# Germination of conidia of *Aspergillus niger* is accompanied by major changes in RNA profiles

M.R. van Leeuwen[1], P. Krijgsheld[2], R. Bleichrodt[2], H. Menke[3], H. Stam[3], J. Stark[3], H.A.B. Wösten[2], and J. Dijksterhuis[1*]

[1]*Applied and Industrial Mycology, CBS-KNAW Fungal Biodiversity Centre, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands*; [2]*Microbiology and Kluyver Centre for Genomics of Industrial Fermentation, Molecular Microbiology, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands*; [3]*DSM Food Specialties, PO Box 1, 2600 MA Delft, The Netherlands*

*Correspondence: Jan Dijksterhuis, j.dijksterhuis@cbs.knaw.nl

**Abstract:** The transcriptome of conidia of *Aspergillus niger* was analysed during the first 8 h of germination. Dormant conidia started to grow isotropically two h after inoculation in liquid medium. Isotropic growth changed to polarised growth after 6 h, which coincided with one round of mitosis. Dormant conidia contained transcripts from 4 626 genes. The number of genes with transcripts decreased to 3 557 after 2 h of germination, after which an increase was observed with 4 780 expressed genes 8 h after inoculation. The RNA composition of dormant conidia was substantially different than all the subsequent stages of germination. The correlation coefficient between the RNA profiles of 0 h and 8 h was 0.46. They were between 0.76–0.93 when profiles of 2, 4 and 6 h were compared with that of 8 h. Dormant conidia were characterised by high levels of transcripts of genes involved in the formation of protecting components such as trehalose, mannitol, protective proteins (*e.g.* heat shock proteins and catalase). Transcripts belonging to the Functional Gene Categories (FunCat) protein synthesis, cell cycle and DNA processing and respiration were over-represented in the up-regulated genes at 2 h, whereas metabolism and cell cycle and DNA processing were over-represented in the up-regulated genes at 4 h. At 6 h and 8 h no functional gene classes were over- or under-represented in the differentially expressed genes. Taken together, it is concluded that the transcriptome of conidia changes dramatically during the first two h and that initiation of protein synthesis and respiration are important during early stages of germination.

**Key words:** *Aspergillus niger*, conidia, germination, transcriptome.

Published online: 17 September 2012; doi:10.3114/sim0009. Hard copy: March 2013.

## INTRODUCTION

Conidia are the main vehicles of distribution for fungi (Navarro-Bordonaba & Adams 1996) and are characterised by a dormant state and transported via different media such as water and air. Air-dispersed conidia possess moderate resistance towards low water activity conditions, high and low temperature, UV radiation and other stressors like reactive oxygen species. The dormancy of these cells is broken upon exposure to water, air, and / or inorganic salts, amino acids and fermentable sugars (Osherov & May 2001, Thanh *et al.* 2005). The environmental conditions are signaled by receptor(s) via Ras/MAPK and cAMP/PKA signal-transduction pathways (Osherov & May 2000, Liebmann *et al.* 2004, Reyes *et al.* 2006, Zhao *et al.* 2006). Upon activation of germination, the disaccharide trehalose and the polyol mannitol are degraded (Witteveen & Visser 1995, Thevelein 1996, d'Enfert *et al.* 1999, Fillinger *et al.* 2001, Ruijter *et al.* 2003, Dijksterhuis *et al.* 2007). As a consequence, glycerol is formed, which is indicative for an active glycolysis (d'Enfert 1997).

The first morphological change in spore germination is isotropic growth. During this process, also called swelling, the diameter of the spore increases two fold or more. It involves water uptake and a decrease in the micro-viscosity of the cytoplasm (van Leeuwen *et al.* 2010). Moreover, molecules are directed to the cell cortex to enable addition of new plasma membrane and cell wall (Bartnicki-Garcia & Lippman 1977, Momany 2002). Isotropic growth is concomitant with metabolic activities such as respiration, and DNA, RNA, and protein synthesis (Mirkes 1974, Osherov & May 2001). Isotropic growth is followed by polarised growth that results in the formation of a germ tube. During this phase, the morphogenetic machinery is redirected to the site of polarisation. This machinery includes the cytoskeleton, the vesicle trafficking system, landmark proteins, signaling pathways and endocytic partners like Rho GTPase modules, polarisome and Arp2/3 complexes (d'Enfert 1997, Momany 2002, Harris & Momany 2004, Harris 2006). Moreover, the lipid composition of the plasma membrane changes by the appearance of sterol-rich domains (Van Leeuwen *et al.* 2008). At later stages of development the growth speed of the germ tube increases and the functional organisation of the hyphal tip area acquires its full potential as judged by zones of endocytosis and exocytosis and the presence of the Spitzenkörper (Taheri-Talesh *et al.* 2008, Köhli *et al.* 2008). By branching and inter-hyphal fusions (Glass *et al.* 2004) a fungal mycelium is established.

Genera of the order Eurotiales (*e.g. Penicillium*, *Aspergillus* and *Paecilomyces*) produce numerous single-celled conidia that are abundant in air samples (McCartney & West 2007). These genera are associated with food spoilage and are able to form a wide panel of mycotoxins (Frisvad *et al.* 2007). In addition, they can act as opportunistic pathogens (Burrell 1991). *Aspergillus niger* is a world-wide food spoiler and can also infect harvested crops (Snowdon 1990). Moreover, it is an important cell factory (Meyer *et al.* 2011). The impact of *A. niger*, the availability of its genome sequence and whole genome microarrays (Pel *et al.* 2007) makes

this an attractive fungal model system. So far, only the asexual stage of *A. niger* has been identified. Formation of conidia involves a complex developmental pathway (Krijgsheld *et al.* 2013). In this study, the transcriptome of conidia of *A. niger* was studied during dormancy and germination. Most changes in the transcriptome occurred early in germination (*i.e.* before isotropic growth). The data show that the transcriptome of dormant conidia is distinct from that of conidia during all stages of germination.

## MATERIALS AND METHODS

### Organism and growth conditions

The *A. niger* strain N402 (Bos *et al.* 1988) and its derivative RB#9.5 were used in this study. The latter strain expresses a gene encoding a fusion of sGFP and the histone protein H2B under regulation of the *mpdA* promoter. For spore isolation, strains were grown for 12 days at 25 °C on complete medium (CM) containing per liter: 1.5 % agar, 6.0 g $NaNO_3$, 1.5 g $KH_2PO_4$, 0.5 g KCl, 0.5 g $MgSO_4$, 4.5 g D-glucose, 0.5 % casamino acids, 1 % yeast extract and 200 µl trace elements (containing per liter: 10 g EDTA, 4.4 g $ZnSO_4 \cdot 7H_2O$, 1.0 g $MnCl_2 \cdot 4H_2O$, 0.32 g $CoCl_2 \cdot 6H_2O$, 0.32 g $CuSO_4 \cdot 5H_2O$, 0.22 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 1.5 g $CaCl_2 \cdot 2H_2O$, and 1.0 g $FeSO_4 \cdot 7H_2O$). Conidia were harvested in ice-cold ACES-buffer (10 mM ACES, 0.02 % Tween-80, pH 6.8). To this end, the colony surface was gently rubbed with a sterile T-spatula and the conidial suspension was filtered through sterile glass wool. Conidia were washed in ice-cold ACES-buffer, resuspended in CM and kept on ice until further processing on the same day.

### Microscopy

Samples of liquid cultures were placed on poly-l-lysine (Sigma) coated cover slips (Van Leeuwen *et al.* 2008). After removal of the medium, the cover slips with the immobilised conidia were placed upside-down onto an object glass overlaid with a thin (< 0.5 mm) layer of 2 % water agar. Any remaining liquid was removed with filter paper. Images were captured with a Zeiss Axioskop 2 plus microscope (Zeiss, Oberkochen, Germany) equipped with a HBO 100 W mercury lamp and a AxioCam MRc (Zeiss, Germany) camera using standard FITC (λ = 450–490 nm, FT510, LP520) filters. A minimal number of 93 cells for each time point was counted for the enumeration of the number of nuclei in dormant and germinating conidia.

### RNA extraction

For isolation of RNA, 3 x $10^9$ conidia were inoculated in 300 ml CM. Three cultures were shaken at 125 rpm at 24 °C for each RNA isolation. At each time point, 15 ml culture medium was sampled from the biological triplicates. Samples were pooled and centrifuged at 5 °C for 5 min at 1100 g. The pellet was frozen in liquid nitrogen and homogenised with the Qiagen Tissuelyser® (2 times 2 min at 30 strokes/sec) in a stainless steel grinding jar that had been cooled with liquid nitrogen (Qiagen, Venlo, The Netherlands). After homogenising, 2 ml RLT buffer (supplied with the Qiagen RNeasy® Maxi kit) was added. All the material of the samples taken at 0 h and 2 h, half of the material taken at 4 h and a third of the material taken at 6 h and 8 h were transferred to a 50 ml Greiner tube. RNA was extracted following the protocol of the RNeasy® Maxi kit (Qiagen) with some modifications. 15 ml RLT buffer supplemented with 170 µl ß-mercaptoethanol was added. After centrifugation (3000 x g, 10 min, 4 ºC) the supernatant was mixed thoroughly with 15 ml 70 % ethanol in a 50 ml Greiner tube and transferred to a RNeasy Maxi column. After centrifugation (3000 g, 5 min, 4 °C) the column was washed with 10 ml RW1 and twice with 10 ml RPE buffer (with 2 and 10 min centrifugation, respectively, at 3000 g at 4 °C). This was followed by addition of 800 µl of RNase-free water to elute the RNA. After 2 min the column was centrifuged at 4 °C for 3 min at 3000 g, followed by an additional elution with 800 µl of RNase-free water. The volume of the aqueous RNA solution was reduced to approximately 100–400 µl with a SpeedVac® (Savant DNA 110). Subsequently, 600 µl of 2 x T & C lysis buffer (Epicentre, Landgraaf, The Netherlands) was added and the mixture was kept on ice. After 5 min, 350 µl of MPC Protein Precipitation Reagent (Epicentre, Landgraaf, The Netherlands) was added, thoroughly mixed, and centrifuged (12.000 x g, 10 min, 4 °C) The supernatant was transferred to a clean micro-centrifuge tube and gently mixed with 1000 µl isopropanol. RNA was precipitated at 12.000 x g (10 min, 4 °C) and the pellet was air-dried for 5 min. The pellet was then resuspended in 100 µl RNase-free water. This was followed by addition of 700 µl of RLT buffer (without ß-mercaptoethanol) and 500 µl 96 % ethanol. RNA was further purified using the RNeasy® Mini kit (Qiagen) according to the RNA Cleanup protocol. The concentration of RNA was measured with the Nanodrop ND-1000 spectrophotometer (NanoDrop Tech., Wilmington, USA). The quality was assayed with an Agilent 2100 Bioanalyzer™, using an RNA Nano LabChip® (Agilent Technology, Palo Alto, CA, USA).

### cDNA labeling, microarray hybridisation and data analysis

cDNA labeling, microarray hybridisation, and scanning were performed at ServiceXS (Leiden, The Netherlands) according to Affymetrix protocols. In brief, 2 µg of total RNA was used to generate Biotin-labeled antisense cRNA with the Affymetrix Eukaryotic One-Cycle Target Labeling and Control reagents. Quality of the cRNA was assayed using the Agilent 2100 Bioanalyzer™. Subsequently, labeled cRNA of biological triplicates for each time point was used for the hybridisation of Affymetrix *A. niger* Genome Genechips (Pel *et al.* 2007). After an automated process of washing and staining, absolute values of expression were calculated from the scanned array using the Affymetrix Command Console v. 1 software. Arrays were globally scaled to a target value (TGT) of 100 using the average signal from all gene features using Microarray Suite v. 5.0. (MAS5.0) in Refiner Array Affymetrix IVT Arrays 5.2 of Genedata Expressionist (Basel, Switzerland). Arrays were then normalised on the median in Genedata Analyst (Basel Switzerland). The array data has been deposited in NCBI's Gene Expression Omnibus (Edgar *et al.* 2002) and is accessible through GEO Series accession number GSE36439 (www.ncbi.nlm.nih.gov/geo/). MAS5.0 detection calls were used to calculate the number of absent / present calls on each probe set. Filtering was performed by setting the value of all samples flagged A or M to a fixed value of 12 (Pepper *et al.* 2007). Genes that had a difference in expression ≥ 2-fold were considered as differentially expressed. Statistical assessment of differential expression between samples was performed with t-tests in Genedata Expressionist. A significance level of 0.01 was used and a Benjamini Hochberg False Discovery rate of p=0.05 (Benjamini & Hochberg 1995) was applied. The Functional Catalogue (FunCat; Munich Information Center for Protein Sequence) was used for functional classification of genes (Ruepp *et al.* 2004).

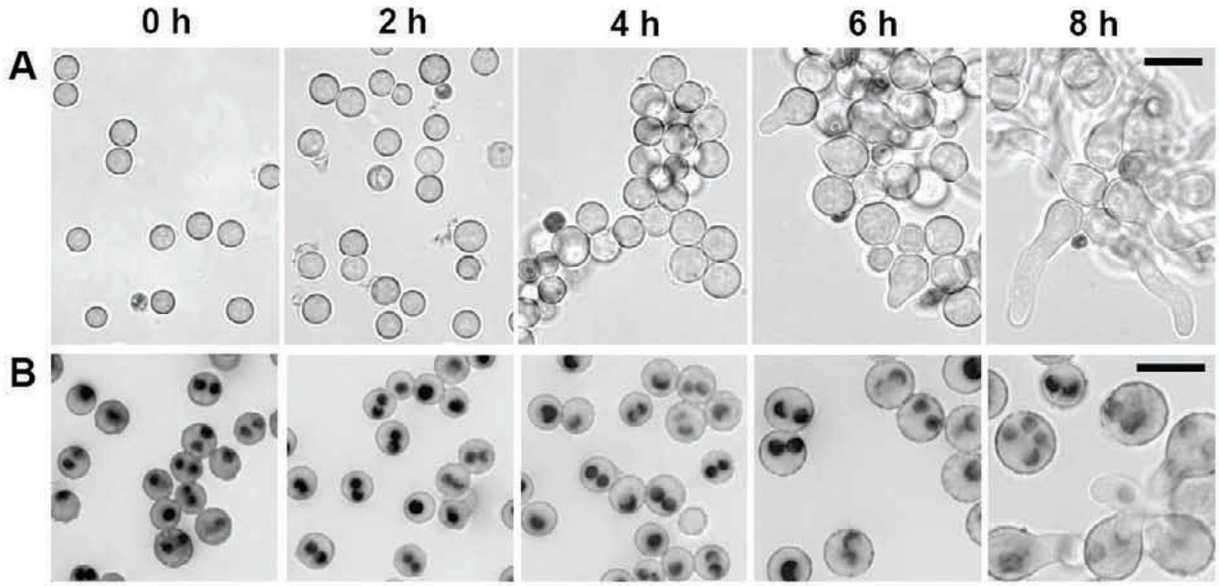



**Fig. 1.** Germination of *A. niger* conidia as observed by bright-field (A) and fluorescence microscopy (λ = 450–490 nm, FT510, LP520) (B). In (B) *A. niger* RB#9.5 was used. This strain expresses a gene encoding a fusion between sGFP and the histone H2B. The fusion protein is targeted to the nucleus. Bar represents 10 µm (A) and 5 µm (B).

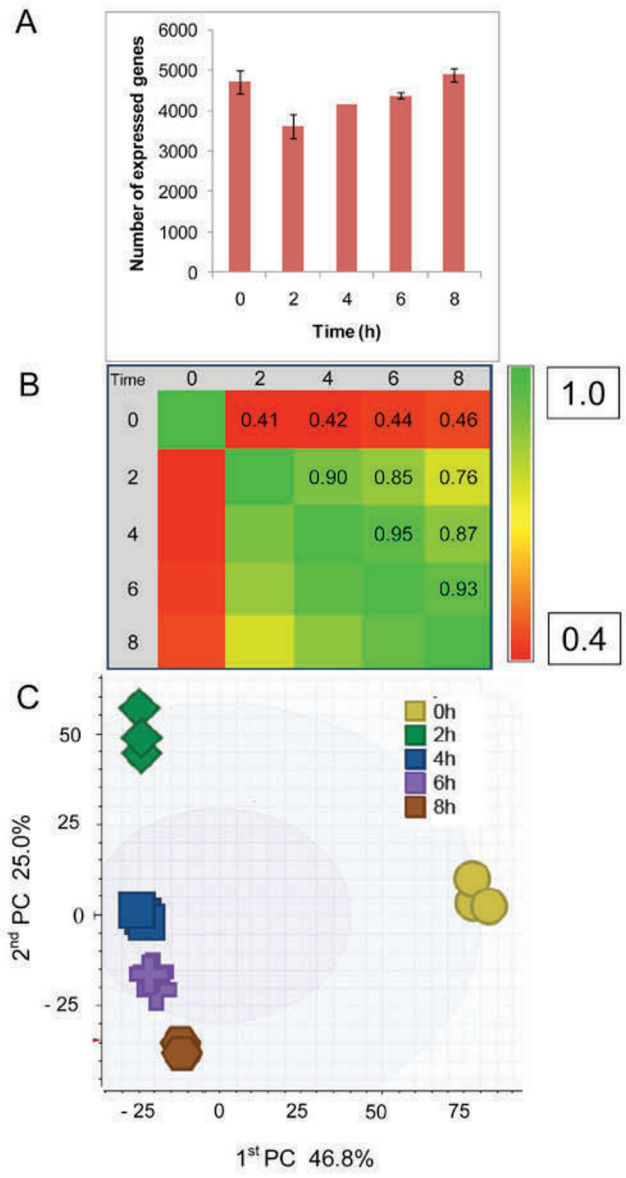



**Fig. 2**. The number of expressed genes during germination of conidia of *A. niger* and the similarity of the RNA profiles of the different stages of germination represented by correlation coefficients (B) and a principal component analysis (C).

## RESULTS

### Conidial germination

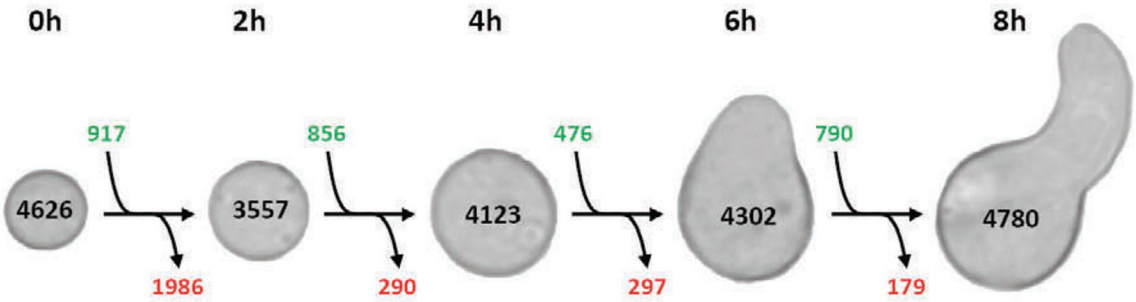
Conidial germination of *A. niger* has a maximal rate between 30 – 34 °C, with more than 90 % germination after 6 h (Abdel-Rahim & Arbab 1985). In this study *A. niger* was grown at 25 °C enabling us to separate the different stages of germination in time (Fig. 1A). Isotropic growth was observed between 2 and 6 h after inoculation and germ tubes were formed between 6 and 8 h. An *A. niger* reporter strain (RB#9.5) expressing a fusion of the H2B histone protein and the sGFP protein under control of the *mpdA* promoter was used to monitor nuclear division (Fig. 1B). Dormant conidia were predominately bi-nucleate (85 %), the remainder being uni-nucleate. Nuclear division was shown to occur between 6 and 8 h of germination. After 8 h, 42 % and 34 % of the germinating conidia contained 3 or 4 nuclei, respectively, 10 % and 14 % of the conidia still had 1 or 2 nuclei, respectively.

### Transcriptional profiling

Most methods for RNA isolation from fungal tissue are based on extraction with phenol or phenol based reagents like TRIzol® (Invitrogen, Breda, The Netherlands). Using this method we were unable to extract RNA from dormant conidia and from conidia during early stages of germination. Therefore, a novel RNA extraction method for conidia of *A. niger* was developed (see Materials and Methods) resulting in high quality intact RNA (see online Supplemental Fig. 1). This method was used to isolate RNA from dormant (0 h) and germinating (2, 4, 6, and 8 h) conidia. RNA from three independent biological replicates were used for hybridisation of *A. niger* Affymetrix microarray chips representing 14,259 open reading frames (Pel *et al.* 2007, Jacobs *et al.* 2009).

MAS5.0 detection calls were used to determine the number of expressed genes. Dormant conidia contained transcripts from 4626 genes (Fig. 2A, 3A). The number of expressed genes decreased to 3 557 after 2 h of germination. This was followed by a gradual increase to 4 780 genes 8 h after inoculation. Correlation of expression showed that the RNA profile of dormant conidia was

**A**




**B**

| | 0 h vs 2 h | 2 h vs 4 h | 4 h vs 6 h | 6 h vs 8 h |
|---|---|---|---|---|
| **up-regulated** | 1161 | 383 | 16 | 73 |
| **down-regulated** | 1959 | 45 | 3 | 16 |

**Fig. 3.** (A) Overview of the global changes in the transcriptome of conidia during germination. Inside the spore the number of expressed transcripts is given. Green and red numbers represent absent to present and present to absent, respectively, between two stages. (B) Number of genes with ≥ 2-fold change between the different stages of germination.

most different when compared to the other samples (Fig. 2B). The correlation coefficient of the profiles at 0 h and 8 h was 0.46. Correlation increased from 0.76 to 0.93 when the profiles of 2 h, 4 h, and 6 h were compared to that of 8 h. A principal component analysis (PCA) showed similar results (Fig. 2C). According to the PCA, the 0 h sample was substantially different from all other time points in that it contributes for the majority of the first principal component while the variation in the other time points was predominantly confined to the second principal component.

## Comparison of gene expression during conidial germination

In dormant conidia, 1986 genes were expressed that were subsequently absent at the 2h time point (Fig. 3A). These numbers were markedly lower (*i.e.* between 179 and 290) when the other stages were compared. A similar trend was obtained when the numbers of down-regulated genes with a fold change ≥ 2 were compared (Fig. 3B). In fact, the differences are even stronger. A number of 1 959 genes were down-regulated between 0 h and 2 h, whereas between 3 and 45 genes were down-regulated when the other stages were compared. The number of up-regulated genes was also highest between 0 h and 2 h (*i.e.* 1161 genes) when compared to the other stages (*i.e.* between 16 and 383 genes). This difference was less when the number of present calls was taken into account (Fig. 3A).

A Fisher exact test showed that transcripts belonging to the functional gene classes protein synthesis and protein fate are over-represented in the RNA profile of dormant spores. Transcripts belonging to the functional category protein synthesis and its subcategories translation and initiation were over-represented in the up-regulated genes at 2 h (Table 1). Moreover, the categories energy (including the sub-category respiration), cell cycle & DNA processing as well as transcription (and notably its sub-categories rRNA synthesis and rRNA processing) were overrepresented in the genes that were up-regulated at 2 h. Furthermore, subcategories of genes involved in nucleotide metabolism were over-represented in the up-regulated genes, while amino acid degradation was under-represented. Taken together, these data indicate that initiation of translation and respiration are key processes for initial stages of germination.

During later stages of germination (between 2 and 8 h of germination) the changes in expression of functional gene classes were smaller. In fact, no functional gene classes are over- or under-represented in the differentially expressed genes at 6 h and 8 h. The categories metabolism and cell cycle and DNA processing were over-represented in the up-regulated genes at 4 h. The latter suggests that the conidium prepared itself for mitosis which occurs a few hours later.

## Specific transcriptional changes associated to conidial germination

### *Regulation*

So far, asexual development has not been studied in *A. niger*. However, its genomic sequence predicts that mechanisms of asexual development are similar, if not identical, to that in *A. nidulans* (Pel *et al.* 2007, Krijgsheld *et al.* 2013). The expression of genes predicted to be involved in regulation of asexual development is given in Table 2. Levels of the master regulator of asexual development *brlA* (An01g10540) were very low in dormant conidia and absent in germinating conidia. Transcription factor genes that are operating more downstream from *brlA* including *medA* (An02g02150), *abaA* (An01g03750) and possibly *hymA* (An02g08420, Karos & Fisher 1999) and *dopA* (An02g08420, Pascon & Miller 2000) were present at higher levels than *brlA* and did show clear higher expression after 2 h (*medA*), a general trend of down regulation (*abaA*) or a general trend in upregulation (*hymA* and *dopA*). In contrast, stuA (An05g00480) was clearly down-regulated when germination was initiated. Genes that are predicted to directly activate genes involved in conidium formation and stress resistance (*i.e. wetA* (An01g08900), *atf1*(An14g06250, An02g07070, An12g10230) and *sakA* (An08g05850)) had high transcript levels in dormant conidia and invariably showed strong down-regulation.

**Table 1.** Over- (E) and under- (S) representation of functional gene classes in the pool of genes that are up- and down-regulated between t = 0 h and t = 2 h and between t = 2 h and t = 4 h after inoculation of conidia of *A. niger*.

| | 0h vs 2h | | 2h vs 4h |
|---|---|---|---|
| | UP | DOWN | UP |
| **01 METABOLISM** | | | E |
| 01.01.10 amino acid degradation (catabolism) | S | | |
| 01.02.01 nitrogen and sulfur utilisation | S | | |
| *01.03 nucleotide metabolism* | E | | |
| 01.03.01 purine nucleotide metabolism | E | | |
| 01.03.04 pyrimidine nucleotide metabolism | E | | |
| 01.05.01 C-compound and carbohydrate utilisation | S | | |
| 01.05.07 C-compound, carbohydrate transport | | | S |
| 01.20.05 biosynthesis of acetic acid derivatives | | | S |
| 01.20.05.01 biosynthesis of acetoacetate, acetone, hydroxybutyric acid | S | | |
| 01.20.35 biosynthesis of secondary products derived from L-phe and L-tyr | S | | |
| 01.20.37 biosynthesis of peptide derived compounds | S | | |
| **02 ENERGY** | E | | |
| 02.11.05 accessory proteins of electron transport and energy conservation | E | | |
| *02.13 respiration* | E | | |
| 02.13.03 aerobic respiration | E | | |
| **03 CELL CYCLE AND DNA PROCESSING** | E | | E |
| 03.01.03 DNA synthesis and replication | | | E |
| 03.03.01 mitotic cell cycle and cell cycle control | | | E |
| **04 TRANSCRIPTION** | E | | |
| 04.01.01 rRNA synthesis | E | | |
| 04.01.04 rRNA processing | E | | |
| 04.05.05 mRNA processing (splicing, 5'-, 3'-end processing) | | S | |
| 04.05.01 mRNA synthesis | S | | |
| **05 PROTEIN SYNTHESIS** | E | S | |
| *05.04 translation* | E | | |
| 05.04.01 initiation | E | | |
| **06 PROTEIN FATE (folding, modification, destination)** | E | E | S |
| 06.07.05 modification by ubiquitination, deubiquitination | S | | |
| 06.13.01 cytoplasmic and nuclear degradation | | | E |
| **11 CELL RESCUE, DEFENSE AND VIRULENCE** | S | | |
| **29 TRANSPOSABLE ELEMENTS, VIRAL AND PLASMID PROTEINS** | S | | |
| **40 SUBCELLULAR LOCALISATION** | E | | |
| **99 UNCLASSIFIED PROTEINS** | S | S | S |

FadA (An08g06130), SfaD (An18g02090) and FlbA (An02g03160) are members of one the signaling pathways that regulates the transition from vegetative growth to conidiation. Their genes are clearly expressed in germinating spores, but transcripts were also shown to be present in dormant spores. Gene *fadA,* which encodes an α-subunit of heterotrimeric G-proteins, was up-regulated after 2 hours of germination. The Gβ-subunit encoded by gene *sfaD* also showed a clear tendency in up-regulation during germination. Interestingly, *flbA*, which represses this signaling pathway showed a trend to down-regulation. Surprisingly, an α-subunit of the heterotrimeric G-proteins namely GanB (An08g05820), which is involved in conidial germination in *A. nidulans,* is lowly expressed in germinating conidia of *A. niger.* It signals via adenylate cyclase (An11g01520) and via the protein kinase PkaC (An02g04270) together with its regulator PkaR (An16g03740, Lafon *et al.* 2005)). The latter genes do exhibit characteristic expression patterns in *A. niger* conidia, including a very high accumulation of transcripts in dormant conidia, a strong drop at 2 hours of germination (but not to zero) and clear tendencies of up-regulation during further germination. This expression pattern is very similar to that of a gene which has a strong similarity to the Gpr1 receptor in yeast (An07g08810), which has a function as a nutrient sensing G-protein coupled receptor (Kraakman *et al.* 1999).

**Table 2.** Expression of regulatory genes in germinating conidia of *A. niger*. The normalised average values of three independent experiments are given. White to black shading indicate expression levels from absent (12 units of expression) to > 7000 expression units. If the outline of the boxes is dashed, the value of gene expression is significantly differentially expressed (> 2-fold) compared to the previous time point. SS = strong similarity; S = similarity; WS = weak similarity. Anid = *Aspergillus nidulans*; Anig = *Aspergilus niger*; Hsap = *Homo sapiens*; Mgri = *Magnaporthe grisea*; Ncra = *Neurospora crassa*; Pans = *Podospora anserina*; Spom = *Schizosaccharomyces pombe*; Scer = *Saccharomyces cerevisiae*.

| Name | Description | Dormant | 2 h | 4 h | 6 h | 8 h |
|---|---|---|---|---|---|---|
| An01g10540 | SS to developmental regulatory protein BrlA - Anid | 17 | 12 | 12 | 12 | 12 |
| An02g02150 | SS to Medusa (MedA) - Anid [truncated ORF] | 20 | 94 | 70 | 54 | 63 |
| An01g03750 | SS to protein AbaA - Anid | 86 | 76 | 61 | 51 | 27 |
| An05g00480 | SS to transcription factor involved in differentiation StuA - Anid | 115 | 12 | 12 | 23 | 60 |
| An02g08420 | SS to hypha-like metulae protein HymA - Anid | 33 | 27 | 60 | 92 | 96 |
| An01g08900 | SS to regulatory protein WetA - Anid | 548 | 26 | 31 | 26 | 26 |
| An14g06250 | WS to transcription factor Atf1+ - Spom | 163 | 23 | 17 | 19 | 26 |
| An02g07070 | SS to transcription factor Atf1 - Spom | 1024 | 42 | 69 | 69 | 69 |
| An12g10230 | S to ATF/CREB-family transcription factor Atf21 - Spom | 262 | 12 | 13 | 13 | 14 |
| An08g05850 | SS to osmotic sensitivity MAP kinase OSM1 (SakA) - Mgris | 1191 | 105 | 293 | 327 | 416 |
| An08g06130 | SS to GTP-binding regulatory protein alpha chain FadA - Anid | 300 | 879 | 588 | 601 | 677 |
| An08g05820 | SS to G protein alpha subunit Mod-D - Pans (ganB) | 66 | 16 | 22 | 29 | 28 |
| An18g02090 | SS to G-protein beta subunit SfaD - Anid | 65 | 95 | 100 | 122 | 144 |
| An02g03160 | SS to developmental regulator FlbA - Anid | 178 | 141 | 103 | 102 | 62 |
| An18g06110 | SS to related a-agglutinin core protein Aga1 - Ncra (RgsA) | 81 | 58 | 47 | 54 | 54 |
| An11g01520 | SS to adenylate cyclase Mac1 - Mgri | 532 | 70 | 78 | 100 | 131 |
| An02g04270 | cAMP-dependent protein kinase catalytic subunit PkaC - Anig | 263 | 17 | 74 | 81 | 100 |
| An16g03740 | cAMP-dependent protein kinase regulatory subunit PkaR - Anig | 800 | 20 | 87 | 128 | 262 |
| An07g08810 | SS to G protein-coupled receptor Gpr1 - Scer | 1800 | 87 | 140 | 171 | 132 |
| An02g01560 | WS to G protein-coupled receptor Edg-4 - Hsap (gprD) | 14 | 18 | 12 | 17 | 16 |
| An01g06290 | SS to hypothetical protein related to VeA - Ncra | 186 | 55 | 72 | 66 | 58 |
| An14g03390 | SS to FluG - Anid | 137 | 58 | 65 | 170 | 300 |
| An02g05420 | SS to putative zinc finger protein (FlbC) - Anid | 25 | 46 | 49 | 43 | 45 |
| An12g08230 | SS to zinc finger protein FlbC - Anid | 22 | 27 | 17 | 22 | 12 |
| An01g04830 | SS to myb-like DNA binding protein FlbD - Anid | 12 | 31 | 12 | 26 | 14 |
| An01g02320 | SS to GTP-binding protein A-ras - Anid | 38 | 152 | 161 | 288 | 359 |
| An05g00370 | SS to Ras-2 protein - Neurospora crassa | 15 | 12 | 12 | 12 | 12 |
| An02g08420 | SS to developm. reg. of asex. and sex. reproduction DopA - Anid | 32 | 20 | 49 | 66 | 107 |
| An01g10790 | SS to hypothetical conidiation-specific protein Con-10 - Ncras | 2061 | 30 | 57 | 100 | 99 |
| An04g02110 | S to Con-8 - Ncras | 7110 | 28 | 49 | 21 | 17 |
| An12g10240 | SS to conidiation-specific protein pCon-10a - Ncras | 2876 | 12 | 12 | 12 | 12 |

Gene *fluG* (An14g03390) encodes a protein that is involved in the production of an extracellular factor that leads to an up-regulation of the transcription factor gene *brlA*. Several *flb*-genes play a role in this upstream regulation of *brlA* and three An genes (An02g05420, An12g08230, An01g04830) are highly similar to these factors. These genes were only lowly expressed (one of the two *flbC* analogues is up-regulated during germination), while fluG exhibits clear tendencies of up-regulation after 8 h of germination. As noted above *brlA* itself is not expressed during germination. Up-regulation of *fluG* may fulfill another role in growth of *A. niger*.

Different studies have stressed the importance of a RasA signaling pathway during germination of *A. nidulans* conidia (Som & Kolaparthi 1994, Osherov & May 2000, Fillinger *et al.* 2001, Harispe *et al.* 2008). A gene similar to a RasA GTP-binding protein (An An01g02320, *rasA*) is strongly up-regulated 2 h after inoculation, while *rasB* (An05g00370) is not expressed at all during germination.

Three *A. niger* genes (An01g10790, An04g02110, An12g10240) are highly homologous to (late) conidiation factors of *N. crassa* (Roberts & Yanofsky 1989). These transcripts show high accumulation in dormant spores of *A. niger*, but were detected at much lower levels at all stages of germination.

### *Compatible solutes*

Trehalose and mannitol are needed to protect proteins and membranes against heat, drought and other stressors. These compatible solutes accumulate in dormant conidia and are degraded during germination (d'Enfert *et al.* 1999, Ruijter *et al.* 2004, Van Leeuwen *et al.* 2013). Conidia of *A. oryzae* and *A. nidulans* contain 0.7–1.4 pg trehalose per spore which is comparable to 2–4 % of the spore wet weight (d'Enfert & Fontaine 1997, Sakamoto *et al.* 2009). Trehalose biosynthesis occurs by the action of trehalose-6-phosphate synthase (TPS). It links UDP-glucose to glucose-6-

**Table 3.** Expression of genes involved in metabolism of compatible solutes. The normalised average values of three independent experiments are given. White to black shading indicate expression levels from absent (12 units of expression) to > 1200 expression units. For further explanation see Table 2. Smut = *Streptococcus mutans*.

| Name | Description | Dormant | 2 h | 4 h | 6 h | 8 h |
|---|---|---|---|---|---|---|
| An08g10510 | trehalose-6-phosphate synthase subunit 1 TpsA - Anig | 871 | 44 | 106 | 175 | 270 |
| An14g02180 | SS to trehalose-6-phosphate synthase TpsB - Anig | 456 | 16 | 59 | 69 | 134 |
| An07g08710 | α, α-trehalose-phosphate synthase 2 TpsB - Anig | 121 | 45 | 100 | 87 | 99 |
| An02g07770 | SS to trehalose synthase TSase - Grifola frondosa | 139 | 22 | 104 | 173 | 494 |
| An13g00400 | SS to reg. sub. treh-6-P synthase/phosphatase complex Tps3 - Scer | 392 | 14 | 19 | 21 | 45 |
| An07g08720 | SS to 123K chain α,α-trehalose-phosphate synthase Tsl1 - Scer | 286 | 19 | 31 | 19 | 27 |
| An11g10990 | SS to TPP of patent WO200116357-A2 - Scer | 96 | 104 | 123 | 136 | 198 |
| An01g09290 | SS to neutral trehalase (TreB) - Anid | 1203 | 17 | 60 | 87 | 178 |
| An01g01540 | SS to α,α-trehalase TreA - Anid | 22 | 31 | 42 | 66 | 329 |
| An02g05830 | SS to mannitol-1-phosphate 5-dehydrogenase MtlD - Smut | 140 | 16 | 27 | 30 | 153 |
| An15g05450 | SS to NADPH-dependent carbonyl reductase S1 - Candida magnoliae | 425 | 84 | 894 | 606 | 862 |
| An03g02430 | SS to mannitol dehydrogenase MtlD - Pseudomonas fluorescens | 645 | 36 | 77 | 119 | 200 |
| An02g07610 | SS to mannitol transporter Mat1 - Apium graveolens | 467 | 12 | 12 | 12 | 12 |

phosphate resulting in trehalose-6-phosphate (d'Enfert *et al.* 1999, Avonce *et al*. 2006). In the next step, the phosphate is removed by trehalose-6-phosphate phosphatase (TPP), which results in the formation of trehalose. Transcripts of *tpsA* (An08g10510), *tpsC* (An14g02180), *tppB* (An13g00400) and *tppC* (An07g08720) were found in dormant conidia (Table 3). Their levels dropped strongly 2 h after inoculation. Expression of *tpsA, tpsC* and *tppB* increased gradually after 2 h, while *tppC* was not up-regulated. Other predicted *tps* and *tpp* genes (*i.e.* An02g07770 and An11g10990) also showed a gradual increase during germination.

The transcript level of the gene encoding neutral trehalase (An01g09290) was high in conidia. This is the major enzyme needed for trehalose degradation during germination (d'Enfert *et al.* 1999). Transcript levels dropped dramatically during early germination and showed a clear increase during isotropic growth. The gene encoding acid trehalase (An01g01540) is involved in extracellular trehalose degradation during vegetative growth (d'Enfert & Fontaine 1997). Transcript levels of this gene are low in dormant conidia but clearly increase during germination. Taken together, transcripts of most trehalose-synthesising and degrading enzymes are relatively abundant in dormant conidia. After a strong decrease of the levels at 2 h, their expression gradually increases.

Mannitol is present in higher amounts than trehalose in *A. niger* conidia and makes up 10–15 % of the dry weight (Witteveen & Visser 1995, Ruijter *et al.* 2003). Mannitol dehydrogenase (MTD) converts mannitol into fructose and vice versa. Fructose enters glycolysis were it is converted via fructose-6-phosphate and fructose-1,6-diphosphate into glyceraldehyde-3-phosphate. Fructose-6-phosphate can be reduced to mannitol-1-phosphate by mannitol-1-phosphate dehydrogenase (MPD) or can enter glycerol metabolism. Transcripts of *mtdA* (An15g05450, R.P. de Vries, personal communication) and *mpdA* (An02g05830) were abundant in dormant spores (Table 3). Like the genes involved in synthesis and degradation of trehalose, levels of *mtdA* and *mpdA* initially strongly dropped, after which they gradually increased during germination.

### *Conidiation, heat shock proteins and other protective factors*

A number of abundant transcripts in dormant conidia are predicted to encode protective proteins (Table 4). The levels of these transcripts have dropped sharply at 2 h. Gene An02g07350 encodes a protein that is homologous to group 3 LEA proteins that protect seeds against drought stress (Chakrabortee *et al*. 2007, Tompa & Kovacs 2010). The putative protective proteins also include dehydrin-like proteins as described in *A. fumigatus* (An13g01110 and An14g05070, Wong Sak Hoi *et al.* 2011) and heat shock proteins. For instance, the protein encoded by An06p01610 is homologous to heat-shock protein Hsp9 of *Schizosaccharomyces pombe*. This protein is also very similar to Hsp12 of *S. cerevisiae* that has been designated as LEA-like and which has been shown to stabilise the plasma membrane (Sales *et al.* 2000). Gene An01g13350 encodes a homologue of the heat shock protein Hsp104, which together with trehalose provides acquired heat resistance when expressed in yeast cells (Elliot *et al.* 1996). A number of 10 other genes predicted to encode heat shock proteins (*e.g.* An15g05410, An07g09990 and An18g00600) also show high accumulation in dormant spores, but some are even further up-regulated at later stages (*e.g.* An16g09260, An11g00550, and An08g05300). Interestingly a transcript (An01g00160) that is predicted to be a regulator of the unfolded protein response (Hac1p in yeast) is also highly present in dormant conidia.

Catalase, superoxidase, glutathione, and thioredoxin also protect conidia by opposing oxidative stress that occurs during air transport or after rewetting of dried spores. Transcripts of genes similar to catalase encoding genes (*i.e.* An01g01830, An12g10720, An09g03130, and An08g08920) were highly present in dormant conidia, but to a much lesser extent in germinating spores. Gene An07g03770, which is predicted to encode a superoxide dismutase, had high mRNA levels in dormant cells. After an initial sharp drop, mRNA levels of this gene increased again 4 h after inoculation. Transcripts of genes involved in the synthesis of glutathione (*i.e.* An02g06560, An01g15190 and An09g06270) were highly represented in dormant conidia, but were lowly expressed in germinating spores. In contrast, genes predicted to encode thioredoxin showed similar levels of transcripts in dormant and germinating conidia. Taken together, mRNA of genes encoding catalase, superoxide dismutase and genes involved in the synthesis of glutathione and thioredoxin are abundant in dormant conidia and show a strong drop after start of germination. These data suggest that stress resistance of conidia may drop strongly very early during germination.

**Table 4.** Expression of genes involved in the production of protective proteins and enzymes involved in the prevention of oxidative stress. The normalised average values of three independent experiments are given. White to black shading indicate expression levels from absent (12 units of expression) to > 6500 expression units. For further explanation see Table 2. Mmar = *Methylobacter marinus*; Nmen = *Neisseria meningitidis*; Pchry = *Penicillium chrysogenum*; Zmay = *Zea mays*.

| Name | Description | Dormant | 2 h | 4 h | 6 h | 8 h |
|---|---|---|---|---|---|---|
| An02g07350 | WS to group 3 Lea protein Mgl3 - Zmay | 4559 | 20 | 122 | 72 | 74 |
| An13g01110 | S to hypothetical protein An14g05070 (dehydrin) - Anig | 550 | 15 | 32 | 19 | 18 |
| An14g05070 | WS to heterokaryon incompatibility protein Het-C (dehydrin) - Ncra | 785 | 12 | 106 | 19 | 13 |
| An06g01610 | SS to the heat shock protein Hsp9p - Spom | 4577 | 80 | 1248 | 525 | 460 |
| An01g13350 | SS to heat shock protein Hsp104 - Scer | 385 | 13 | 28 | 40 | 61 |
| An15g05410 | SS to heat-shock protein Hsp30- Anid | 1275 | 31 | 27 | 32 | 30 |
| An18g06650 | SS to heat shock protein Hsp30 - Anid | 610 | 37 | 93 | 118 | 97 |
| An07g09990 | SS to heat shock protein Hsp70 - Ajellomyces capsulata | 4139 | 1247 | 2733 | 2140 | 1895 |
| An03g00400 | S to the heat shock protein Hsp42 - Scer | 61 | 23 | 28 | 21 | 19 |
| An11g08220 | S to heat shock protein Hsp70 patent WO200034465-A2 - Nmen | 476 | 12 | 12 | 12 | 12 |
| An18g00600 | SS to heat shock protein Hsp30 - Ncras [truncated ORF] | 513 | 12 | 12 | 38 | 12 |
| An11g00550 | SS to chaperonin Hsp10 - Scer | 303 | 537 | 704 | 647 | 685 |
| An08g05300 | SS to heat shock protein Hsp70 Pss1+ - Spom | 251 | 913 | 803 | 692 | 558 |
| An12g04940 | SS to mitochondrial heat shock protein Hso60 - Scer | 522 | 1011 | 1211 | 1158 | 1036 |
| An16g09260 | SS to DnaK-type molecular chaperone Ssb2 - yeast Scer | 3585 | 6676 | 6741 | 6299 | 6103 |
| An08g03480 | SS to the mitochondrial heat shock protein Hsp78p - Scer | 618 | 57 | 201 | 217 | 361 |
| An01g00160 | S to regulator of unfolded protein response (UPR) Hac1p - Scer | 999 | 219 | 244 | 441 | 619 |
| An08g08920 | SS to catalase C CatC - Anid | 78 | 24 | 56 | 29 | 29 |
| An09g03130 | SS to catalase CatA - Anid | 3106 | 24 | 34 | 29 | 37 |
| An12g10720 | SS to catalase Cat - Methanosarcina barkeri | 1978 | 12 | 12 | 12 | 12 |
| An01g01830 | SS to catalase/peroxidase CpeB - Streptomyces reticuli | 555 | 27 | 49 | 49 | 61 |
| An07g03770 | SS to Cu,Zn superoxide dismutase SodC - Afum | 3091 | 12 | 529 | 856 | 1380 |
| An02g06560 | SS to glutathione S-transferase IsoJ - Rhodococcus sp. | 379 | 12 | 49 | 75 | 185 |
| An01g15190 | SS to glutathione-dependent formaldehyde dehydrogenase Fdh - Mmar | 568 | 12 | 12 | 12 | 12 |
| An09g06270 | SS put.glutath.-depend. formald. dehydrogen. SPBC1198.01 - Spom | 6489 | 26 | 398 | 239 | 323 |
| An16g06100 | S to glutathione S-transferase Gst1 - Ascaris suum | 64 | 12 | 28 | 18 | 12 |
| An13g02540 | SS to glutathione S-transferase Gtt1 - Scer | 150 | 14 | 16 | 39 | 176 |
| An02g08110 | SS to glutathione peroxidase Hyr1 - Scer | 251 | 80 | 132 | 193 | 337 |
| An01g02500 | SS to thioredoxin - Anid | 1002 | 385 | 1141 | 1642 | 2018 |
| An01g08570 | SS to thioredoxin reductase TrxB - Pchry | 77 | 139 | 148 | 135 | 128 |
| An15g07230 | S to mitochondrial thioredoxin of patent WO9832863-A2 - Rattus sp. | 194 | 22 | 20 | 12 | 15 |
| An03g02980 | SS to thioredoxin - Anid | 65 | 154 | 206 | 191 | 242 |

### *Cell wall modulation*

Conidia of *A. niger* possess a relatively thick, layered cell wall, of which the pigmented outer cell wall is shed during germination (Tiedt 1982). The spores contain complex melanin pigments (see Krijgsheld *et al.* 2012). Transcripts of three genes (An 03g03750, An09g05730, An14g05350) of the melanin synthesis pathway (Jorgenson *et al.* 2010) were present in dormant conidia but disappeared during germination. Transcripts of five out of seven genes that code for proteins with similarity to hydrophobins are highly accumulated in dormant conidia and drop strongly upon activation of germination.

Transcripts of genes encoding cell wall degrading and synthesising enzymes were present at every stage of germination (Table 5). A transcript similar to a GPI-anchored chitinase ChiA (An09g06400, Yamazaki *et al*. 2008) was most highly expressed 6–8 h after inoculation. This enzyme is associated with polarised growth in *A. nidulans,* which makes sense while germ tubes were formed 6–8 h after inoculation. Gene An04g1430 which also has strong similarity to ChiA was highly expressed 8 h after inoculation but transcripts were also abundant in dormant conidia. This suggests an active role during other processes, for instance during spore formation. Chitin synthases are the counterparts of chitinases and reported to be present in the fungal cell (Horiuchi 2009). Transcripts of An07g05570 (*chs1, A. nidulans*), An09g04010 (*chsC, A. fumigatus*) and An12g10380 (*chsC, A. fumigatus*) accumulated 2 h after inoculation, whereas transcripts of An02g02340 and 02360 were present at all stages. The latter genes were similar to *csmA*, a class V chitin synthase with a myosin motor domain, which has been associated with hyphal tip growth (Takeshita *et al*. 2005).

Transcripts of two genes that encode glucanases that degrade glucan in the cell wall were observed during germination; one gene (An08g10740) is up-regulated during germination while the other (An12g09130) showed its highest accumulation in dormant

**Table 5.** Expression of genes involved in the production of enzymes involved in cell wall synthesis or processing. The normalised average values of three independent experiments are given. White to black shading indicate expression levels from absent (12 units of expression) to > 7100 expression units. For further explanation see Table 2. Afum = *Aspergillus fumigatus;* Cabi = *Candida albicans*; Ccin = *Coprinopsis cinerea*, Cmin = *Coniothyrium minitans;* Pbra = *Paracoccidioides brasiliensis*, Tree = *Trichoderma reesei.*

| Name | Description | Dormant | 2 h | 4 h | 6 h | 8 h |
|---|---|---|---|---|---|---|
| An14g05350 | SS to yellowish-green 1 Ayg1 - Afum | 111 | 24 | 25 | 15 | 12 |
| An09g05730 | SS to polyketide synthase Alb1 - Afum | 46 | 12 | 12 | 17 | 21 |
| An03g03750 | SS to brown 2 Abr2 - Afum | 59 | 14 | 14 | 16 | 19 |
| An03g02360 | S to the spore-wall fungal hydrophobin DewA - Anid | 1286 | 32 | 15 | 12 | 12 |
| An03g02400 | SS to the spore-wall fungal hydrophobin DewA - Anid | 3110 | 89 | 60 | 36 | 34 |
| An04g08500 | SS to rodletless protein RodA - Anid | 252 | 19 | 16 | 17 | 18 |
| An12g05020 | S to hydrophobin Hfbl - Tree | 12 | 12 | 12 | 12 | 64 |
| An07g03340 | SS to hydrophobin Hyp1 - Afum | 812 | 92 | 63 | 48 | 35 |
| An08g09880 | WS to hydrophobin Coh1 - Ccin | 159 | 17 | 12 | 12 | 12 |
| An09g06400 | SS to chitinase ChiA - Anid | 32 | 35 | 170 | 805 | 1321 |
| An04g01430 | WS to the chitinase ChiA - Anid | 987 | 28 | 123 | 310 | 1341 |
| An06g01000 | SS to protein related to chitinase 3 precursor - Ncra | 68 | 162 | 768 | 588 | 634 |
| An07g05570 | SS chitin synthase Chs1 - Anid | 20 | 157 | 40 | 37 | 52 |
| An09g04010 | SS to chitin synthase ChsC - Afum | 145 | 345 | 134 | 128 | 160 |
| An12g10380 | SS to chitin synthase C ChsC - Afum | 100 | 830 | 372 | 344 | 411 |
| An02g02340 | SS to the chitin synthase with a myosin motor-like domain CsmA - Anid | 226 | 188 | 232 | 227 | 281 |
| An02g02360 | SS to CsmA - Anid [truncated ORF] | 76 | 174 | 163 | 175 | 213 |
| An09g02290 | SS to chitin synthase ChsE - Anid | 29 | 12 | 14 | 46 | 107 |
| An08g10740 | SS to ZmGnsN3 glucanase of patent WO200073470-A2 - Zmay | 177 | 785 | 473 | 597 | 617 |
| An12g09130 | S to glucanase ZmGnsN3 of patent WO200073470-A2 - Zmay | 333 | 24 | 135 | 205 | 454 |
| An06g01550 | SS to glucan synthase Fks - Pbra | 181 | 2417 | 1597 | 1490 | 1737 |
| An17g02120 | SS to 1,3-beta-glucan synthase Gs-1 - Ncra | 68 | 132 | 111 | 120 | 119 |
| An09g03070 | SS to alpha-glucan synthase Mok1 - Spom | 432 | 558 | 481 | 333 | 357 |
| An04g09890 | SS to cell wall alpha-glucan synthase Ags1 - Spom | 14 | 105 | 45 | 17 | 19 |
| An11g07660 | S to exo-1,3-beta-glucanase Xog - Cabi | 71 | 33 | 52 | 77 | 117 |
| An03g05290 | S to glucan 1,3-beta-glucosidase Bgl2 - Scer | 23 | 614 | 215 | 134 | 151 |
| An07g04650 | S to exo-beta-1,3-glucanase Bgl2 - Scer | 233 | 12 | 12 | 12 | 29 |
| An19g00090 | SS to the exo-beta-1,3-glucanase Cmg1 - Cmin | 36 | 67 | 213 | 628 | 1297 |
| An16g06800 | SS to endoglucanase EglB - Anig | 34 | 30 | 30 | 51 | 127 |
| An03g06220 | SS to beta (1-3) glucanosyltransferase Gel3 - Afum | 12 | 12 | 12 | 27 | 98 |
| An16g02850 | SS to cell wall glycosidase Crh1 - Scer | 69 | 62 | 52 | 112 | 142 |
| An01g11010 | SS to the cell wall protein Crh1 - Scer | 54 | 1256 | 78 | 84 | 364 |
| An07g01160 | SS to cell wall protein Utr2 - Scer | 12 | 108 | 102 | 83 | 109 |
| An07g07530 | SS to cell wall protein Utr2 - Scer | 80 | 795 | 692 | 660 | 793 |
| An08g03580 | SS to 1,3-beta-glucanosyltransferase Bgt1 - Afum | 7135 | 319 | 104 | 75 | 55 |
| An10g00400 | SS to beta(1-3)glucanosyltransferase Gel1 - Afum | 36 | 716 | 455 | 480 | 751 |
| An16g07040 | S to beta-1,3-glucanosyltransferase Bgt1 - Afum [truncated ORF] | 14 | 82 | 481 | 1148 | 2037 |

conidia and 8 h old germlings. A gene similar to a glucan synthase of *Paracoccidioides brasiliensis* (Sorais *et al*. 2010, An06g01550) was highly up-regulated 2 h after inoculation and levels remained high up to 8 h. Another gene that is predicted to encode a glucan synthase (An09g03070, similar to *mok1* of *S. pombe*, Katayama *et al*. 1999) had similar expression levels throughout germination. Other genes shown in Table 5 are related to cell wall processing. Their encoding proteins make cross-links between 1,6- and 1,3 glucans and between glucans and chitin (see Fontaine *et al.* 1997, Rodríguez-Peña *et al.* 2000, Cabib 2009). These genes had accumulated transcripts in dormant conidia (An08g03580), after 2 h or 8 h. During all stages of germination, specific activities of enzymes can be seen, but most are highly expressed after 2 h.

## DISCUSSION

In this study the transcriptome of dormant and germinating conidia of the fungus *A. niger* was studied. In fact, this is the first report describing a whole genome expression analysis of dormant and germinating conidia within the class Eurotiomycetes. This class contains, among others, the genera *Aspergillus* and

*Penicillium*. The data show that the RNA profile of dormant conidia is substantially different when compared to all other stages of germination, each of which is characterised by a typical morphology. A transcriptome reorganisation was shown to take place before the stage of isotropic growth, after which RNA profiles changed gradually. These changes are illustrated by the correlation coefficients of the profiles of dormant conidia and germinating conidia 8 h after inoculation (0.46) and those of conidia 2, 4 and 6 h after inoculation with that of 8 h after inoculation (0.76 to 0.93).

## Dormant conidia

About half of the 14 253 genes are expressed in a vegetative mycelium of *A. niger* (Levin *et al.* 2007), while approximately 40 % of the genes are active in the aerial structures (*i.e.* aerial hyphae, conidiophores, conidiospores) (Bleichrodt *et al.* 2013). Transcripts of 33 % of the genes were detected in dormant conidia, which is in good agreement with the finding that 42 % and 27 % of the genes had transcripts in dormant conidia of *Fusarium graminearum* (Seong *et al.* 2008) and *Aspergillus fumigatus* (Lamarre *et al.* 2008). The lower complexity of the RNA in conidia when compared to the vegetative mycelium and the aerial structures is explained by the fact that these spores represent a single cell type. In contrast, vegetative mycelium and aerial structures consist of different types of hyphae and cells (Krijgsheld *et al*. 2013). For instance, the vegetative mycelium consists of hyphae that differ in age, in morphology and in the environmental conditions they are exposed to. In fact, even expression profiles of neighboring hyphae are highly different (Vinck *et al.* 2005, 2011, De Bekker *et al.* 2011). It would be interesting to assess to which extent dormant spores are also heterogenic with respect to their RNA profiles.

Lamarre *et al.* (2008) showed that the RNA profile of dormant conidia of *A. fumigatus* only changes marginally during a storage period of one year. It is thought that the mRNAs in dormant conidia function as a pool of pre-packed mRNAs primed for translation (Osherov *et al.* 2002, Lamarre *et al.* 2008). This would enable the conidium to respond quickly and specific after the onset of germination. Indeed, a Fisher exact test showed that transcripts belonging to the functional gene classes protein synthesis and protein fate were overrepresented in the RNA profile of dormant spores. It should be noted that a large number of transcripts are not expected to have a role during germination but rather would have functioned during the formation of the conidia. This would explain why more than 40 % of the genes (*i.e.* 1986 out of 4626) with transcripts in dormant conidia are no longer active 2 h after inoculation. In *A. niger* this set includes genes predicted to protect the conidium against dehydration, freezing, heat, UV-radiation, and other stressors such as reactive oxygen species. For instance, transcripts of genes predicted to encode protective proteins such as the LEA-like proteins (Battaglia *et al.* 2008) and dehydrins (Wong Sak Hoi *et al.* 2011) had highly accumulated in conidia. Also transcripts of genes predicted to be involved in the synthesis and degradation of compatible solutes were specific for dormant spores. Another example is the genes involved in the fortification of the spore cell wall such as the genes encoding hydrophobins and genes involved in pigmentation. Transcripts of the genes of transcription factors that are important for spore formation and stress resistance were found in dormant spores but were absent after breaking of dormancy.

## Germinating conidia

Lamarre *et al.* (2008) studied changes in expression profiles during early germination of *A. fumigatus* conidia by means of macro-arrays that covered approximately 3 000 genes. Differential expression of near 800 genes (80 % being up-regulated) was observed after 30 min of germination at 37 °C. In our study, a whole genome expression analysis was performed during early germination. The first two h after inoculation (*i.e.* before the stage of isotropic growth) is characterised by disappearance of transcripts. As mentioned above, transcripts of 1986 genes were no longer detected. On the other hand, 917 genes became active. Transcripts belonging to the functional gene classes protein synthesis and its subcategories translation and initiation were over-represented in the up-regulated genes. Moreover, the categories energy (including the sub-category respiration), cell cycle & DNA processing as well as transcription (with its sub-categories rRNA synthesis and rRNA processing) were over-represented. On the other hand, genes involved in mRNA processing were under-represented in the up-regulated genes. Taken together, the composition of the RNA profiles of the dormant conidium and conidia 2 h after inoculation indicate that protein synthesis is key during early germination. A similar phenomenon has been described for *A. fumigatus* (Lamarre *et al.* 2008). The importance of protein synthesis in early stages of germination is also indicated by the fact that the protein synthesis inhibitor cycloheximide prevents isotropic growth, while inhibitors of the cytoskeleton and DNA- and RNA synthesis had no effect (Osherov & May 2000).

In this study, the distinct morphological changes that occur during germination are not correlated with the highest change in the transcriptome. This is of interest as Kasuga *et al*. (2005) concluded that transcriptional and morphogenetic change during conidial germination are highly coupled in case of *N. crassa.* Two h after inoculation, transcripts of only 3 557 genes were present in the conidia of *A. niger*. This number increased to 4 780 8 h after inoculation. Differential expression of genes was relatively low. Between 16 and 383 genes were ≥ 2-fold up-regulated between 2 and 4 h, 4 and 6 h, and 6 and 8 h. On the other hand only 3–45 genes were ≥ 2-fold down-regulated during these stages. The minor changes in gene expression is also illustrated by the fact that during 2 and 8 h post-inoculation only the categories metabolism and cell cycle and DNA processing were over-represented in the up-regulated genes between 2 and 4 h. The latter suggests that the conidium prepared itself for mitosis which indeed occurred a few hours later. Taken together, germination of *A. niger* is typified by one large transcriptional transition (*i.e.* during the first two h after inoculation). Germination in the protozoan *Dictyostelium discoidium* is also characterised by such a transcriptional transition (Xu *et al.* 2004).

Further studies on conidial germination should provide mechanisms underlying the transition of the RNA profile early during spore germination. Perturbation of early germination with natamycine showed that transcriptome reorganisation occurs to a similar scale despite the presence of the antifungal (Van Leeuwen *et al.* 2013). This suggests that transcriptome reorganisation is a relatively endogenous process that plays an important role in the transition from a stabilised fungal conidium towards a vegetative growing cell.

## ACKNOWLEDGEMENTS

MRVL was supported by The Netherlands Technology Foundation (STW) Open technology project UBC.6524. The authors thank Ferry Hagen, Timon Wyatt and Frank Segers (at the CBS-KNAW Fungal Biodiversity Centre), Jan Grijpstra (Utrecht

University) for advice during this study. Eefjan Breukink, Yvonne te Welscher and Ben de Kruijff (Department of Biochemistry of Membranes, Utrecht University, The Netherlands) are thanked for fruitful discussions throughout this study.

## REFERENCES


Abdel-Rahim AW, Arbab HA (1985). Factors affecting spore germination in *Aspergillus niger*. *Mycopathologia* **89**: 75–79.

Avonce N, Mendoza-Vargas A, Morett E, Iturriaga G (2006). Insights on the evolution of trehalose biosynthesis. *BMC Evolutionary Biology* **6**: 109–120.

Bartnicki-Garcia S, Lippman E (1977). Polarization of cell wall synthesis during spore germination of *Mucor rouxi*. *Experimental Mycology* **1**: 230–240.

Battaglia M, Olvera-Carrillo Y, Garciarrubio A, Campos F, Covarrubias AA (2008). The enigmatic LEA proteins and other hydrophilins. *Plant Physiology* **148**: 6–24.

Bekker C de, Bruning O, Jonker MJ, Breit TM, Wösten HAB (2011).Single cell transcriptomics of neighboring hyphae of *Aspergillus niger. Genome Biology* **12**: R71.

Benjamini Y, Hochberg Y (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society, Series B (Methodological)* **57**: 289–300.

Bleichrodt R, Vinck A, Krijgsheld P, Leeuwen MR van, Dijksterhuis J, Wösten HAB (2013). Cytoplasmic streaming in vegetative mycelium and aerial structures of *Aspergillus niger. Studies in Mycology* **74**: 31–46.

Bos CJ, Debets AJM, Swart K, Huybers A, Kobus G, Slakhorst SM (1988). Genetic analysis and the construction of master strains for assignment of genes to six linkage groups in *Aspergillus niger*. *Current Genetics* **14**: 437–443.

Burrell R (1991). Microbiological agents as health risks in indoor air. *Environmental Health Perspectives* **95**: 29–34.

Cabib E (2009). Two novel techniques for determination of polysaccharide cross-links show that Crh1p and Crh2p attach chitin to both β (1-6)- and β (103) glucan in the *Saccharomyces cerevisiea* cell wall. *Eukaryotic Cell* **11**: 1626–1636.

Chakrabortee S, Boschetti C, Walton LJ, Sarkar S, Rubinsztein DC, Tunnacliffe A (2007) Hydrophilic protein associated with desiccation tolerance exhibits broad protein stabilization function. *PNAS* **104**: 18073–18078.

D'Enfert C (1997). Fungal spore germination: insights from the molecular genetics of *Aspergillus nidulans* and *Neurospora crassa*. *Fungal Genetics and Biology* **21**: 163–172.

D'Enfert C, Fontaine T (1997). Molecular characterization of the *Aspergillus nidulans* treA gene encoding an acid trehalase required for growth on trehalase. *Molecular Microbiology* **24**: 203–216.

D'Enfert C, Bonini BM, Zapella PD, Fontaine T, da Silva AM, Terenzi HF (1999). Neutral trehalases catalyse intracellular trehalose breakdown in the filamentous fungi *Aspergillus nidulans* and *Neurospora crassa*. *Molecular Microbiology* **32**: 471–483.

Dijksterhuis J, Nijsse J, Hoekstra FA, Golovina EA (2007). High viscosity and anisotropy characterize the cytoplasm of fungal dormant stress-resistant spores. *Eukaryotic Cell* **6**: 157–170.

Edgar R, Domrachev M, Lash AE (2002). Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Research* **30**: 207–210.

Elliott B, Haltiwanger RS, Futcher B (1996). Synergy between trehalose and Hsp104 for thermotolerance in *Saccharomyces cerevisiae*. *Genetics* **144**: 923–933.

Fillinger S, Chaveroce MK, Dijck P van, Vries RP de, Ruijter G, Thevelein J, d'Enfert C (2001). Trehalose is required for the acquisition of tolerance to a variety of stresses in the filamentous fungus *Aspergillus nidulans*. *Microbiology* **147**: 1851–1862.

Fontaine T, Hartland RP, Diaquin M, Simenel C, Latgé JP (1997). Differential patterns of activity displayed by two exo-β-1,3-glucanases associated with the *Aspergillus fumigatus* cell wall. *Journal of Bacteriology* **179**: 3154–3163.

Frisvad JC, Thrane U, Samson RA (2007). Mycotoxin producers. In: *Food Mycology: A multifaceted approach to fungi and food*. Dijksterhuis J, Samson RA, eds. CRC press, Taylor & Francis group, Boca Raton: 135–160.

Glass NL, Rasmussen C, Roca MG, Read ND (2004). Hyphal homing, fusion and mycelial interconnectedness. *Trends in Microbiology* **12**: 135–141.

Harris SD, Momany M (2004). Polarity in filamentous fungi: moving beyond the yeast paradigm. *Fungal Genetics and Biology* **41**: 391–400.

Harris SD (2006). Cell polarity in filamentous fungi: shaping the mold. *International Reviews of Cytology* **251**: 41–77.

Harispe L, Portela C, Scazzocchio C, Peñalva MA, Gorfinkiel L (2008). Ras GTPase-Activating Protein Regulation of Actin Cytoskeleton and Hyphal Polarity in Aspergillus nidulans. *Eukaryotic Cell* **7**: 141–153.

Horiuchi H (2009) Functional diversity of chitin synthases of *Aspergillus nidulans* in hyphal growth, conidiophore development and septum formation. *Medical Mycology* **47** (Supplement I): 547–552.

Jacobs DI, Olsthoorn MMA, Maillet I, *et al.* (2009). Effective lead selection for improved protein production in *Aspergillus niger* based ion integrated genomics. *Fungal Genetics and Biology* **46**: S141–S152.

Jørgensen TR, Park J, Arentshorst M, Welzen AM van, Lamers G, *et al.*(2011). The molecular and genetic basis of conidial pigmentation in *Aspergillus niger. Fungal Genetics and Biology* **48**: 544–553.

Karos M, Fischer R (1999). Molecular characterization of HymA, an evolutionarily highly conserved and highly expressed protein of *Aspergillus nidulans*. *Molecular and General Genetics* **260**: 510–521.

Kasuga T, Townsend JP, Tian C, Gilbert LB, Mannhaupt G, Taylor JW, Glass NL (2005). Long-oligomer microarray profiling in *Neurospora crassa* reveals the transcriptional program underlying biochemical and physiological events of conidial germination. *Nucleic Acid Research* **33**: 6469–6485.

Katayama S, Hirata D, Arellano M, Pérez P, Toda T (1999). Fission yeast α-glucan synthase Mok1 requires the actin cytoskeleton to localize the sites of growth and plays an essential role in cell morphogenesis downstream of protein kinase C function. *Journal of Cell Biology* **144**: 1173–1186.

Köhli M, Galati V, Boudier K, Roberson RW, Philippsen P (2008). Growth-speed-correlated localization of exocyst and polarisome components in growth zones of *Ashbya gossypii* hyphal tips. *Journal of Cell Science* **121**: 3878–3889.

Kraakman L, Lemaire K, Ma P, Teunissen AW, Donaton MC, Dijck P van, Winderickx J, Winde JH de, Thevelein JM (1999). A *Saccharomyces cerevisiae* G-protein coupled receptor, Gpr1, is specifically required for glucose activation of the cAMP pathway during the transition to growth on glucose. *Molecular Microbiology* **32**: 1002–1012.

Krijgsheld P, Bleichrodt RJ, Veluw GJ van, Wang F, Müller WG, *et al*. (2013). Development of *Aspergillus*. *Studies in Mycology* **74**: 1–29.

Lafon, A, Seo, JA, Han, KH, Yu, JH, d'Enfert, C (2005). The heterotrimeric G-protein GanB(alpha)-SfaD(beta)-GpgA(gamma) is a carbon source sensor involved in early cAMP-dependent germination in *Aspergillus nidulans*. *Genetics* **171**: 71–80.

Lamarre C, Sokol S, Depeaupuis JP, Henry C, Lacroix C, Glaser P, Coppée JY, Francois JM, Latgé JP (2008). Transcriptomic analysis of the exit from dormancy of *Aspergillus fumigatus* conidia. *BMC Genomics* **9**: 417–422.

Leeuwen MR van, Smant W, Boer W de, Dijksterhuis J (2008). Filipin is a reliable *in situ* marker of ergosterol in the plasma membrane of germinating conidia (spores) of *Penicillium discolor* and stains intensively at the site of germ tube formation. *Journal of Microbiological Methods* **74**: 64–73.

Leeuwen MR van, Doorn TM van, Golovina EA, Stark J, Dijksterhuis J (2010). Water- and air-distributed conidia differ in sterol content and cytoplasmic microviscosity. *Applied and Environmental Microbiology* **76**: 366–369.

Leeuwen MR van, Krijgsheld P, Wyatt TT, Golovina EA, Menke H, Dekker A, Stark, J, Stam H, Bleichrodt RJ, Wösten HAB, Dijksterhuis J (2013). The effect of natamycin on the transcriptome of conidia of *Aspergillus niger*. *Studies in Mycology* **74**: 71–85.

Levin AM, Vries RP de, Conesa A, Bekker C de, Talon M, Menke HH, Peij NN van, Wösten HAB (2007). Spatial differentiation in the vegetative mycelium of *Aspergillus niger*. *Eukaryotic Cell* **6**: 2311–2322.

Liebmann B, Muller M, Braun A, Brakhage AA (2004). The cyclic AMP-dependent protein kinase a network regulates development and virulence in *Aspergillus fumigatus*. *Infection and Immunity* **72**: 5193–5203.

Meyer V, Wu B, Ram AF (2011). *Aspergillus* as a multi-purpose cell factory: current status and perspectives. *Biotechnology Letters* **33**: 469–476.

McCartney A, West J (2007). Dispersal of fungal spores through air. In: *Food Mycology: A multifaceted approach to fungi and food.* Dijksterhuis J, Samson RA, eds. CRC press, Taylor & Francis group, Boca Raton: 65–81.

Mirkes PE (1974). Polysomes, ribonucleic acid, and protein synthesis during germination of *Neurospora crassa* conidia. *Journal of Bacteriology* **117**: 196–202.

Momany M (2002). Polarity in filamentous fungi: establishment, maintenance and new axes. *Current Opinion in Microbiology* **5**: 580–585.

Navarro-Bordonaba J, Adams TH (1996). Development of conidia and fruiting bodies in Ascomycetes. In: *The Mycota. I. Growth, Differentiation and Sexuality* Wessels JGH, Meinhardt F, eds. Springer-Verlag, Berlin: 333–349.

Osherov N, May GS (2000). Conidial germination in *Aspergillus nidulans* requires RAS signaling and protein synthesis. *Genetics* **155**: 647–656.

Osherov N, May GS (2001). The molecular mechanisms of conidial germination. *FEMS Microbiology Letters* **199**: 153–160.

Osherov N, Mathew J, Romans A, May G (2002). Identification of conidial-enriched transcripts in *Aspergillus nidulans* using suppression subtractive hybridization. *Fungal Genetics and Biology* **37**: 197–204.

Pascon RC, Miller BL (2000). Morphogenesis in *Aspergillus nidulans* requires Dopey (DopA), a member of a novel family of leucine zipper-like proteins conserved from yeast to humans. *Molecular Microbiology* **36**: 1250–1264.

Pel HJ, Winde JH de, Archer DB, Dyer PS, Hofmann G, *et al.* (2007). Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88. *Nature Biotechnology* **2**: 221–231.

Pepper SD, Saunders EK, Edwards LE, Wilson CL, Miller CJ (2007). The utility of MAS5 expression summary and detection call algorithms. *BMC Bioinformatics* **8**: 273.

Reyes G, Romans A, Nguyen CK, May GS (2006). Novel mitogen-activated protein kinase MpkC of *Aspergillus fumigatus* is required for utilization of polyalcohol sugars. *Eukaryotic Cell* **5**: 1934–1940.

Rodríquez-Peña RN, Cid V, Arroyo J, Nombela C (2000). A novel family of cell-wall related proteins regulated differently during the yeast life cycle. *Molecular and Cellular Biology* **20**: 3245–3255.

Roberts AN, Yanofsky C (1989) Genes expressed during conidiation in *Neurospora crassa*: characterization of con-8. *Nucleic Acids Research* **17**: 197–214.

Ruepp A, Zollner A, Maier D, Albermann K, Hani J, Mokrejs M, Tetko I, Guldener U, Mannhaupt G, Munsterkotter M (2004). The FunCat, a functional annotation scheme for systematic classification of proteins from whole genomes. *Nucleic Acids Research* **32**: 5539–5545.

Ruijter GJG, Bax M, Patel H, Flitter SJ, Vondervoort PJL van de, Vries RP de, Kuyk PA van, Visser J (2003). Mannitol is required for stress tolerance in *Aspergillus niger* conidiospores. *Eukaryotic Cell* **2**: 690–698.

Ruijter GJG, Visser J, Rinzema A (2004). Polyol accumulation by *Aspergillus oryzae* at low water activity in solid-state fermentation. *Microbiology* **150**: 1095–1101.

Sakamoto K, Iwashita K, Yamada O, Kobayashi K, Mizuno A, Akita O, Mikami S, Shimoi H, Gomi K (2009). *Aspergillus oryzae* atfA controls conidial germination and stress tolerance. *Fungal Genetics and Biology* **46**: 887–897.

Sales K, Brandt W, Rumbak E, Lindsey G (2000). The LEA-like protein HSP 12 in *Saccharomyces cerevisiea* has a plasma membrane location and protects membranes against desiccation and ethanol induced stress. *Biochimica et Biophysica Acta* **1463**: 267–278.

Seong KY, Zhao X, Xu JR, Güldener U, Kistler HC (2008). Conidial germination in the filamentous fungus *Fusarium graminearum*. *Fungal Genetics Biology* **45**: 389–399.

Snowdon AL (1990). A color atlas of post-harvest diseases and disorders of fruit and vegetables: General introduction and fruits. Wolfe Scientific, London, UK.

Som T, Kolaparthi VSR (1994). Developmental decisions in *Aspergillus nidulans* are modulated by Ras activity. *Molecular and Cellular Biology* **14**: 5333-5348.

Sorais F, Barreto L, Leal JA, Bernabé M, San-Blas G, Niño-Vega GA (2010). Cell wall glucan synthases and GTPases in *Paracoccidioides brasiliensis*. *Medical Mycology* **48**: 35–47.

Taheri-Talesh N, Horio T, Araujo-Bazán L, Dou X, Espeso EA, Peñalva MA, Osmani SA, Oakley BR (2008). The tip growth apparatus of *Aspergillus nidulans*. *Molecular Biology of the Cell* **19**: 1439–1449.

Takeshita N, Ohta A, Horiuchi H (2005). CsmA, a class V chitin synthase with a myosin motorlike domain, is localized through direct interaction with the actin cytoskeleton in *Aspergillus nidulans*. *Molecular Biology of the Cell* **16**: 1961–1970.

Tiedt LR (1993). An electron microscope study of conidiogenesis and wall formation of conidia of *Aspergillus niger*. *Mycological Research* **12**: 1459–1462.

Thanh NV, Rombouts FM, Nout MJR (2005). Effect of individual amino acids and glucose on activation and germination of *Rhizopus oligosporus* sporangiospores in tempe starter. *Journal of Applied Microbiology* **99**: 1204–1214.

Thevelein JM (1996). Regulation of trehalose metabolism and its relevance to cell growth and function. In: *The Mycota. III. Biochemistry and Molecular Biology*. Brambl R, Marzluf GA, eds. Springer-Verlag, Berlin: 395–420.

Tompa P, Kovacs D (2010). Intrinsically disordered chaperones in plants and animals. *Biochemistry and Cell Biology* **88**: 167–174.

Vinck A, Terlou M, Pestman WR, Martens EP, Ram AF, Hondel CA van den, Wösten HA (2005). Hyphal differentiation in the exploring mycelium of *Aspergillus niger*. *Molecular Microbiology* **58**: 693–699.

Wong Sak Hoi JW, Lamarre C, Beau R, Meneau I, Berepiki A, Barre A, Mellado E, Read ND, Latgé JP. (2011) A novel family of dehydrin-like proteins is involved in stress response in the human fungal pathogen *Aspergillus fumigatus*. *Molecular Biology of the Cell* **22**: 1896–906.

Witteveen CFB, Visser J (1995). Polyol pools in *Aspergillus niger*. *FEMS Microbiology Letters* **134**: 57–62.

Xu Q, Ibarra M, Mahadeo D, Shaw C, Huang E, Kuspa A, Cotter D, Shaulsky G (2004). Transcriptional transitions during *Dictyostelium* spore germination. *Eukaryotic Cell* **5**: 1101–1110.

Yamazaki H, Tanaka A, Kaneko J, Ohta A, Horiuchi H (2008). *Aspergillus nidulans* ChiA is a glycosylphosphatidylinositol (GPI)-anchored chitinase specifically localized at polarized growth sites. *Fungal Genetics and Biology* **45**: 963–972.

Zhao W, Panepinto JC, Fortwendel JR, Fox L, Oliver BG, Askew DS, Rhodes JC (2006). Deletion of the regulatory subunit of protein kinase A in *Aspergillus fumigatus* alters morphology, sensitivity to oxidative damage, and virulence. *Infection and Immunity* **74**: 4865–4874.