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Technological Advancement

Conidial germination in the filamentous fungus *Fusarium graminearum*

Kye-Yong Seong [a], Xinhua Zhao [b], Jin-Rong Xu [b], Ulrich Güldener [c], H. Corby Kistler [a,d,*]

[a] *Department of Plant Pathology, University of Minnesota, St. Paul, MN 55108, USA*
[b] *Department of Botany and Plant Pathology, Purdue University, West Lafayette, IN 47907, USA*
[c] *Institute for Bioinformatics, GSF National Research Center for Environment and Health, 85764 Neuherberg, Germany*
[d] *USDA ARS Cereal Disease Laboratory, University of Minnesota, 1551 Lindig Street, St. Paul, MN 55108, USA*

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Abstract

The ascomycetous fungus *Fusarium graminearum* is an important plant pathogen causing Fusarium head blight disease of wheat and barley. To understand early developmental stages of this organism, we followed the germination of macroconidia microscopically to understand the timing of key events. These events, recorded after suspension of spores in liquid germination medium, included spore swelling at 2 h, germination tube emergence and elongation from conidia at 8 h and hyphal branching at 24 h. To understand changes in gene expression during these developmental changes, RNA was isolated from spores and used to interrogate the *F. graminearum* Affymetrix GeneChip. RNAs corresponding to 5813 genes were detected in fresh spores and 5146, 5249 and 5993, respectively, in spores incubated in germination medium after 2, 8 or 24 h ($P < 0.001$). Gene expression data were used to predict the cellular and physiological state of each developmental stage for known processes. Predictions were confirmed microscopically for several previously unreported developmental events such as manifestation of peroxisomes in fresh spores and nuclear division resulting in binuclear cells within macroconidia prior to spore germination. Knowledge of stage-specific gene expression and changes in gene expression levels between developmental stages are an important first step to understanding the molecular mechanisms responsible for spore germination and development.
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Keywords: *Gibberella zeae*; Transcriptome; Development

1. Introduction

Fusarium graminearum (O'Donnell et al., 2004) is an ascomycetous fungus that is the major cause of *Fusarium* head blight (FHB) on wheat and barley as well as stalk and ear rot disease on maize (Stack, 2003). This fungus not only causes crop yield and quality losses, but also contaminates grain with mycotoxins rendering it unfit for food or feed.

The fungus infects plants by way of asexual conidia or sexually derived ascospores. Spores are dispersed to host plants largely by wind or rain and often over long distances (Gilbert and Fernando, 2004). The spores deposited on or inside floral tissue germinate and initiate the infection cycle. *F. graminearum* does not form any special structure, such as an appressorium, for penetration of the plant surface and does not appear to penetrate directly through the epidermis. Rather, germinated spores develop hyphae on the exterior surfaces of florets and glumes, and gain access through stomata and other sites within the inflorescence that are susceptible to penetration (Bushnell et al., 2003).

In general, fungal spores are specialized reproductive structures that may be highly resistant to harsh environmental conditions ensuring fungal survival and be important for dispersing fungi to exploit new niches including susceptible plant tissue. Therefore, spore maturation and germination are essential developmental steps in the fungal life cycle and critical for plant colonization. Because of the critical role spores play in disease initiation, it is important

* Corresponding author. Address: USDA ARS Cereal Disease Laboratory, University of Minnesota, 1551 Lindig Street, St. Paul, MN 55108, USA. Fax: +1 651 649 5054.
E-mail address: hckist@umn.edu (H.C. Kistler).

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to study the molecular mechanisms of spore development and germination in order to gain an understanding of these processes that may be useful to develop strategies to control pathogenic fungi.

The molecular and genetic mechanisms of fungal spore germination have been reviewed (D'Enfert, 1997; Osherov and May, 2001) and studies on gene expression during spore germination and maturation also have been reported. Transcripts of select genes have been shown by northern hybridization to be present in fresh conidia of *Neurospora crassa* while their expression levels were reduced in dormant conidia (Sachs and Yanofsky, 1991). More comprehensive gene expression studies of spore germination also have been conducted for *N. crassa* (Kasuga et al., 2005) and *Ustilago maydis* (Zahiri et al., 2005) that demonstrated changes in expression of hundreds of genes over a germination time course. However, in each case, only a fraction of the genome was monitored for each fungus.

Fusarium graminearum is one of more than 40 fungi for which a complete genome sequence is now available (Galagan et al., 2005; Cuomo et al., 2007). With the genome sequence, it has been possible to design an oligonucleotide microarray that can be used to explore the transcriptome of the organism (Güldener et al., 2006b). The array contains multiple oligonucleotide probes representing all predicted genes derived from the whole genome shotgun assembly of *F. graminearum* (Cuomo et al., 2007). In order to understand molecular events during conidial maturation and germination, gene expression profiles were monitored in fresh and germinating conidia using the 18K feature *F. graminearum* Affymetrix GeneChip. Our goals were to (1) create a comprehensive data set for understanding gene expression during spore germination, (2) functionally validate expression profiles by using them to predict morphological, ultrastructural and physiological changes in developing conidia and (3) develop targets for further functional analysis. Inference from gene expression data has allowed us to discover novel processes previously undescribed in germinating *F. graminearum* macroconidia.

2. Materials and methods

2.1. Strains and culture conditions

F. graminearum strain NRRL 31084 (PH-1) was grown at 25 °C in liquid complete medium (CM) (Harris et al., 1994). Spores (macroconidia) were prepared as described previously (Seong et al., 2005). Spores grown in liquid medium for 5 days were harvested by centrifugation at 3500 rpm for 5 min and washed twice with sterile distilled water prior to RNA extraction. Spores were then immediately submerged in liquid nitrogen for RNA extraction. As spores at all time points were treated identically, differences in hybridization signal between treatments were assumed to be due to treatments and not to subsequent handling steps.

2.2. Light microscopy and histological visualization

For spore germination studies, PH-1 macroconidia were observed using an Olympus IX70 Inverted Microscope at 400× magnification. For lipid and glycogen detection, fresh PH-1 macroconidia were suspended in CM for 4 h with gentle shaking. Lipid droplets in germinating conidia were visualized by staining with a Nile Red solution consisting of 50 mM Tris/maleate buffer, pH 7.5, with 20 mg ml^{-1} polyvinylpyrrolidone and 2.5 μg ml^{-1} Nile Red Oxazone (Sigma) (Greenspan et al., 1985; Weber et al., 1999). The fluorescence of the lipid droplets was viewed with a Nikon E800 epifluorescence microscope (excitation at 490 nm). Glycogen was stained with a solution consisting of 60 mg ml^{-1} KI and 10 mg ml^{-1} I_2 in distilled water (Weber et al., 1998; Thines et al., 2000), and was visualized using bright-field optics.

Nuclear division in germinating macroconidia was observed by DAPI staining. Fresh conidia of PH-1 were suspended in CM for 0, 2, 4, and 8 h with gentle shaking. Conidia were collected by centrifugation, washed with PBS buffer (pH 7.2) and then resuspended in PBS containing 0.1% Triton X-100. Cells were then fixed with PBS paraformaldehyde (3.7% w/v) and stained with 10 μg ml^{-1} 4′6-diamidino-2-phenylindole (DAPI, Sigma). The cell nuclei were observed using a Nikon E800 epifluorescence microscope at UV excitation wavelength. The percentage of conidia with 2 (or 4) nuclei per cell in at least one cell was determined.

2.3. Electron microscopy

Spores were soaked in 5 ml of fixative solution (2% paraformaldehyde (v/v), 2% glutaraldehyde (v/v), and 50 mM sodium phosphate buffer) and incubated for 1 h at 4 °C for fixation. The fixed spores were washed with distilled water three times and were soaked in potassium permanganate solution (1.5% w/v) for 20 min. The samples were washed with distilled water until the supernatants became clear. The samples were soaked in 2% (w/v) uranyl acetate solution for 15 min and washed three times with distilled water. Then, the samples were embedded in preheated 2% (w/v) low melting temperature agarose. Agar blocks containing treated spores were cut into cubes 1mm per side and dehydrated with serial treatment in ethanol solution (25%, 50%, 75%, 95% and 100% ethanol in water). Each serial treatment was for 10 min. Dehydrated agarose blocks were transferred to ethanol-resin solutions (2:1, ethanol:resin; 1:2, ethanol:resin, respectively), and incubated for 30 min in each solution. The blocks were then incubated in a 100% resin solution for 3 h. Every hour the resin was replaced with fresh solution. Blocks were transferred to BEEM capsules and cured for 3 days at 60 °C. Sections 60 nm thick were cut from blocks on a RMC MT7000 ultramicrotome and post-stained for 20 min in 3% (w/v) uranyl acetate followed by 3 min in 3% (w/v) Sato lead

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stain. Stained sections were viewed on a FEI CM12 transmission electron microscope operated at 60 kV.

2.4. Extraction of RNA

Total RNA was isolated from macroconidia, and resulting cultures, at four developmental stages (Fig. 1): (1) fresh spores (0 h) directly obtained from cultures, (2) activated spores (2 h), 2 h after suspension of fresh spores in liquid CM, (3) germinating spores (8 h), 8 h after suspension in CM, and (4) hyphae (24 h), 24 h after spore suspension in CM. While spores did not develop in a wholly synchronous manner, time points were chosen to maximize the number of cells present in one developmental stage without contamination with those in the subsequent stage. Tissue was prepared by immersion and grinding in liquid nitrogen and following extraction with Trizol™ reagent (Invitrogen, Carlsbad, CA) and RNeasy Mini Total RNA extraction kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Prior to labeling, total RNA quality was assayed with an Agilent 2100 Bioanalyzer™ (Palo Alto, CA).

2.5. RNA labeling and hybridization

Ten micrograms of total RNA was treated according to the conventional Affymetrix eukaryotic RNA labeling protocols. Synthesis of cDNA was conducted with total RNA and T7-Oligo (dT) primer (Proligo, Boulder, CO) using the SuperScript™ Double-Stranded cDNA Synthesis Kit (Invitrogen, Carlsbad, CA). The cDNA was purified with an Affymetrix GeneChip Sample Cleanup Module (Affymetrix, Santa Clara, CA). To produce biotinylated cRNA, the cDNA was transcribed *in vitro* using the Enzo BioArray™ HighYield™ RNA Transcript Labeling Kit (Enzo Life Sciences, Farmingdale, NY) in the presence of biotinylated UTP and CTP. The biotin-labeled cRNA was purified with an Affymetrix GeneChip Sample Cleanup Module (Affymetrix, Santa Clara, CA). Fifteen micrograms of labeled RNA was chemically fragmented using the Affymetrix GeneChip Sample Cleanup Module (Affymetrix, Santa Clara, CA), and used for hybridization. One *Fusarium graminearum* GeneChip (Güldener et al., 2006b) each was used for the three biological replications in each experiment (0, 2, 8 and 24 h) and at each replication for the time points of germination process. Chip hybridizations, washes, and reading were conducted in the Biomedical Image Processing Facility at the University of Minnesota following standard Affymetrix procedures (http://www.bipl.ahc.umn.edu/affymetrix.html).

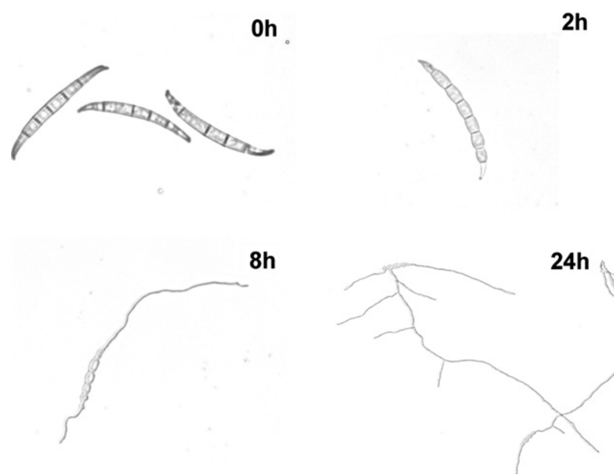

Fig. 1. Light micrographs of *F. graminearum* macroconidia at the four time points used for microarray analysis.

2.6. Analysis of microarray hybridization data

The hybridization signal was scanned with a Genepix 4000b dual laser scanner (AXON Instrument, Foster City, CA) and quantified with software GenePixPro3. The CEL files obtained were loaded into the Gene Expressionist 6.1 Refiner software (Genedata, San Francisco, CA). Chip defective area and outliers were masked while the files were processed for further analysis. Robust Multichip Analysis (RMA) algorithm was used for condensing the data as implemented in the Analyst segment of Gene Expressionist version 6.1. Data were normalized using RMA global normalization algorithm with the whole genes. A presence/absence test was conducted with the detection quality P-value < 0.001. A given gene was called present only if it was detected in at least two GeneChips. Gene expression levels between treatments were compared by t-tests using a significance level of 0.001. Data from microarray experiments are stored at PLEXdb <http://www.plexdb.org> under Accession No. FG7: *Fusarium* gene expression profiles during conidial germination stages.

2.7. Gene annotation and categorization

Gene prediction, annotation and assignment to functional category has been described previously (Güldener et al., 2006a). Genes identified at or between particular developmental stages were categorized for function and the hypergeometric distribution was used to calculate the cumulative probability of each single category, drawing from the population of total genes in that category found in the genome as a whole. Two custom categories of genes also were considered: cell wall associated proteins and transporter proteins. Predicted cell wall associated proteins consisted of predicted glucosyl-phosphotidylinositol (GPI) anchored proteins (Fankhauser and Maser, 2005), as well as manually annotated sets of predicted chitin synthases, mannosyl transferases, and β-1,6 and β-1,3 glucan synthase orthologs from yeast (Lussier et al., 1997). Transporter proteins were annotated from proteins containing predicted major facilitator superfamily (IPR007114) and ABC transporter (IPR003439) Interpro domains as well and from BlastX matches to known transporters.

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3. Results

3.1. Light microscopic observation of spore germination

To follow morphological changes occurring during spore germination, macroconidia of *F. graminearum* were suspended in liquid complete medium (CM) and observed by light microscopy. Macroconidia consist of an elongated apical cell, a pedicellate foot cell, and 1–5 intercalary cells (average = 3). Within 2 h of incubation, approximately 80% of macroconidia had clearly swollen as evident by the convex nature of the cell walls between septa (Fig. 1). During this time, the average middle intercalary cell increased in width from 3.6 ± 0.2 to 5.1 ± 0.3 μm but germination was not evident. Germ tubes first appeared in spores by 3 h. By 8 h, over 90% of macroconidia had at least one germ tube (Fig. 1) from terminal cells, intercalary cells, or both, but no hyphal branching was observed in germ tubes at 8 h. By 24 h, germination tubes had become branched hyphae, consistent with normal mycelial growth.

To determine if there was a preferential pattern of germ tube emergence from macroconidia, we observed 595 germinating spores after 8 h of incubation. Most spores (62%) produced only a single germ tube. Spores were only slightly more likely to have germinated from one or both terminal cells (58%) than from one or more intercalary cells (51%). However, since each spore contains more intercalary cells than terminal cells, on a cell-by-cell basis, terminal cells were about 2.5 times more likely to have germinated than intercalary cells. A total of 36% of macroconidia produced two germination tubes, which consists of 26.7% of spores germinating from both terminal cells, 7.6% germinating from one terminal and one intercalary cell, and only 1.7% germinating from two intercalary cells. Therefore, for conidia forming more than one germ tubes, there was a strong preference for germination from each terminal cell.

Based on light microscopy studies, four time points were chosen for gene expression analysis to represent particular milestones in spore development (Fig. 1). Developmental stages were: (1) fresh spores (0 h) directly obtained from actively growing cultures, (2) activated spores (2 h), 2 h after incubating fresh spores in liquid CM, (3) germinating spores (8 h), 8 h incubation, and (4) hyphae (24 h), germ tubes developing into branching hyphae after 24 h incubation.

3.2. Qualitative and quantitative differences in genes detected during spore development

In order to determine changes in gene expression during spore development, total RNA was obtained from the four developmental stages and used for microarray hybridization assays using the *F. graminearum* Affymetrix GeneChip (Güldener et al., 2006b). Total genes detected and genes specifically expressed at each developmental stage were noted and categorized according to predicted function (Table 1 and Supplemental Tables A–H). Genes significantly up- or down-regulated 2-fold or more between developmental stages were also noted and categorized according to predicted function (Table 2 and Supplemental Tables I–N).

A surprisingly high number of genes were detected in the fresh spores; out of a total of 13,969 genes annotated in *F. graminearum*, 5813 were detected in fresh spores at a detection P-value < 0.001 (Table 1 and Supplemental Table A). Differences in the number of detected genes among developmental stages can be attributed to overall differences in abundance of transcripts. While it was anticipated that fewer genes would be detected in spores (presumed to be metabolically quiescent), the number of genes detected in fresh spores actually was very similar to that of hyphae (24 h) and was greater than the number in activated (2 h) or germinating spores (8 h). Despite differences in overall numbers, the majority of genes detected at any particular developmental stage were not restricted to that stage and the number of shared genes was a similar percentage of the total. Nevertheless, each

Table 1
Genes expressed at each of five developmental stages

	Developmental stage			
	0 h	2 h	8 h	24 h
Total genes expressed (Supplemental Table)	5813 (A-0 h total)	5146 (B-2 h total)	5249 (C-8 h total)	5993 (D-24 h total)
Stage-specific genes (Supplemental Table)	627 (E-0 h specific)	260 (F-2 h specific)	185 (G-8 h specific)	484 (H-24 h specific)

Table 2
Genes up- or down-regulated between developmental stages

	Developmental stages		
	0–2 h	2–8 h	8–24 h
Number of genes up-regulated (Supplemental Table)	1805 (I-0–2 h up)	1301 (J-2–8 h up)	954 (K-8–24 h up)
Number of genes down-regulated (Supplemental Table)	1616 (L-0–2 h down)	1265 (M-2–8 h down)	947 (N-8–24 h down)

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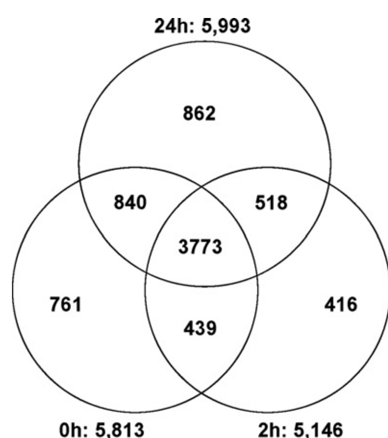

Fig. 2. Venn diagram summarizing the number of genes expressed in fresh spores (0 h), swollen spores (2 h) and hyphae (24 h).

stage contained hundreds of potentially stage-specific transcripts (Fig. 2).

3.3. Gene expression in fresh spores

Of the 5813 genes detected in fresh spores, 627 were found only at that developmental stage (Supplemental Table E). Compared to the genome as a whole, these 627 were significantly enriched ($P < 0.0001$) for genes corresponding to MIPS category 99, Unclassified Proteins. Indeed, 477 of the 627 genes unique to fresh spores (76%) were unclassified, undoubtedly reflecting our highly limited knowledge of genes specifically expressed in spores.

Gene expression profiles of fresh spores were examined for physiologically relevant sets of genes. Among those genes with significantly higher levels of expression in fresh spores compared to activated spores (2 h incubation, Supplemental Table I) are categories of genes involved in peroxisome formation (MIPS 42.19) and peroxisomal transport (MIPS 20.09.10), including orthologs of peroxin (PEX) proteins of *Saccharomyces cerevisiae*, which are involved in peroxisome biogenesis and function (Fig. 3). Peroxisomes are organelles within which degradation of long chain- and branched-fatty acid occurs by β-oxidation. Genes involved in oxidation of fatty acids (MIPS 02.25) also were significantly up-regulated in fresh spores including genes for components of peroxisomal β-oxidation: acyl-CoA oxidase (fg02287), long chain specific acyl-CoA dehydrogenase (fg10285, and acyl-CoA dehydrogenases (fg05140 and fg10790), multifunctional β-oxidation protein (fg09643), acetylacyl-CoA thiolases (fg04243 and fg09503) and peroxisomal Lon protease (fg09725). In order to validate the inference that peroxisomes may play an important role at this developmental stage, ultrastructural examination of fresh spores revealed an abundance of peroxisomes, more than observed at any other stage examined (Fig. 3). Peroxisomes tended to be concentrated at spore septa. Histological staining of macroconidia for lipids confirmed that fresh spores contained numerous lipid bodies preferentially located at septa that dissipate during germination (Fig. 4).

Differential expression of the genes involved in the glyoxylate cycle also was detected in fresh spores including the key components isocitrate lyase (fg09896) and malate synthase (fg08700). Therefore, it may be inferred that fresh spores degrade fatty acids to acetyl-CoA by β-oxidation for use in energy generation through the TCA cycle and the glyoxylate cycle. This process not only may provide metabolic energy for maturing conidia but also may generate glucose for further biosynthesis of cellular materials. In support of this idea is the observation that enzymes involved in synthesis of glucose from acetyl-CoA (gluconeogenesis) also are differentially expressed in fresh spores. Three enzymes specific for gluconeogenesis are preferentially expressed in fresh spores (>2× greater expression at 0 h versus 2 h): pyruvate carboxylase (fg07075), fructose-1,6-bisphosphatase (fg09280), and phosphoenolpyruvate carboxykinase (fg08601). The gene encoding glucose-6-phosphatase, another enzyme specific to gluconeogenesis, was not found in the genome assembly. Additionally, an ortholog of CAT8 (fg09921), which encodes a transcription factor involved in de-repression of gluconeogenetic enzymes in *S. cerevisiae* (Hiesinger et al., 2001), was highly expressed in fresh spores.

Fresh spores may also obtain glucose by degradation of storage polysaccharides. High expression levels for several predicted glucanases and glycosidases, which do not appear to be secreted based on TargetP analysis, were detected in fresh spores, including several β-glucosidases (fg04953, fg06605, fg07274, fg02632, fg04913, and fg00166), an α-glucosidase (fg03462), and a β-1,3-glucanase (fg05401). These observations suggest that long chain carbohydrates may be degraded to generate glucose and other sugars in fresh spores. To confirm the degradation of storage carbohydrates in germinating spore, conidia were stained for glycogen using potassium iodide. While fresh conidia stained deeply for glycogen, staining diminished noticeably after germination (Fig. 4).

3.4. Massive gene induction and repression upon spore activation (2 h)

Transcripts corresponding to a total of 5146 genes were detected in conidia incubated for 2 h (Table 1 and Supplemental Table B). Of these, 260 genes were specifically expressed at this germination stage (Supplemental Table F) and these were enriched for genes involved in transcription (MIPS category 11; $P = 2.49\text{E–}006$). Indeed, changes in transcription levels dominated the transition from fresh spores at 0 h to swollen spores at 2 h, a process we will refer to as "spore activation."

Spore activation marked the largest change in gene expression throughout the germination time-course. A total of 3421 genes were up- or down-regulated more than 2-fold upon activation compared to fresh spores (Table 1); 1805 genes were up-regulated (Supplemental Table I) and

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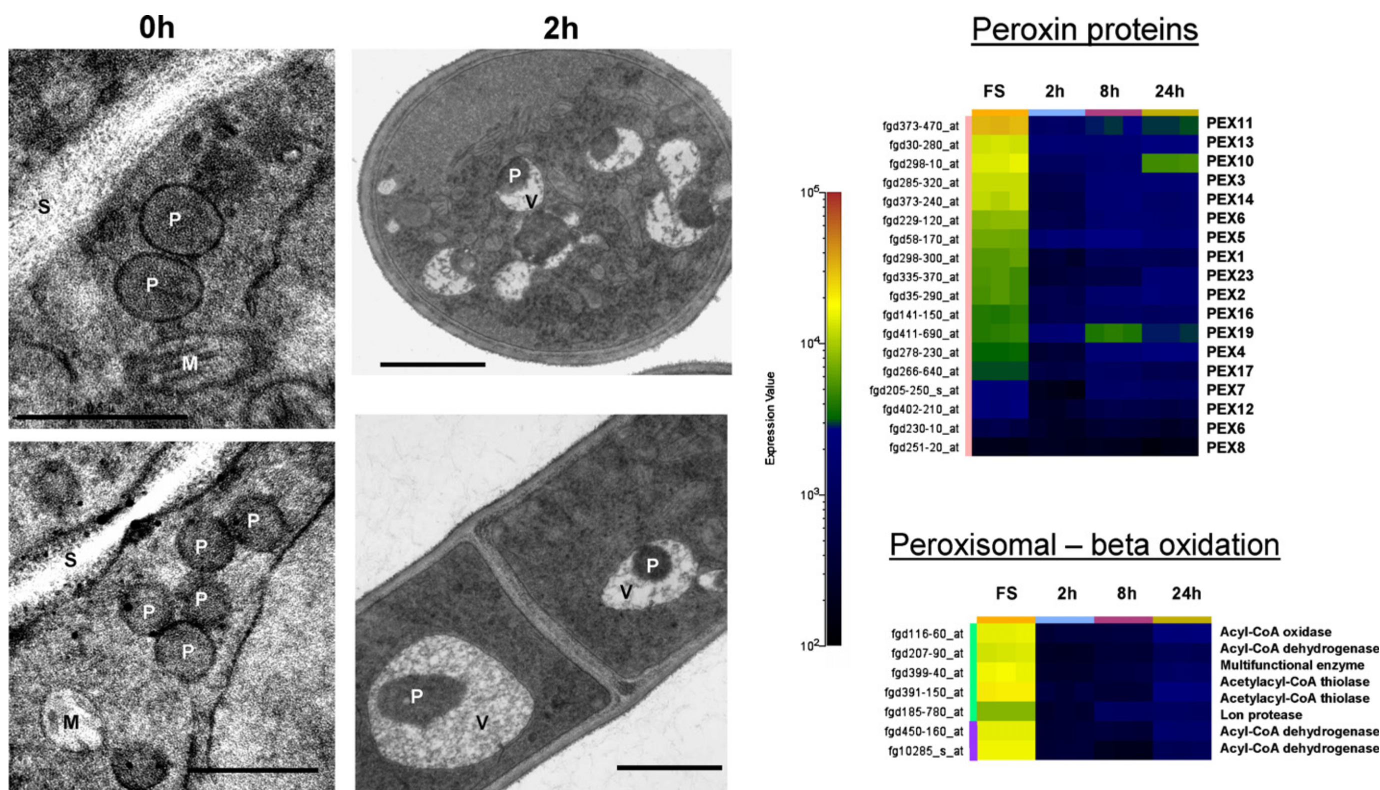

Fig. 3. (Left) Electron micrographs of fresh (0 h) and swollen (2 h) spores illustrate the presence of peroxisomes (P) located near septa (S) or within vacuoles (V), respectively. Bars measure 0.5 μm at 0 h and 1.0 μm at 2 h. (Right) Heat maps show the relative expression levels of PEX genes and genes for enzymes involved in peroxisomal β-oxidation as indicated by the scale to the left. Columns are expression values at each time point in triplicate. Rows are mean values of probe sets representing each gene. FS = fresh spores at 0 h.

1616 genes were down-regulated (Supplemental Table L). Prominent among those genes up-regulated were those involved in metabolism (MIPS category 01; $P = 0.0$), transcription (MIPS category 11; $P = 2.58E–087$), protein synthesis (MIPS category 12; $P = 8.24E–204$) and cell cycle and processing (MIPS category 10; $P = 1.68E–049$). Shifts in metabolism greatly involved utilization of nitrogen sources available in the germination medium as the most significantly up-regulated metabolic genes were for amino acid metabolism (MIPS category 01.01; $P = 3.80E–018$) and nucleotide metabolism (MIPS category 01.03; $P = 2.82E–037$). The machinery of transcription and translation was also robustly induced, including ribosome biogenesis (MIPS category 12.01; $P = 5.51E–145$) and RNA synthesis (MIPS category 11.02; $P = 1.52E–030$).

Up-regulated genes belonging to the cell cycle and processing category were dominated by those functioning in DNA synthesis and replication (MIPS category 10.01.03; $P = 2.63E–016$) and mitotic cell cycle control (MIPS category 10.03.01; $P = 2.56E–025$), suggesting that DNA replication and mitosis occurs early in conidium germination. Nuclear division prior to spore germination has not been reported for *F. graminearum*. To confirm this unexpected result, DAPI- and Calcofluor-treated conidia were observed to determine the number of nuclei per cell at various time points. While at 0 h nearly all spores (97.9 ± 0.9%) contain cells with only a single nucleus, at 2 h, most spores (73.1 ± 7.6%) have one or more cells containing two or more nuclei or appear to be undergoing karyokinesis (Fig. 4). Surprisingly, the binucleate cells persist in 79.0 ± 8.2% and 82.9 ± 6.7% of macroconidia at 4 and 8 h, respectively. Gene expression results therefore may be used to correctly predict the cell cycle state of the fungus.

Down-regulated during spore activation were genes for fatty acid oxidation (MIPS category 02.25; $P = 6.96E–010$) and peroxisome formation (MIPS category 42.19; $P = 1.97E–005$). As metabolism shifts from internal lipid metabolism to utilization of sugars and amino acids in the germination medium, peroxisomes also appear to undergo autophagocytosis in swollen spores (Fig. 3).

3.5. Gene expression associated with spore germination (8 h)

A total of 5249 genes were detected in RNA isolated from germinated conidia incubated in CM for 8 h (Table 1; Supplemental Table C). Among 185 genes that were specifically expressed at this developmental stage (Supplemental Table G), many belong to categories of the cell cycle and DNA processing (MIPS category 10; $P = 0.00017$), especially mitotic cell cycle and cell cycle control (MIPS category 10.03.01; $P = 0.00017$) as well as cell type

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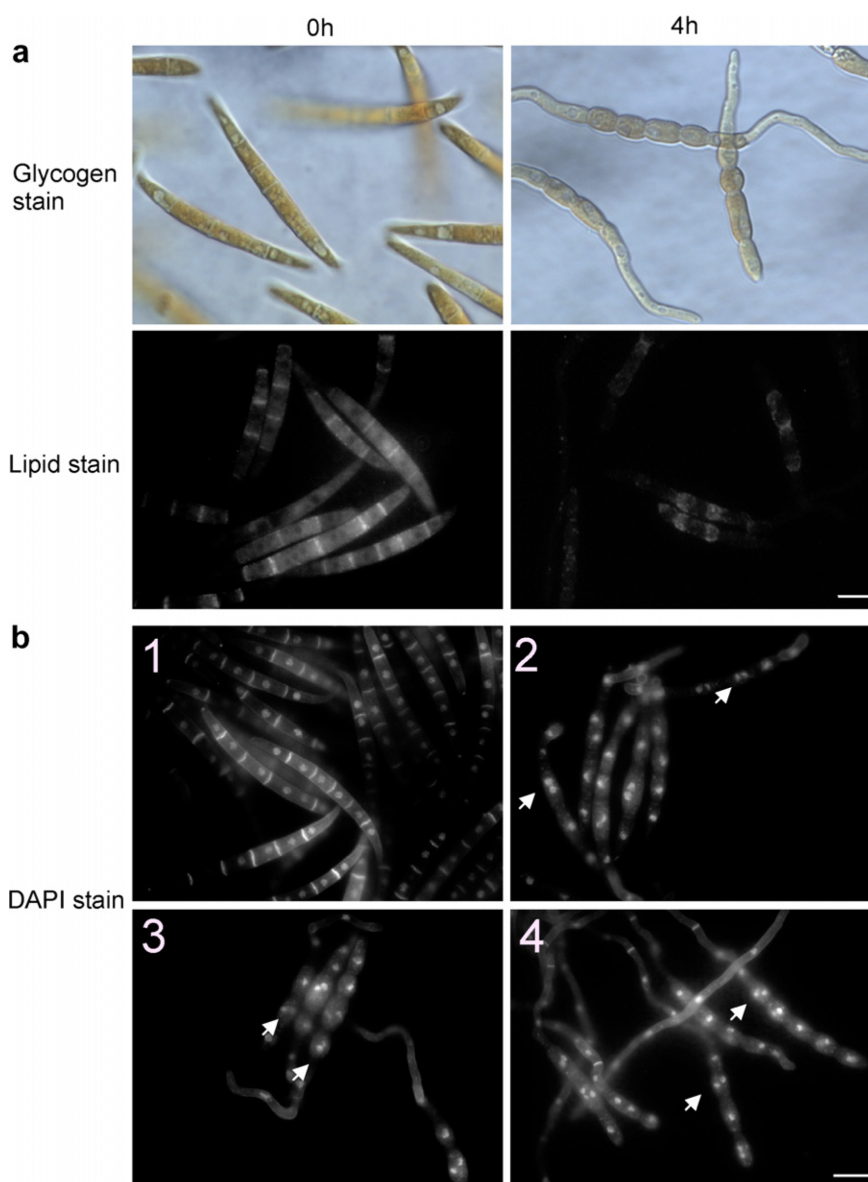

Fig. 4. (a) *F. graminearum* macroconidia stained with KI for glycogen (top) and with Nile Red for lipid (bottom) in fresh spores (0 h) or at 4 h. (b) DAPI stained macroconidia showing nuclei in fresh spores (1), at 2 h (2), 4 h (3) and 8 h (4). Arrows in (B2–B4) point out representative binuclear cells.

differentiation (MIPS category 43; $P = 0.04$) including genes for budding, cell polarity and filament formation (MIPS category 43.01.03.05; $P = 0.017$). Genes in the latter category are consistent with the establishment of polarized growth characteristic of this developmental stage.

A total of 2566 genes were significantly up- or down-regulated in the transition between activated (2 h) and germinated (8 h) spores (Table 1); 1301 genes were up-regulated (Supplemental Table J) and 1265 genes were down-regulated (Supplemental Table M). Among those categories of genes significantly up-regulated during germination were metabolism (MIPS category 01; $P = 0.0$), protein fate (MIPS category 14; $P = 2.85$E–055), cellular transport (MIPS category 20; $P = 1.10$E–025), biogenesis of cellular components (MIPS category 42; $P = 1.20$E–022), cell type differentiation (MIPS category 43; $P = 1.64$E–016), cell fate (MIPS category 40; $P = 1.91$E–012), energy (MIPS category 02; $P = 2.99$E–014), and cell rescue, defense and virulence (MIPS category 32; $P = 1.46$E–013). Among those genes most highly up-regulated for metabolism and energy are those for C-compound and carbohydrate utilization (MIPS category 01.05.01; $P = 4.10$E–013) and glycolysis and gluconeogenesis (MIPS category 02.01; $P = 2.20$E–008), reflecting the shift from lipid-based metabolism within conidia to metabolism in the glucose containing germination medium. Among the genes up-regulated involving protein fate are those associated with proteasomal degradation and the ubiquitin/proteasomal pathway (MIPS category 14.13.01.01; $P = 2.41$E–027), suggesting a large turnover of proteins associated with the transition from spore to hypha. Among those up-regulated genes associated with cellular transport and

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biogenesis of cellular components were genes involved in vacuole formation (MIPS category 42.25; $P = 1.76E–007$) and vacuolar transport (MIPS category 20.09.13; $P = 2.37E–018$), which display a pattern of elevated expression in germinating conidia. Taken together these results imply conidial germination may involve the processes of both proteosomal and vacuolar proteolysis.

The 1265 genes down-regulated between 2 and 8 h (Supplemental Table M) reflect largely the diminishment of transcription (MIPS category 11; $P = 9.26E–082$) and protein synthesis (MIPS category 12; $P = 6.46E–040$) after the large burst of activity associated with spore activation at 2 h. Many down-regulated genes related to ribosome synthesis belong to those categories involved in ribosome biogenesis (MIPS category 12.01; $P = 1.48E–025$), including rRNA synthesis (MIPS category 11.02.01; $P = 2.19E–023$) RNA processing (MIPS category 11.04.01; $P = 4.66E–081$), and ribosomal proteins (MIPS category 12.01.01; $P = 4.85E–005$). Down-regulation of the synthesis of transcriptional machinery involved gene categories including mRNA synthesis (MIPS category 11.02.03; $P = 6.01E–018$) and processing (MIPS category 11.04.03; $P = 3.68E–023$).

3.6. Gene expression associated with mycelium and hyphal branching (24 h)

The largest number of genes expressed at any developmental stage was at 24 h where 5993 genes were detected (Table 1; Supplemental Table D), of which 484 were determined to be unique to this developmental stage (Supplemental Table H). Most stage-specific genes were not over-represented at 24 h with the exception of a few transporters such as those involved in ion transport (MIPS category 20.01.01; $P = 0.007$), genes involved in nitrogen metabolism, and unclassified proteins (MIPS category 99; $P = 0.0001$).

Between 8 and 24 h, hyphae from germinated conidia continue to expand and begin to laterally branch (Fig. 1). However, only 1901 genes were up- or down-regulated between 8 and 24 h, the smallest number between any developmental stages examined. A total of 954 genes were up-regulated and 947 genes were down-regulated. The most significantly up-regulated categories of genes included those involved in oxidation of fatty acids (MIPS category 02.25; $P = 9.05E–006$) and oxidative stress response (MIPS category 32.01.01; $P = 2.99E–005$) as well as various transport related genes including those for ion transport (MIPS category 20.01.01; $P = 5.17E–006$) and ABC transporters (MIPS category 20.03.25; $P = 2.99E–005$).

Differences in gene expression between 8 and 24 h were greater for those genes that were down-regulated than those that were up-regulated. Genes associated with protein synthesis and transcription continued to be reduced, following the trend in expression for these genes between 2 and 8 h. Among those categories showing reduced expression were genes involved in ribosome biogenesis (MIPS category 12.01; $P = 3.95E–042$) and ribosomal proteins (MIPS category 12.01.01; $P = 3.85E–046$) as well as mRNA synthesis (MIPS category 11.02.03; $P = 1.83E–008$). A large percentage of down-regulated genes also were related to cell cycle and DNA processing (15.4%) (MIPS Category 10; $P = 6.23E-044$), biogenesis of cellular components (10%) (MIPS category 42; $P = 6.77E–015$), and cell type differentiation (8.0%) (MIPS category 43; $P = 2.99E–020$). The later category includes numerous genes involved in fungal budding, cell polarity, and filament formation (MIPS category 43.01.03.05; $P = 5.22E–006$).

4. Discussion

Spore developmental processes are critical for the reproduction and survival of fungi. By way of conidial formation and dispersal, fungi proliferate, expand in range and can explore different niches. Once dispersed, dormant fungal spores detect appropriate activation signals and initiate the process of germination. Although various efforts have concentrated on identifying and defining the role of genes involved in specific stages of fungal development (Loo, 1976; Osherov and May, 2000), most of the molecular mechanisms determining the fate of the fungal developmental stages remain to be elucidated.

Spore germination for a plant pathogenic fungus may be seen as a critical developmental step in pathogenesis. Germination must occur at both the correct time and location in order for infection to be successful. For *F. graminearum*, germination on the emergent spikes of wheat and barley during the time plants are undergoing antithesis is necessary for the initiation of disease.

Consistent with the findings of a recent study (Harris, 2005), we have found that macroconidia of *F. graminearum in vitro* preferentially germinate at terminal cells. However, the frequency with which both terminal cells germinate ("bipolar germination") described by Harris is much higher than we observed in this study. Differences in spore preparation and germination medium between the studies may explain differences in the rate of germination from terminal cells. Nevertheless our overall conclusion, that terminal cells preferentially germinate compared with intercalary cells, is the same as that of Harris. For our microarray study, in order to maximize germination rates and synchronicity with which spores germinate, we chose to use a complete medium in which these characteristics prevail. However, future germination studies will focus either on germination on plant surfaces or in media made from plant extracts that may mimic the chemical composition of the plant infection court. Evidence from other plant pathogenic fungi suggests that germination characteristics may differ between saprophytic growth on artificial media and during pathogenic interactions (Barhoom and Sharon, 2004).

In this study, we monitored the entire transcriptome of *F. graminearum* during spore developmental stages using

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a *F. graminearum* Affymetrix GeneChip (Güldener et al., 2006b). Our goal was to focus on the expression of several categories of genes in order to deduce the metabolic state and potential molecular mechanisms for spore development. Identifying genes with stage-specific expression patterns or altered expression levels between developmental stages is central to understanding molecular mechanisms regulating development processes. Although even genome-wide monitoring of RNA abundance gives only a limited view of overall cellular activity during spore germination, a previous study of spore germination in *N. crassa* (Kasuga et al., 2005) has established that such analysis leads to an accurate assessment of ongoing developmental processes.

Indeed we have found that gene expression data present a coherent snapshot of the cellular and physiological state of each developmental stage for a number of known processes and are consistent with changes in gene expression previously reported for spore development in other fungi. For example, using a 3.3 K feature *N. crassa* microarray (Kasuga et al., 2005) expression data were obtained at eight time points during spore germination. While differences in experimental design prevent a side-by-side comparison of results for the 1154 orthologous genes found on both the *Fusarium* and *Neurospora* microarrays, all significantly over-represented functional categories of genes up-regulated during the earliest stages of spore germination (0–1 h) in *N. crassa* (Kasuga et al., 2005) were the same categories over-represented among up-regulated genes at the earliest stages of *F. graminearum* germination (0–2 h) (Supplemental Table I). These functional categories include heat shock proteins (MIPS category 32.01.05), amino acid metabolism (MIPS category 01.01), transcription (MIPS category 11), cell cycle and DNA processing (MIPS category 10), ribosome biogenesis (MIPS category 12.01), energy (MIPS category 2) and cellular transport, transport facilities and transport routes (MIPS category 20). Our results therefore are consistent with the results obtained for *N. crassa* spore germination. However, because of our greatly increased gene sample size as well as greater signal sensitivity and quantitative capabilities of the Affymetrix platform, many more significant functional categories were identified by this study and with greater levels of significance.

As pointed out by Kasuga et al., "...the utility of mRNA profiling depends upon its correlation with and implications for functional measurements of cell biology, biochemistry and organismic development...." To this end we have found metabolic and cellular changes occurring during spore development may be anticipated by knowledge of gene expression changes. The clearest example of this was the inference from gene expression data that peroxisome formation was occurring in fresh spores, predicted by the strong expression of a suite of genes involved in peroxisome biogenesis and function. Peroxins encoded by PEX genes are involved in peroxisome biogenesis have been extensively studied in yeast with over 20 PEX genes currently described (van der Klei and Veenhuis, 2006). In *F. graminearum*, 12 othologs of PEX proteins were identified and were expressed and up-regulated in fresh spores (Fig. 3). Among the functions of peroxisomes are to sequester oxidative steps in lipid metabolism including β-oxidation of fatty acids, as well as steps in the synthesis of sterols, isoprenoids, ether-phospholipids, and polyunsaturated fatty acids (Wanders and Tager, 1998). The observation that enzymes involved in peroxisomal β-oxidation, in additional to PEX genes, are greatly up-regulated in fresh spores compared to other developmental stages, presents a coherent expectation that peroxisomes may be synthesized within fresh spores and be important for their metabolism. This expectation was met as structures consistent with peroxisomes were found to be most abundant during ultrastructural examination of fresh spores (Fig. 3). Taken together, these observations imply that spores, newly dislodged from conidiophores, are undergoing a shift in energy metabolism toward lipid catabolism. Mycelium upon which spores are produced, derive their energy from sugars and amino acids obtained from the growth medium. However, once conidia are fully formed, further energy must be derived from internal reserves, which for fungi, include lipids and polysaccharides such as glycogen (Bianchi and Turian, 1967; Mims et al., 1995).

Also predicted by gene expression data was nuclear division in macroconidia prior to spore germination. It has been reported that *F. graminearum* forms binucleate cells *in planta* during vegetative colonization of vascular tissue within the host (Guenther and Trail, 2005). However, to our knowledge, the formation of binucleate cells in conidia of *Fusarium* preceding germination is an unprecedented observation. Our microarray and staining data suggest that nuclei in conidial cells may be arrested in the G1 stage, and a round of DNA synthesis and mitosis is necessary before germination. Nuclear division in intercalary conidium cells appears to be uncoupled from cytokinesis. However, because the *F. graminearum* is homothallic, it may allow for the formation of cells that are homokaryotic dikaryons even if karyogamy is not imminent. Recently, using a transformant expressing a GFP-His1 construct (Freitag et al., 2004), we have also observed binucleate compartments in vegetative hyphae grown in CM (unpublished data).

We hypothesize that stage-specific gene expression and changes in gene expression levels between developmental stages observed in this study will be fundamental to understanding the molecular genetic basis for spore germination and development. In the future, functional analysis will focus on three categories of genes that may be critical to improve our knowledge of spore germination: cellular transporters, cell wall proteins, and transcription factors.

Genes for predicted transmembrane transporter proteins may be important to fungal spores as they may function as receptors to sense signals for spore germination from the environment. Alternatively RNAs for transporter genes may exist in spores as pre-formed messages allowing the cells to respond rapidly to the appropriate environmental

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cue leading to activation from dormancy. Genes for transporters expressed in spores appear to be mostly for the major facilitator superfamily but also include some ABC transporters. Interestingly, the major facilitator superfamily has undergone evolutionary expansion and is the second most abundant class of protein in the genome of *F. graminearum* with a total of 294 predicted genes (Cuomo et al., 2007). Overall it appears that more permeases and transporters are expressed in fresh spores and in hyphae, when nutrient-limiting conditions may exist, than for activated spores when the fungus may be in a more nutrient-rich environment. Remarkably, transcripts of 216 genes annotated for permeases or transporters were detected in fresh spores. Out of these 216, 29 were detected only in fresh spores (Supplemental Table O). A majority of these are predicted permeases for sugars or other carbon compounds. These results suggest that fresh spores are metabolically active and metabolize internal metabolites but also may also be capable of taking up additional nutrients from the environment.

Consistent with a role as sensors of such environmental cues, predicted choline transporters related to *HNM1* of *S. cerevisiae* (Nikawa et al., 1990) also are expressed in fresh spores (fg01762 and fg02264). Choline is abundant in anthers of wheat and suggested to be one of two growth stimulants in anthers during the infection cycle of *F. graminearum* (Strange et al., 1972). As an excellent potential carbon and nitrogen source during early stages of *F. graminearum* infection, choline uptake may be essential for *F. graminearum* infection during anthesis.

In addition to transporters, structural proteins including cell wall biosynthetic enzymes, cell wall associated structural proteins and septins might be expected to differ among developmental stages in germinating spores. Preferential expression of chitin synthase I (*CHS1*—fg10327), chitin synthase IV (*CHS4*—fg01272), a probable wall-related mannosyltransferase and a gene related to spore coat protein SP96 (fg03840) were noted in fresh spores compared to any other growth stage tested. Likewise putative surface proteins predicted to be both secreted, based on signal peptide sequence (Emanuelsson et al., 2000), and wall-associated, by virtue of GPI anchor motifs (Fankhauser and Maser, 2005), are differentially regulated during spore development. Of a total of 69 predicted secreted GPI proteins, eight are predominantly expressed in fresh spores. Among these eight GPI-proteins are a predicted glycine-rich cell wall protein (fg00347), a calcineurin-like phosphoesterase (fg02069), and a fasciclin domain (putative cell adhesion domain) protein (fg06592). The fact that genes apparently involved in cell wall biogenesis and architecture were up-regulated in fresh spores implies that the cell walls of these spores were not fully formed and may be undergoing further development in response to aging.

This study presents a first look at genome-wide gene expression changes occurring during spore germination in *F. graminearum*. We have validated inferences made from gene expression profiles by linking genes with known function to structural and physiological elements associated with those functions. Future studies will focus on stage-specific gene expression that may be controlling or critical to the success of each developmental stage. For example we have identified at least 50 predicted transcription factors that are preferentially expressed at certain times during spore development. Systematic site-directed mutagenesis will be used to determine if these elements are essential for each developmental stage. Also to be examined by mutagenesis will be genes for predicted trans-membrane transporters that exhibit stage-specific expression. We hypothesize that such proteins may transmit signals triggering or potentiate spore germination. Fundamental knowledge on the initiation and regulation of spore germination may lead to novel disease control measures aimed at interfering with this vital process necessary for disease initiation and fungal survival.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fgb.2007.09.002.

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