface, a germ tube emerges. In the presence of appropriate chemical and physical signals, the end of germ tube differentiates into a highly melanized dome-shaped infection structure called an appressorium (Dean 1997). A narrow penetration peg emerges from the appressorium at the plant interface, penetrates the rice cuticle, and grows into the tissues below (Howard and Valent, 1996). The accumulation of glycerol in the appressorium generates sufficient turgor pressure to drive the penetration peg through the rice cuticle (Balhadere and Talbot, 2001; Howard et al., 1991; de Jong et al., 1997). External signals such as hydrophobicity (Lee and Dean, 1993a; Lee and Dean, 1994), surface hardness (Xiao et al., 1994), and cutin monomers (Gilbert et al., 1996) are known to trigger appressorium development.

A number of genes involved in the induction and function of appressoria have been identified by mutant analysis (Balhadere et al., 1999; Chumley and Valent, 1990; DeZwaan et al., 1999; Shi et al., 1998; Sweigard et al., 1998; Talbot et al., 1993) and expression pattern analysis (Lee and Dean, 1993b; Kamakura et al., 1999; Kamakura et al., 2002). Genes involved in signaling pathways also have been isolated and shown to be involved in regulating appressorium formation (Choi and Dean, 1997; Dean, 1997; Fang and Dean, 2000; Liu and Dean, 1997; Mitchell and Dean, 1995; Xu and Hamer, 1996; Xu et al., 1998). However, knowledge of the molecular basis of conidial germination and appressorium formation remains superficial. Thus, a global or system-wide inspection of genes involved would be highly important (Yoder and Turgeon, 2001).

Single pass, partial sequencing of either 5' or 3' ends of complementary DNA (cDNA) clones to generate a set of expressed sequence tags (ESTs) represents a relatively inexpensive and rapid procedure for finding genes and

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generating information about their expression in organisms. Recently, the EST strategy has been applied to gene identification from a number of fungal plant pathogens (Keon et al., 2000; Soanes et al., 2002; Thomas et al., 2001). EST data for *Botytis cinerea* (www.genoscope.cns.fr), *Cladosporium fulvum* (www.ncbi.nlm.nih.gov/dbEST), and *M. grisea* (www.ncbi.nlm.nih.gov/dbEST) are available online. Initial assessment and analysis of expressed sequences from the *Phytophthora* species, which are oomycetaceous plant pathogens, have recently been published (Kamoun et al., 1999; Qutob et al., 2000). EST analysis was applied to identify host plant genes and fungal pathogen genes involved in host-pathogen interactions (Kim et al., 2001; Kruger et al., 2002; Rauyaree et al., 2001) Because of the lack of genome sequence data for plant pathogenic fungi in the public database, EST approach to gene discovery remains a valid tool in obtaining gene information. As the body of information increases, the value of comparative analysis of the genes involved during pathogenicity becomes more practical and realistic.

Identification of genes showing disease-related expression patterns is needed to accelerate the dissection of the disease process and for the development of rational ways to control the disease. The study of differences in gene-expression patterns is one of the most promising approaches for understanding mechanisms of differentiation and development. Differential cDNA screening and the more advanced technique of subtractive cDNA hybridization have a great potential to identify genes involved in pathogenicity. Differential expression of genes involved in the infection process has been described in phytopathogenic fungi. A number of genes expressed in germinated spores have been isolated from several obligate pathogens: a rust fungus, *Puccinia graminis* (Liu et al., 1993); a powdery mildew fungus, *Erysiphe graminis* (Justesen et al., 1996); and a downy mildew fungus, *Bremia lactucae* (Judelson and Michelmore, 1990). Genes expressed during the formation of an infection structure have been isolated in *Uromyces appendiculatus* (Bhairi et al., 1989; Xuei et al., 1992). The function of these genes remains to be further studied. The *CAP20* gene of *Colletotrichum gloeosporioides* was found to be expressed preferentially during appressorium formation. Gene disruption analysis revealed that *CAP20* was essential for fungus pathogenicity (Hwang et al., 1995). In *Ustilago fabae*, synthesis and secretion of host cell-wall-degrading enzymes and chitin deacetylase were found to be dependent on infection structure development (Deising et al., 1995). A number of *in planta*-induced genes have been isolated from *Cladosporium fulvum* (Wubben et al., 1994), *Phytophthora infestans* (Pieterse et al., 1991, 1993), and *Uromyces fabae* (Hahn and Mendgen, 1997; Hahn et al., 1997). The corn smut gene *mig1* from *Ustilago maydis* was identified and characterized. This gene was highly stimulated after fungal penetration of maize tissue (Basse et al., 2000).

In addition to differential hybridization method, subtractive hybridization technology has been known as a powerful tool in isolating novel genes and in understanding molecular mechanism (Gold et al., 2001). Suppression subtractive hybridization greatly reduces the complexity of the original technique (Daitchenko et al., 1996) and has been developed as a kit by Clontech (Palo Alto, California). Suppression subtractive hybridization has been applied to clone potato genes involved in the early stage of hypersensitive response to *Phytophthora infestans* (Birch et al., 1999), using as tester mRNA purified from infected leaves of a resistant cultivar undergoing the hypersensitive reaction, and as driver mRNA from infected leaves of a susceptible cultivar. Suppression subtractive hybridization technology is currently being employed in several studies on plant pathogenic fungi, and Xiong et al. (2001) recently reported defense-related rice genes identified through two different cloning strategies, differential screening and suppression subtractive hybridization.

In this paper, global approaches to discover genes involved during appressorium formation of rice blast fungus have been described. These systemic ways provide a global view of gene activities during infection structure development and allow the identification of novel genes likely involved in appressorium formation.

Appressorium Stage EST Sequencing

A directional appressorium stage cDNA library was constructed using RNA isolated from germinated conidia on an appressorium-inductive surface. Single pass sequencings on 2,325 cDNA clones were determined using the dye terminator sequencing method. Raw nucleotide sequence was processed using the software Phred and Phrap (Ewing and Green, 1998; Gordon et al., 1998; http://www.phrap.org) for base calling, vector masking, and contig assembly. Contig assembly generated a set of 1,449 unique sequences from the full set of 2,325 ESTs. To identify the putative function of the cDNAs, each edited EST sequence was queried against the GenBank database using the BlastX algorithm through the web site of the National Center for Biotechnology Information (NCBI). Consequently, BlastX revealed that 1051 ESTs (45%) matched significantly ($E < 10^{-5}$) to known protein sequences in the GenBank database. The most redundant ESTs showed homology to ribosomal proteins with a frequency of 4.6%, followed by UVI-1, MAS3 protein, elongation factor, and 14-3-3 protein. Ribosomal proteins and elongation factor are thought to be house-keeping proteins and are expressed abundantly. A unique set of EST clones with significant homologs was

grouped according to their putative function. For the redundant ESTs, the clone with the most significant match was selected for listing (www.fungalgenomics.ncsu.edu). Clones showing homology to ribosomal protein and hypothetical protein are not included.

1051 ESTs with significant matches to known protein sequences were categorized into 11 groups by their putative function. A functional classification scheme was devised based on the Expressed Gene Anatomy Database (EGAD) (White and Kerlavage, 1996). The pathogenesis category was additionally applied to emphasize the genes directly related to features of fungal plant pathogens. All major functional categories were represented by at least 11 ESTs. The majority of the identified ESTs were related to metabolism, protein synthesis, and cell division and growth. This implies that the appressorium formation stage is very active in metabolism and protein synthesis, which was not unexpected. A large portion of ESTs was placed into the cell signaling, cell growth, and division. This suggests that high degree of interaction for surface recognition, along with the active morphological development, is a prerequisite during the early stages of development leading to infection. The least frequent group of ESTs was that involved in cell defense. 185 ESTs were grouped in a hypothetical category because the function of each protein has not been characterized. A total of 105 ESTs with functions that could not be fit easily into any category were assigned to the unclassified category.

ESTs during the Early Stage of Infection Process

The advantage of analysis of ESTs derived from certain interesting developmental stages is that the putative function of each gene expressed with the biochemical processes occurring in the cell at a certain step during the development can be connected. Hence, it is possible to discuss the putative functions of genes identified through analysis of ESTs with specific processes that occur during appressorium formation.

As physical stimuli, hydrophobicity and hard surface were suggested to be a critical parameter in germination and appressorium formation of *M. grisea* (Lee and Dean, 1994; Xiao et al., 1994). This study identified ESTs of *MPG1* encoding a hydrophobin-like protein, which is required for full pathogenicity and appressorium formation (Talbot et al., 1996). Hydrophobin protein is highly redundant in this EST dataset. Also, calmodulin is induced by hard surface contact in *M. grisea*, and inhibition of calmodulin prevents conidial germination (Liu and Kolattukudy, 1999). Also identified were a calmodulin gene (6B04), calmodulin dependent kinase (1P07), and calmodulin dependent protein phosphatase (3G14) in the current EST dataset. This calcium signaling pathway may regulate germ tube emergence and appressorium formation in *M. grisea*.

Ubiquitin-dependent protein degradation is also required in germination and appressorial differentiation of *M. grisea* and other pathogenic fungi (Liu and Kolattukudy, 1998; McCafferty and Talbot, 1998). A number of genes encoding ubiquitin-like protein (3C13), ubiquitin conjugating enzyme (2K21), ubiquitin regulatory domain protein (5O19), ubiquitin biosynthesis methyltransferase (5L05), and ubiquitin ligase (6L05) were identified. *PUB4*, encoding poly-ubiquitin (McCafferty and Talbot, 1998), was expressed highly in response to environmental stress (UV, heat, or cold).

Another gene, *PTH11* encoding a novel transmembrane protein 2J21, was also identified (EST 2J21). *PTH11* is involved in host surface recognition and the *pth11* mutants were non-pathogenic due to a defect in appressorium differentiation (DeZwaan et al., 1999). A *PTH11* homolog also has been identified from EST analysis of *Blumeria graminis* during appressorium differentiation (Thomas et al., 2001) indicating that *PTH11* may have a similar role in both fungi for host recognition.

Although the cDNA library for this EST analysis was constructed from the appressorium developmental stage on an artificial surface, the expression of genes involved in cell wall degradation was detected. This study identified several EST clones encoding proteins that could cause degradation of the rice cuticle. Cutinase binding protein (3J01), cutinase transcription factor (2A03), and cuticle degradating serine proteinase (4H20) were identified. The importance of cuticular degradation in *M. grisea* during the infection process is not clear since appressorium-mediated mechanical penetration has been considered the main source of penetration in this fungus (Sweigard et al., 1992). Cutinase and other esterases in the matrix surrounding uredospores of the broad bean rust fungus, *Uromyces viciae-fabae*, were shown to be involved in attachment by forming an adhesion pad with the underlying plant cuticle (Deising et al., 1992).

Dynamic cell wall modification is supposed to happen during conidial germination and germling differentiation to appressoria in *M. grisea* (Hamer and Talbot, 1998). The identification of genes encoding beta-1,3-exoglucanse (4F22), glucan beta glucosidase (6F12), and chitinase (1D08, 2I21) may suggest that the turnover of major cell wall components is important for proper morphogenetic changes.

Germination and appressorium formation also require the formation of new cell membrane and cell walls. Genes encoding cell membrane protein (2I24), ergosterol biosynthesis protein (5A04), cell wall protein (5C21), cell wall biogenesis protein (2F03), and chitin synthase (5I16) were identified. In *U. maydis*, mutants lacking chitin synthase displayed significant reduction in growth rate, chitin content, and chitin synthase activity, especially in the mycelium form.

Virulence to corn also was reduced in these mutants (Xoconostle-Cazares et al., 1997).

A number of genes in the signal transduction pathway were also identified. The involvement of cAMP signaling in development and pathogenicity of *M. grisea* has been previously reported (Lee and Dean, 1993; Mitchell and Dean, 1995; Choi and Dean, 1997). This study identified EST (5N07) of *rpkA* encoding cAMP-dependent protein kinase regulatory subunit gene, which is known to be involved in the cAMP-dependent signal pathway associated with appressorium formation and function (Gilbert and Dean, unpublished, GenBank AF015753). The involvement of MAP kinase pathway has been well examined in *M. grisea* (Xu, 2001) and a number of MAP kinase genes were identified (Xu and Hamer, 1996; Xu et al., 1998; Dixon et al., 1999). EST (1M18) of *OSM1* encoding osmotic sensitivity MAP kinase was identified. This gene was found to regulate the response to hyperosmotic stress, but was dispensable for appressorial development in *M. grisea* (Thines et al., 2000).

As another signal transduction pathway, calcium/calmodulin signaling also appears to be involved in germ tube emergence and appressorium formation in *M. grisea* (Lee and Lee, 1998; Liu and Kolattukudy, 1999). ESTs (1P07, 5D06) in the dataset matched the calcium/calmodulin-dependent protein kinase. EST (5I17) encoding serine/threonine protein phosphatases were also detected in the EST data set. These proteins are important mediators of fungal proliferation and development, as well as signal transduction and infection-related morphogenesis in *M. grisea* (Dickman and Yarden, 1999).

In the current appressorium stage EST dataset, there are genes encoding proteins such as glycerol-3-phosphate dehydrogenase (2A10), glucose transporters (5O03), membrane ATPase (1K11), and glycogen phosphorylase (5F16). These genes may play an important function for generating turgor pressure for functional appressoria. The gene encoding glycerol-3-phosphate dehydrogenase is involved in glycerol biosynthesis. The role of glycerol in turgor generation during appressorium formation was discussed by de Jong et al. (1997) and Money (1997). Glucose transporters may act as glucose sensors to initiate shifts in intracellular glucose levels and may regulate turgor pressure generation along with membrane ATPase (Money 1997). Recently, the role of CPKA-dependent degradation by glycogen phosphorylase and amyloglycosidase, and PMK1 dependent mobilization of triacylglycerol during appressorium formation was suggested to be a key process in turgor generation (Thines et al., 2000; Weber et al., 2001).

A requirement of melanization during infection process has been examined in a number of plant pathogenic fungi like *M. grisea* and *Colletotrichum* species (Bell and Wheeler, 1986). In *M. grisea*, the turgor pressure inside appressorium was maintained by a specialized cell wall layer containing melanin (Howard and Ferrari, 1989; de Jong et al., 1997). Tetrahydroxynaphthalene reductase (THNR) and scytalone dehydratase are involved in melanin synthesis and are required for normal appressorium function (Vidal-Cros et al., 1994; Motayama et al., 1998). This study identified a THNR gene (2N15) which is abundantly detected (7 of 1051significant matches) in this EST dataset.

Also identified in this study were a number of genes supposed to be involved in penetration and colonization in plant cells. EST (4O10) encoding PTH2 was detected and this gene was identified through random insertional mutagenesis and shown to be involved in pathogeniciy (Sweigard et al., 1998). *PTH2* encodes carnitine acetyltransferase and this enzyme is required to allow activated fatty acids to traverse the mitochondrial membrane for oxidation. This gene was suggested to be necessary for *M. grisea* to colonize within plant cells (Sweigard et al., 1998).

Plants develop defense mechanisms to recognize pathogens and protect them from their attack. Production of reactive oxygen is often associated with disease resistance of plants (Nurnberger and Scheel, 2001). In the rice-rice blast interaction, reactive oxygen production caused hypersensitive reaction (HR) and greatly reduced disease lesions against a virulent race of the rice blast fungus (Higa et al., 2001; Ono et al., 2001). For successful penetration and colonization in host cell by pathogens, the pathogens must overcome the toxic effects of reactive oxygen from plant. A number of genes encoding superoxide dismutase (3F11), catalase/peroxidase (4P01), and multicopper oxidase (6G21) were identified and these types of enzymes are known to detoxify reactive oxygen.

In addition to the breakdown of toxic reactive oxygens and other antifungal components produced by the plant, fungal pathogens may avoid toxins by actively exporting them from the cell. The identification of EST encoding multidrug transporter (6N06) may reflect the potential active efflux of toxic compounds of rice during infection process. However the gene *ABC1*, encoding the previously characterized membrane transporter (Urban et al., 1999), was not detected in this dataset.

Infection structure specific protein, encoded by *mif23*, was found to be expressed highly during appressorium formation (Lee and Dean, 1993b), and 3 ESTs (2D06, 3F08, 4M19) of *mif23* were recognized in this study. The function of this gene during appressorial differentiation remains to be further characterized.

The identification of ESTs showing homology to the genes related to pathogenicity in other plant pathogenic fungi is extremely interesting since these genes may have similar functions in the rice/rice blast pathosystem. For example, a homolog (4K05) to *TRI11* encoding isotricho-

dermin C-15 hydroxylase, a member of cytochrome P-450 superfamily of *Fusarium sporotrichioides*, was identified. Disruption of *TRI11* results in an altered trichothecene production, which is characterized by the accumulation of isotrichodermin – a trichothecene pathway intermediate (Alexander et al., 1998). In other *Fusarium* species, total trichothecene toxin-producing capacity of the isolates is considered to be a decisive component of pathogenicity (Mesterhazy et al., 1999). Although there is no report on the production of trichothecene in *M. grisea*, the detection of trichothecene homolog in EST dataset during appressorium formation may suggest a possible role of this gene on pathogenicity of *M. grisea*.

Another interesting homolog (5E15) identified was *PKS1* encoding polyketide synthase in *Cochliobolus heterostrophus*. *PKS1* is involved in production of the virulence factor T-toxin. When *PKS1* in race T was inactivated by targeted gene disruption, T-toxin production and high virulence were eliminated, indicating that *PKS1* is required for fungal virulence (Yang et al., 1996).

EST data in this study revealed (5A11, 6A11) showing homology against *CAP20* of *C. gloeosporioides*. This gene was shown to be involved in appressorium formation and pathogenicity (Hwang et al., 1995). Another EST (6L03) showed homology to a two-component histidine kinase of *Glomerella cingulata*. In yeast, two-component histidine kinase regulates the osmosensing MAP kinase cascade (Maeda et al., 1994).

Limitation of EST Analysis

The EST approach to gene expression analysis has several limitations. Since the source of the cDNA library depends on the type of cells or developmental stages, not all genes can be covered by a limited number of libraries. Another potential problem is redundant sequencing. In a given cell, the majority of genes are expressed at lower levels and they constitute only a small portion of the total transcripts, whereas, a small number of highly expressed genes constitute a large portion of the total transcripts (Cohen, 1997). Therefore, direct screening of standard cDNA libraries will primarily identify highly expressed genes (Adams et al., 1992). This problem can be solved by constructing normalized cDNA libraries to reduce the high-abundance copies and to increase the representation of the low-abundance copies. A normalized appressorium stage cDNA library is being constructed for *M. grisea* (Junseop Jeong, personal communication).

Differential Hybridization

The cDNA clones from the appressorium stage were arrayed in a high density format on Hybond N+ nylon membranes. A total of 18,432 clones are double-spotted on a single membrane. The combination of patterns and location of squares and fields on the nylon filter membrane provide the means to identify particular cDNA clones. A double spot pattern is used to eliminate false signals following hybridization. After spotting, clones were allowed to grow on the membrane laid on LB agar containing ampicillin (100 mg/L) at 37°C for 20 hours and fixed to the nylon membrane. Detailed information is available at the following web site (www.genome.clemson.edu). Appressoria cDNA library membranes were used for differential hybridization by comparing the hybridization signal profile between appressorium stage and vegetative stage of fungus development. Total RNAs from both stages were used for cDNA synthesis and probes were prepared from each cDNA population. All hybridizations were carried out in a standard hybridization solution at 65°C. Visual comparison of the signal intensity of individual clones to the two different probes lead to identification of differentially expressed genes. The cDNA clones showing differential signal to cDNA probe of appressorium stage were selected as appressorium stage up-regulated and were further analyzed by sequencing and Northern analysis.

A total of 621 clones were identified as showing appressorium stage up-regulated expression from differential hybridization. The 5' end sequences of all the selected clones were obtained using the T3 primer. Raw sequence data were processed in Phred and Phrap software, masked for vector sequence, and assembled into contigs. Fifty-five contigs were assembled from 621 clones identified from the differential hybridization analysis. The consensus sequence of these contigs was queried by BlastX for similarity search at the amino acid level. Out of the 55 contigs, 42 showed significant ($E < 10^{-5}$) homology to known proteins. The most redundant contig (*MAS1*) containing 77 clones showed moderate homology ($E = 10^{-19}$) to gEgh16, a protein identified from germinating conidia of *E. graminis*. Interestingly, another sequence contig (*MAS3*) containing 53 clones showed high homology to gEgh16. Comparison of these two sequence contigs revealed that they shared about 40% identity in nucleotide sequence. Three known genes of *M. grisea* were also identified: tetrahydroxynaphthalene reductase (15 clones, Vidal-Cros et al., 1994), hydrophobin *MPG1* (14 clones, Talbot et al., 1993), and transcriptional regulator *CON7* (2 clones, GenBank AF015771). In addition, there were homologs to CAP20, a protein known to be uniquely expressed during appressorium formation in *C. gloeosporioides* (Hwang et al., 1995) and snodprot1, a protein isolated from *Septoria nodorum* during infection of wheat (GenBank 074238).

Suppression Subtractive Hybridization

A subtracted cDNA probe was created using cDNA from the appressorium stage (tester) and cDNA from the vegetative stage (driver) using a cDNA subtraction kit (Clontech, USA). The subtracted cDNA was labeled and used to screen the appressorium stage cDNA library membrane by hybridization. cDNA clones showing clear hybridization signals to the subtracted probe were selected for sequencing and Northern analysis. A total of 133 cDNA clones were identified as showing appressorium stage up-regulated expression from suppression subtractive hybridization analysis. Based on the BlastX analysis of 133 clones, 66 clones showed significant matches with known proteins. Forty-two clones out of 66 matched with MAS1 and were further analyzed.

Functional Characterization of Genes Identified

Identification of fungal genes involved in infection process is one of the most critical steps leading to the understanding of fungal infection mechanisms in plant-pathogen interaction. In this study, different strategies were applied to identify genes involved during appressorium formation-related early development in *M. grisea.* With an aid of global approaches, several genes possibly involved during early infection process were identified for further analysis. Two of them, named MAS1 and MAS3, were mainly studied for their function and their role as a new virulence factor was reported recently (Xue et al., 2002). Other genes including CAP20 homolog, *Magnaporthe* pisatin demethylase, and endochitinase etc. are under intensive characterization. The cDNA clones used in this study are also applied to microarray analysis and being used for monitoring their expression pattern. Recently, whole genome sequencing of *M. grisea* was completed and made accessible through the website www://genome.wi.mit.edu/annotation/fungi/magnaporthe/. As a post-genome approach, functional genomics is considered as a next major stream in *Magnaporthe* genomics. A massive characterization of function of *Magnaporthe* genes is being achieved in high throughput way using *Agrobacterium* mediated transformed mutant lines (Lee Y-H personal communication). These integrated efforts in *Magnaporthe* research community will accelerate means to find a novel method to manage one of the major fungal pathogen.

Data Access

All sequences are deposited at the GenBank (accession number: AI068363 to AI069420). Access to the sequence data is also available at NCSU (www.fungalgenomics.ncsu.edu).

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