EC Accepts, published online ahead of print on 19 December 2008
Eukaryotic Cell doi:10.1128/EC.00330-08

Global gene expression analysis during germination in the Chytridiomycete *Blastocladiella emersonii*

Silvia M. Salem-Izacc[#], Tie Koide, Ricardo Z. N. Vêncio[+] and Suely L. Gomes[*]

Departamento de Bioquímica, Instituto de Química,

Universidade de São Paulo, São Paulo, Brazil

Running title: Gene expression during *B. emersonii* germination

*Corresponding author. Mailing address: Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, Av. Prof. Lineu Prestes 748, 05508-000, São Paulo, SP, Brazil. Phone: 55-11-3091-3826. Fax: 55-11-3091-2186. E-mail: sulgomes@iq.usp.br.

[#]On leave from Instituto de Ciências Biológicas, Universidade Federal de Goiás, GO, Brazil. Present address: [+]Departamento de Genética, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brazil

ABSTRACT

Blastocladiella emersonii is an aquatic fungus of the Chytridiomycete class. During germination, the zoospore, a motile non-growing cell, goes through a cascade of morphological changes that culminates with its differentiation into the germling cell, capable of coenocytic vegetative growth. Transcriptome analyses of *B. emersonii* cells were carried out during germination induced under various environmental conditions. Microarray data analyzing 3,563 distinct *B. emersonii* genes revealed that 26% of them are differentially expressed during germination in nutrient medium, in at least one of the time points investigated. Over 500 genes are upregulated during the time course of germination under those conditions, most being related to cell growth, including genes involved in protein biosynthesis, DNA transcription, energetic metabolism, carbohydrate and oligopeptide transport, and cell cycle control. On the other hand, several transcripts stored in the zoospores are downregulated during germination in nutrient medium, such as genes involved in signal transduction, amino acid transport and chromosome organization. In addition, germination induced in the presence of nutrients was compared with that triggered either by adenine or potassium ions in inorganic salt solution. Several genes involved in cell growth, induced during germination in nutrient medium, do not show increased expression when *B. emersonii* zoospores germinate in inorganic solution, suggesting that nutrients exert a positive effect on gene transcription. Transcriptome data also revealed that most genes involved in cell signaling show the same expression pattern irrespective of the initial germination stimulus.

INTRODUCTION

Blastocladiella emersonii is a saprobic aquatic fungus, belonging to the *Chytridiomycete* class, located at the base of the fungal phylogenetic tree. Its life cycle is characterized by two distinct stages of cell differentiation: the germination and the sporulation. Under adverse nutritional conditions, the fungus stops growing and enters the sporulation stage, which after a series of morphological changes culminates with the production and release of the zoospores, motile cells that are important for the survival and dispersal of the organism. The zoospore is a wall-less cell that neither grows nor divides and can swim for hours using its endogenous reserves. In the presence of appropriate stimuli the zoospore germinates undergoing a series of drastic morphological and biochemical changes. In the first 15 min of germination the zoospore retracts its single polar flagellum and forms a thin cell wall of chitin, becoming a round cell. By this time, it is observed a cellular efflux of calcium (8), mobilization of cellular glycogen (45), and a decrease in lipid contents (34). These events do not require concomitant translation or transcription and are directed by proteins and mRNAs stored in the zoospore (20, 23, 30, 37, 38). Next, the round cell converts into a vegetative cell, the germling cell, with the formation of a germ tube that elongates and begins to branch at approximately 60 min, giving rise to a rhizoidal system through which nutrients are absorbed (41). Conversion of the round cell into a vegetative cell is accompanied by a large stimulation of protein synthesis. This increase in protein synthesis seems to be mediated by the mobilization of stored mRNA into polysomes between 15 and 30 min after induction of germination (10, 32).

Germination is a controlled process that responds to environmental stimuli. In *B. emersonii,* germination can be induced either by addition of nutrient medium or in an

inorganic solution containing 50 mM KCl (35). Van Brunt and Harold (44) suggested that potassium ions trigger encystment by causing membrane depolarization. Many other inducers have been identified, such as cyclic AMP (cAMP), cyclic GMP (cGMP), N-(alkylated) xanthines (adenine and adenosine), all of them inducing germination with a high degree of synchrony (9, 11, 31). Soon after germination is triggered, a transient increase in intracellular cAMP levels is detected (43), and a concomitant activation of the cAMP-dependent protein kinase is observed (1). All the enzymes of the cAMP metabolism are developmentally regulated in the fungus, with very low activities during exponential growth, increasing during sporulation and reaching maximal levels in the zoospores (6, 15, 24). Together these data suggest an important role for cAMP and protein phosphorylation in the germination process.

Calcium, another important second messenger in signaling pathways of eukaryotic cells, was also found to play a role during *B. emersonii* germination. As mentioned above, during zoospore encystment a large efflux of calcium is observed. In addition, lanthanum, which blocks the uptake and efflux of Ca^{2+}, inhibits germination completely when added at the time of induction (8). Calcium was also found to be both necessary and sufficient for sporulation of *B. emersonii*, and low levels of Ca^{2+} were observed to enhance the stability of zoospores (2, 35, 36). Calmodulin (CaM), a small acidic protein whose primary role is to serve as an intracellular Ca^{2+} receptor modulating the activity of numerous intracellular proteins involved in diverse signaling pathways (49), is found in *B. emersonii* zoospores (7, 33). Furthermore, pharmacological agents known to antagonize CaM action were shown to inhibit germination if added at the time of induction, indicating an important role for Calcium and CaM during this morphogenetic transition (33). These observations indicate that the molecular mechanisms involved in triggering germination should involve multiple

sensors and pathways, each one possibly sensitive to a certain environmental stimulus, working in a particular combination.

To complement the studies concerning the morphological and biochemical events associated with *B. emersonii* germination, it is important to establish a comprehensive evaluation of the genes that have their expression modulated during this process. In order to achieve this purpose we constructed a 9,216-element array containing 3,563 distinct EST sequences, obtained from cDNA libraries constructed with RNA from cells at different stages of the life cycle of the fungus (28), and from cells under heat or cadmium stress (5). The microarrays were used to study the global changes in transcript levels in cells isolated at different times after induction of germination in nutrient medium. More than 900 genes were differentially expressed during germination in nutrient medium in at least one of the time points analyzed, corresponding to 26 % of the genes in the microarrays. We also analyzed the differences between germination triggered in nutrient medium and in inorganic solution where the effective inducers were either adenine or potassium ions. Analysis of these data allowed us to identify specific genes and gene sets whose expression seem to be related to the biochemical and morphological changes associated with *B. emersonii* germination.

MATERIALS AND METHODS

Culture conditions and germination induction

Cultures of *B. emersonii* were maintained on plates containing 0.13% peptone, 0.13% yeast extract, 0.3% glucose and 1.5% agar. For RNA extraction, zoospores were inoculated (3.0×10^5 cells/ml) in defined DM3 liquid medium (24) and grown for 16 h at 17 °C with agitation (150 rpm). After this period of growth, vegetative cells were collected

by filtration through a Nitex cloth, rinsed and resuspended in sporulation solution (SE: 1 mM Tris-maleate buffer, pH 6.8, containing 1 mM $CaCl_2$) at a density of 1.0×10^6 cells/ml. Vegetative cells were incubated at 27ºC, with agitation, until the zoospores were completely released (3.5 to 4.0 h). The new zoospores were separated from the empty vegetative cells by filtration and collected by centrifugation (1000 x *g*, 4ºC), suspended in 1 ml of SE and either frozen on liquid nitrogen for total RNA extraction or inoculated into a proper solution for germination.

The solutions used to induce germination were: DM3 defined medium; germination solution (1 mM Tris-maleate buffer, pH 6.8, containing 1 mM $CaCl_2$, 10 mM $MgCl_2$ and 50 mM KCl); or germination solution without KCl, containing 2.5 mM adenine. Zoospores were inoculated in the appropriate solution at a density of 1.0×10^6 cells/ml and incubated at 27ºC with agitation. The progress and synchrony of germination were monitored by taking samples at different times and examining cell types under a light microscope. Cells were collected by vacuum filtration at different times of germination and frozen in liquid nitrogen for total RNA extraction.

RNA isolation

Total RNA was isolated using Trizol LS (Life Technologies) and the integrity of the RNA was checked through agarose-2.2 M formaldehyde gel electrophoresis, followed by ethidium bromide staining and RNA visualization under UV light. RNA samples were isolated from zoospores and from germinating cells at 30, 60 and 90 minutes after induction of germination in DM3 medium and at 30 and 60 minutes after induction of germination in either germination solution or germination solution without KCl, in the presence of 2.5 mM adenine.

PCR amplification and array printing

Microarray chips were designed to contain 3,563 distinct EST sequences obtained from cDNA libraries constructed with RNA from cells at different stages of the life cycle of the fungus (28), and cells exposed to heat shock or cadmium stress (5). *B. emersonii* plasmid clones were amplified in 100 μl PCR reactions (40 cycles, annealing at 51ºC), directly from bacterial clones in culture, using T7 and SP6 primers. Samples were visualized on 1% agarose gels to inspect PCR amplification quality and quantity. PCR products were purified by filtration using 96-well Millipore Multiscreen filter plates and eluted in 10 mM Tris-HCl solution at pH 8.0. Purified PCR products were mixed with an equal volume of dimethyl sulfoxide (DMSO) in 384 well V-bottom plates. Microarrays were constructed by arraying cDNA fragments on DMSO optimized, metal-coated glass slides (type 7 star, Amersham Biosciences) using the Generation III Microarray Spotter (Molecular Dynamics/Amersham Pharmacia Biotech). Each cDNA fragment was spotted at least in duplicate (i.e., technical replicates). Following printing, the slides were allowed to dry and the spotted DNA was bound to the slides by UV cross-linking (50 mJ).

All DNA fragments spotted on the slides were previously sequenced in order to confirm their putative identity. About 87% of the fragments had their identity validated and the others were re-annotated after BlastX against a local database constructed with sequences from Swiss-Prot and TrEMBL (http://www.expasy.org) associated to the Gene Ontology terms (http://www.geneontology.org). All the best hits identified had E-values smaller than 10^{-6}.

Probe preparation and hybridization, and data analysis

Ten micrograms of total RNA were reverse transcribed and labeled using a CyScribe Post-Labeling kit (GE Healthcare), according to manufacturer's instructions. Briefly, the RNA was reverse transcribed using the enzyme CyScript in the presence of modified amino allyldUTP, an optimized nucleotide mix, buffer, dithiothreitol, and random primers. The resulting amine-modified cDNA was then chemically labeled in the amino allyl group by using CyDye *N*-hydroxysuccinimide esters in 0.1 M sodium bicarbonate (pH 9.0). The products of the labeling reactions were purified in Millipore Multiscreen filtering plates to remove unincorporated labeled nucleotides.

Microarrays were co-hybridized with the fluorescent labeled probes. Hybridizations were performed overnight at 42ºC. The slides were then washed in 1×SSC and 0.2% SDS (10 min, 55ºC), twice in 0.1×SSC and 0.2% SDS (10 min, 55ºC), and in 0.1×SSC (1 min, at room temperature). Slides were rinsed briefly in milli-Q water and dried under a nitrogen stream. The hybridizations were performed as displayed in Table 1. Each experimental condition was analyzed with three independent biological experiments. Since each slide carried two replicates of the arrayed genes, a total of six intensity readings were generated for each gene in the microarray. Slides were scanned with a Generation III ScannerTM (Molecular Dynamics) adjusting the photomultiplier tube (PMT) to 700 for both channels.

Fluorescence mean intensity and surrounding median background from each spot were obtained with ArrayVision v6.0 (Imaging Research, Inc). Data from clones that generated poor quality PCR fragments (no amplification or unspecific bands) or poor quality spots (visually inspected) were excluded. Normalization was carried out by LOWESS fitting on an M-versus-S plot, where M is the fluorescence log ratio of the test sample relative to the control condition, $M=\log_2(\text{test/control})$, and S is the log-mean fluorescence intensity, $S = \log2_2(½ \text{ test} + ½ \text{ control})$ (19).

Determination of differentially expressed genes

We used intensity-dependent cutoff values for classifying a gene as differentially expressed based on self-self hybridization experiments (46). In this type of hybridization, the same cDNA sample is labeled independently with both Cy3 and Cy5 dyes to estimate the experimental noise. Two distinct self-self experiments were performed, one with RNA from zoospores and another with RNA from germinating cells. The HTself program available on the web (http://blasto.iq.usp.br/~rvencio/HTself) was used to determine the intensity-dependent cutoff curves. These curves delimit the boundaries of intrinsic experimental noise and, therefore, genes which do not show statistically significant variation in their expression levels. Using these intensity-dependent cutoff values we were able to determine which genes were differentially expressed during germination. Genes that presented at least 80% of replicates with expression ratios above or below the cutoff limits determined by self-self hybridization experiments were considered as upregulated or downregulated, respectively.

Clustering analysis and determination of overrepresented functional gene categories in each group

Differentially expressed genes presenting the complete time course profile (0, 30, 60 and 90 min of germination in nutrient medium) were clustered in ten groups according to their expression patterns using the K-means algorithm implemented in the SpotWhatR software (19). To characterize each K-means group based on functional gene categories, we measured the level of statistical association between "being in a given group" and

"belonging to a functional category," using the BayGO method (47). We considered that a gene category was overrepresented if the value of the statistical significance was smaller than 0.05.

Validation of microarray data by quantitative real-time RT-PCR

To evaluate the reliability of the array-based data, 7 genes were randomly selected and their expression levels analyzed by quantitative RT-PCR. Appropriate primers were designed using the Primer Express 2.0 Software (Applied Biosystems). Quantitative real time RT-PCR experiments (qRT-PCR) were performed using the GeneAmp 5700 Sequence Detection System (Applied Biosystems) equipment and the Platinum SYBR Green qPCR SuperMix UDG kit (Invitrogen). The thermocycling conditions comprised an initial step at 50°C for 2 minutes, followed by 95°C for 10 minutes and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The specificity of the amplified products was evaluated by analysis of the dissociation curves generated by the equipment. Two independent RNA samples were used for each gene analyzed. The gene encoding a putative mitochondrial RNA helicase-like protein was used as the calibrator gene in all experiments. The determination of the expression ratios was carried out using the method ΔΔCT, described by Livak and Schmittgen (22).

Microarray data accession number

The microarray data discussed in this work have been deposited in NCBI's Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO series accession number GSE12883 (http://www.ncbi.nih.gov/geo/query/acc.cgi?acc=GSE12883).

RESULTS AND DISCUSSION

Global Gene Expression Analysis

To investigate global changes in gene expression during *B. emersonii* germination process, we constructed cDNA microarrays containing 3,563 putative unique genes, which were identified in 12 distinct cDNA libraries prepared using RNA isolated from cells at different stages of the life cycle of the fungus (28), and from cells exposed to heat shock or cadmium stress (5). The PCR amplified cDNAs spotted on the arrays were re-sequenced in order to confirm their identity and were classified according to Gene Ontology (GO) terms, as shown in Figure 1.

Differentially expressed genes during germination were investigated in time course experiments, using RNA samples isolated from cells collected 30, 60 and 90 min after inoculation of zoospores in nutrient medium DM3. The reference sample was zoospore RNA, which was considered the time zero of germination. Competitive microarray hybridizations were carried out with RNA isolated from three independent biological experiments. We also compared germination in nutrient medium with germination induced in inorganic salt solution, triggered either by adenine or potassium ions. In these experiments we considered germination in nutrient medium as the reference sample. The different competitive hybridizations carried out are described in Table 1. A particular gene was classified as differentially expressed during germination in comparison with the reference sample if at least 80% of the replicates were outside the credibility intervals

1 defined in self-self hybridization experiments obtained by the software HTself (46), using
2 at least 3 valid replicates, as described in Materials and Methods.

3 **Gene expression profiles during germination in nutrient medium**

4 Analysis of competitive hybridization data according to the criteria described above
5 revealed a total of 535 genes upregulated and 389 genes downregulated, in at least one of
6 the time points analyzed, when cells germinating in nutrient medium are compared to
7 zoospores. Differentially expressed genes were then clustered according to their expression
8 profile into ten groups using the K-means algorithm. Gene clusters were classified into GO
9 functional categories and a search for overrepresented categories in each group was carried
10 out using BayGO software (47), as described in Materials and Methods. Figure 2 shows the
11 expression profiles obtained through K-means clustering and the corresponding
12 overrepresented gene categories.

13 To validate the expression profiles obtained in microarray experiments, quantitative
14 real time RT-PCR (qRT-PCR) assays were performed for 7 randomly selected genes using
15 cell samples of two independent biological time-course experiments of germination in
16 nutrient medium. To normalize the data, the gene encoding a mitochondrial RNA helicase-
17 like protein was used, as its expression showed no change in the microarray experiments in
18 all conditions tested. Figure 3 shows the comparison of the results from microarrays and
19 qRT-PCR experiments for time points 30, 60 and 90 min of germination in nutrient
20 medium, using as reference zoospore RNA (time zero). All genes analyzed by qRT-PCR
21 confirmed the same expression profile of the microarrays.

22

Genes upregulated during germination in nutrient medium

Genes classified as upregulated during *B. emersonii* germination were clustered in groups K1 to K5 and the categories overrepresented in these groups according to BayGO are shown in Figure 2. The complete list of genes in groups K1 to K5 and their expression ratio are found in Supplemental Table 1S. The main biological processes observed among the upregulated genes are those necessary for cell growth and maintenance, including gene transcription, protein biosynthesis, energy metabolism, nutrient transport and cell cycle control. These results are in agreement with the events that are common to the germination processes described for other fungi (3, 27, 48).

The best-represented functional category observed among the upregulated genes is protein biosynthesis, which includes genes encoding ribosomal proteins, translation initiation and elongation factors (K1, K3 and K4), as well as genes related to protein folding (K2) and ribosome biogenesis (K1). Altogether, 56 genes involved in protein biosynthesis were upregulated during germination in nutrient medium in the time points analyzed. The induction of *B. emersonii* genes involved in ribosome biogenesis during germination in nutrient medium coincides temporally with the increase of polysome formation previously observed (10, 30) and is probably related to the sudden increase in the rate of protein synthesis that occurs early in germination using the pre-formed mRNAs stored in the zoospores (30). The ability of fungal spores to store pre-packaged mRNA has also been described in *Aspergillus nidulans* and *Neurospora crassa* (14, 27). The conidia of these fungi show high levels of free ribosomes that associate with pre-synthesized RNAs

forming polysomes in the presence of a carbon source and the stored mRNA are primed for rapid activation and translation.

Among the upregulated genes, we also detected genes involved in carbohydrate and oligopeptide transport, responsible for nutrient uptake from the environment (K4). This group also includes genes related to cell cycle regulation that control cell entry into S phase and mitosis. As expected, several genes involved in the energy metabolism pathway were also induced during the first 90 min of germination in nutrient medium: glycolysis (K3 and K5), tricarboxylic acid cycle (K5) and fatty acid biosynthesis (K5) genes. Some of these genes encode key enzymes in energetic metabolism, like pyruvate kinase, citrate synthase and isocitrate dehydrogenase (Supplemental Table 1S).

As depicted in Figure 2, genes in groups K4 and K5 show the highest induction levels. Genes in these groups play a role in important biological processes, as protein biosynthesis, nutrient uptake and energetic metabolism, and are among the 20 most highly induced genes (Supplemental Table 1S, marked with an asterisk). In addition, a large number of genes (147 genes) with no putative function assigned were classified as upregulated during germination in nutrient medium, suggesting their role in the germination process and constituting a first characterization of these genes (Supplemental Table 1S).

Several recent microarray studies have also investigated differentially expressed genes during germination in fungi, as *Trichophyton rubrum* (21), *Neurospora crassa* (16) and *Ustilago maydis* (50). Most of the genes upregulated in these organisms correspond to the same functional categories observed during *B. emersonii* germination, as for instance, protein biosynthesis and primary metabolism involved in energy production of the cell.

Genes downregulated during germination in nutrient medium

The early morphological events of *B. emersonii* germination have been shown to occur in the absence of transcription and data indicate that the mRNAs necessary to begin the germination process are probably synthesized in the final stages of sporulation and stored in the zoospores (30). Among these transcripts we would expect to find those present in the zoospores and downregulated during germination in nutrient medium. The expression profiles of genes clustered in groups K6 to K10 encompass such transcripts. The functional categories overrepresented among the genes in groups K6 to K10 according to BayGO are shown in Figure 2, and the complete set of genes are listed in Supplemental Table 2S. Some of the overrepresented functional categories observed among these genes are discussed bellow.

Signal transduction

Zoospores must sense the environment in order to recognize the appropriate conditions to germinate. So, it is imperative that they possess the necessary means to sense and respond to changes in the environment, choosing the best moment to germinate. In *B. emersonii* zoospores, we detected transcripts encoding proteins involved in different signaling pathways (groups K7 and K9 and Supplemental Table 2S): a Gsα subunit, a cGMP phosphodiesterase, a soluble guanylate cyclase (its transcript levels do not change

during germination in nutrient medium, but increase during germination induced by adenine; data not shown), proteins of the MAP kinase pathway, phosphatases PP2A and calcineurin (a phosphatase that is stimulated by calmodulin), a Ras GTPase, and also the catalytic and regulatory subunits of the cAMP dependent protein kinase (PKA). These data corroborate previous biochemical studies indicating that germination in *B. emersonii* seems to be controlled by more than one signaling pathway (see Introduction section).

The regulatory and catalytic subunits of the PKA of *B. emersonii* were previously shown to present their highest levels in zoospores, decreasing to almost undetectable levels during germination (25), which is consistent with the transcript profiles of the corresponding genes reported here. Cyclic GMP phosphodiesterase activity was also determined throughout the life cycle of the fungus (42), and shown to present the same pattern of variation observed for the PKA activity. Thus, the expression profile for the cGMP phosphodiesterase transcript found here is in agreement with the changes in enzyme activity during germination.

Protein phosphatase 2A affects a variety of biological processes in the cell such as transcription, cell cycle progression and cellular morphogenesis (17). This phosphatase is also involved in many aspects of cellular function including the regulation of metabolic enzymes and proteins involved in signal transduction (26). PP2A has been previously investigated in *B. emersonii* (4). The authors demonstrated that its activity is developmentally regulated, increasing during sporulation and reaching maximum levels in zoospores. They also showed that the protein phosphatase inhibitor okadaic acid induces encystment but inhibits germ tube formation. Microarray data revealed that the transcript encoding the regulatory subunit of PP2A is present in zoospores and is severely

downregulated during germination in nutrient medium. In addition, the transcript encoding the PP2A inhibitor is highly induced during germination. Thus, in zoospores, where there is an arrest in the cell cycle, PP2A regulatory subunit transcript levels are high and PP2A inhibitor transcript levels are low, whereas during germination, when the cell cycle is resumed, the opposite behavior is observed. These results agree with the hypothesis that this PP2A negatively controls *B. emersonii* germination. Interestingly, previous work has shown that in fission yeast, PP2A negatively regulates entry into mitosis (18).

Amino acid transporters

Several transcripts encoding amino acid transporters are stored in *B. emersonii* zoospores and are downregulated during germination in nutrient medium (K9 and Supplemental Table 2S). It is known that nutrients, besides their role in metabolism, also exert regulatory effects that seem to be mediated by nutrient sensors present in the cell membrane. In *Saccharomyces cerevisiae*, for instance, the Gap1 protein is an amino acid permease that acts not only as a transporter, but also as an amino acid sensor. The activity of Gap1 is enhanced in cells in the absence of nitrogen, but when cells are supplemented with ammonium, L-glutamine or L-glutamate this protein is inactivated both at the transcriptional and post-transcriptional levels (13, 39, 40). Downregulation of these amino acid transporter genes in germination triggered by nutrient medium suggests a similar role for these genes in *B. emersonii*.

Histones and histone deacelylase

Another interesting observation is a significant decrease in the expression levels of genes encoding histones during *B. emersonii* germination in nutrient medium (group K10; Supplemental Table 2S). Furthermore, the transcript for a putative histone deacetylase is present in zoospores and downregulated during germination in nutrient medium, when unblocking of transcription occurs (Supplemental Table 2S). Removal of acetyl groups from histone tails, an event catalyzed by histone deacetylases, is a means of implementing gene silencing (12). The presence of histone deacetylase transcripts in the zoospores is a hint that chromatin structure could be responsible for the inhibition of replication and transcription observed in these cells. So, the repression of the genes encoding histones and a histone deacetylase during germination in nutrient medium could be required to resume DNA replication and transcription.

In addition, a large number of genes (a total of 192) with no putative function assigned were also downregulated during germination (Supplemental Table 2S), thirteen of them being among the 20 most highly repressed genes (Supplemental Table 2S, marked with an asterisk).

Comparison of germination induced in nutrient medium and in inorganic solution

To investigate if different genetic pathways are utilized during germination occurring in the absence of nutrients, we compared germination in growth medium with that induced in inorganic salt solution, triggered either by adenine or potassium.

Morphologically, the early events of germination during the first 60 min, as the retraction of the flagellum, synthesis of the cell wall, germ tube formation and initial branching, are the same under the three conditions tested (data not shown). However, 90 min after induction in the absence of nutrients cells fail to grow and begin to show dark spots in the cytoplasm (not shown).

Comparison of global gene expression profiles of cells germinating in nutrient medium with those germinating in inorganic salt solution was carried out using competitive microarray hybridizations. In these experiments we used RNA obtained from cells isolated 30 and 60 min after induction of germination, according to the hybridization scheme presented in Table 1B.

A high number of differentially expressed genes were observed during germination triggered by adenine or potassium relative to germination in the presence of nutrients. Figure 4 shows the number of genes upregulated or downregulated in each case. These data suggest that the control of expression of a large number of genes during germination respond to the presence of nutrients in the medium.

It is noteworthy that several genes involved in cell signaling pathways (Table 2) are not differentially expressed when we compare germination induced in nutrient medium with germination triggered in inorganic salt solution. These results suggest that many cell signaling pathways are activated independently of the initial stimulus triggering the germination process.

Microarray expression data obtained in germination assays triggered by adenine (30 and 60 min) were also validated using qRT-PCR experiments. In this case the RNA used as

reference was the corresponding time points of germination induced in nutrient medium. The two methodologies were compared by considering the direction of gene expression modulation for each transcript. As shown in Supplemental Table 3S, 13 of the 14 experimental points evaluated showed the same direction of gene expression modulation, indicating 92.9 % of coincidence between microarray and qRT-PCR data. In general, qRT-PCR experiments showed expression ratios higher than those observed in the microarrays, probably indicating a higher sensitivity of former approach. The Spearman correlation obtained considering all experimental data was 0.86, indicating a relatively strong correlation between microarray and qRT-PCR results.

Upregulated genes during germination in inorganic salt solution relative to germination in nutrient medium

Microarray hybridization analysis with RNA from cells isolated 30 min after induction of germination in the presence of adenine or potassium revealed 42 genes with higher transcript levels in both cases when compared to cells germinating in nutrient medium (Figure 4). Among these genes, 26 presented a putative identification and are listed in Table 3. Interestingly, most of these transcripts were already detected in the zoospores and their levels decreased during germination in nutrient medium, as observed in the time course germination assays.

A total of eight genes upregulated in cells germinating in inorganic solution when compared to nutrient medium encode amino acid transporters (Table 3). The corresponding transcripts are present in the zoospores, with their levels decreasing during germination in

nutrient medium but remaining high in germination triggered by adenine or potassium. These results support the idea that these amino acid transporters could play a role in nutrient sensing and that their expression would be negatively controlled by the presence of amino acids or other nutrients in the medium. Interestingly, Seong *et al.* (29) have described a similar finding during germination of the filamentous fungus *Fusarium graminearum.* These authors observed that more permeases and transporters are expressed in fresh spores and in hyphae, when nutrient-limiting conditions may exist, than in activated spores when the fungus is in a more nutrient-rich environment. Thus, expression amino acid transporter genes in both *Blastocladiella* and *Fusarium* are repressed in the presence of nutrients.

Five genes involved in chromosome organization and biogenesis show the same expression profile as the amino acid transporter genes (Table 3). This finding may be related to alterations in chromatin structure, which are necessary for the unblocking of transcription and DNA replication that occurs during germination in nutrient medium and results in important alterations both in the control of the cell cycle and in the transcriptome of *B. emersonii*. Our results indicate that histones could be involved in the regulation of DNA transcription, acting selectively in the unblocking of the genes related to cell growth that are induced only in nutrient medium.

Genes implicated in fatty acid metabolism are also upregulated in cells germinating in inorganic solution when compared to nutrient medium, indicating that *B. emersonii* cells must use their endogenous lipid reserves in order to germinate in the absence of nutrients (Table 3). We also detected the induction of the gene encoding a $H^{(+)}$-ATPase, which generates the electrochemical gradient required for ion balance, nutrient uptake and energy

production in fungi; the gene for a carbonic anhydrase, related to carbon utilization and intracellular pH regulation; and the gene encoding a mitochondrial homocitrate synthase (Table 3). Homocitrate synthase is an enzyme that catalyses the first reaction of the α-aminoadipate pathway, which is responsible for lysine biosynthesis in some fungi. Lysine may be converted to acetyl-CoA, which then enters the tricarboxylic acid cycle. This observation suggests that when germination proceeds in the absence of nutrients the fungus needs to synthesize metabolic precursors for energy production.

A larger number of genes are upregulated after 60 min of germination in the absence of nutrients, 68 of them being detected in the presence of both adenine and potassium (Supplemental Table 4S). Among them we observed genes related to DNA catabolism, proteolysis, response to toxins and apoptosis. Interestingly, 13 genes in Supplemental Table 4S are also upregulated in response to heat shock and/or cadmium stress, six of them without a match in public databases (5). This finding indicates that at this time point cells germinating in the absence of nutrients begin to express genes involved in stress responses.

Downregulated genes during germination in inorganic salt solution relative to germination in nutrient medium

Several genes were downregulated in cells germinating in inorganic salt solution in comparison to those germinating in the presence of nutrients. Consistently, these genes are mainly related to cell growth processes such as glycolysis, protein biosynthesis, nucleotide biosynthesis, transcription and transport (Table 4). As observed, these genes were induced during germination in the presence of energy rich compounds in the induction medium

(Figure 2, K1-K5), indicating that nutrients exert a positive regulatory effect on the transcription of these genes.

FINAL REMARKS

Analysis of stage-specific gene expression and alterations in gene expression levels related to fungal life cycle stages are central to understand the molecular mechanisms responsible for germination and other developmental processes in fungi. The data presented here provide important insights into the molecular mechanisms of fungal germination at the cellular level, and contribute to a better understanding of regulation of gene expression related to this morphological transition in *B. emersonii*.

The question concerning the signal transduction pathways involved in triggering *B. emersonii* germination process, for instance, was investigated by analyzing the expression pattern of the corresponding genes. Data revealed that a large number of distinct transcripts related to signal transduction are observed in zoospores and found to present the same expression profile during the initial stages of germination with all the inducers investigated. These results indicate that many signaling pathways are activated irrespective of the initial stimulus used to trigger this developmental stage.

The functional categories overrepresented among the genes upregulated during *B. emersonii* germination were found to coincide with those involved in the process of germination of other fungi. However, genes involved in cellular growth, including genes of protein biosynthesis and energetic metabolism, were only upregulated in germination

induced in the presence of nutrients. In addition, many genes downregulated during germination in nutrient medium are kept at high expression levels in germination triggered in inorganic salt solution. These results indicate that nutrients exert an important role in the regulation of many genes during the germination process.

Furthermore, many transcripts encoding proteins without a match in public databases were found to be differentially expressed in our experiments, and thus constitute an important initial functional characterization of the corresponding genes.

Acknowledgements

This work was supported by grants to S.L.G. from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP). S.M.S.-I. was a doctoral fellow of Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), T.K. was a doctoral fellow of FAPESP, and R.Z.N.V. was a doctoral fellow of Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). S.L.G. was partially supported by CNPq.

REFERENCES

1. **Behrens, M. M., and J. C. C. Maia.** 1986. Differentiation specific activation of cyclic AMP-dependent protein kinase in *Blastocladiella emersonii.* Biochem. Intern. **12**:503-512.

2. **Coutinho, E. C., and L. C. Correa.** 1999. The induction of sporulation in the aquatic fungus *Blastocladiella emersonii* is dependent on extracellular calcium. FEMS Microbiol. Lett. **179**:353-359.

3. **d'Enfert, C.** 1997. Fungal spore germination: insights from the molecular genetics of *Aspergillus nidulans* and *Neurospora crassa*. Fungal Genet. Biol. **21**:163-172.

4. **Etchebehere, L. S., M. N. Simon, R. B. Campanhã, P. D. Zapella, M. Véron, and J. C. Maia.** 1993. Developmental regulation of hexosamine biosynthesis by protein phosphatases 2A and 2C in *Blastocladiella emersonii***.** J. Bacteriol. **175**:5022-5027.

5. **Georg, R. C., and S. L. Gomes.** 2007. Transcriptome analysis in response to heat shock and cadmium in the aquatic fungus *Blastocladiella emersonii*. Eukaryot. Cell. **6**:1053-1062.

6. **Gomes, S. L., L. Mennucii, and J. C. C. Maia,** 1978. Adenylate cyclase and cyclic AMP metabolism during cytodifferentiation of *Blastocladiella emersonii***.** Biochim. Biophys. Acta 541: 190-198.

7. **Gomes, S. L., L. Mennucci, and J. C. C. Maia.** 1979. A calcium-dependent protein activator of mammalian cyclic nucleotide phosphodiesterase from *Blastocladiella emersonii*. FEBS Lett. **99**:39-42.

8. **Gomes, S. L., L. Mennucci, and J. C. C. Maia.** 1980. Calcium efflux during germination of *Blastocladiella emersonii*. Dev. Biol. **77**:157-166.

9. **Gomes, S. L., L. Mennucci, and J. C. C. Maia.** 1980. Induction of *Blastocladiella emersonii* germination by cyclic adenosine 3', 5'-monophosphate. Cell Differ. **9**:169-179.

10. **Gong, C., and J. S. Lovett.** 1977. Regulation of protein synthesis in *Blastocladiella* zoospores: Factors for synthesis in nonsynthetic spores. Exp. Mycol. **1**:138-151.

11. **Gottschalk, W. K., and D. R. Sonneborn.** 1982. Phenotypic dissections of the *Blastocladiella emersonii* zoospore's developmental choice. *Dev. Biol.* **93**:165-180.

12. **Grunstein, M.** 1990. Histone function in transcription. Annu. Rev. Cell Biol. **6**:643-676.

13. **Hein, C., and B. André.** 1997. A C-terminal di-leucine motif and nearby sequences are required for $NH_4^{(+)}$-induced inactivation and degradation of the general amino acid permease, Gap1p, of *Saccharomyces cerevisiae*. Mol. Microbiol. **24**:607-616.

14. **Hollomon, D. W.** 1970. Ribonucleic acid synthesis during fungal spore germination. J. Gen. Microbiol. **62**:75-87.

15. **Juliani, M. H., M. R. Brochetto, J. C. Da Costa Maia.** 1979. Changes in cyclic AMP binding and protein kinase activities during growth and differentiation of *Blastocladiella emersonii*. Cell Differ. **8**:421-430.

16. **Kasuga, T., J. P. Townsend, C. Tian, L. B. Gilbert, G. Mannhaupt, J. W. Taylor, and N. L. Glass.** 2005. Long-oligomer microarray profiling in *Neurospora crassa* reveals the transcriptional program underlying biochemical and physiological events of conidial germination. Nucleic Acids Res. **33**:6469-6485.

17. **Kinoshita K., T. Nemoto, K. Nabeshima, H. Kondoh, H. Niwa, and M. Yanagida.** 1996. The regulatory subunits of fission yeast protein phosphatase 2A (PP2A) affect cell morphogenesis, cell wall synthesis and cytokinesis. Genes Cells. **1**:29-45

18. **Kinoshita, N., H. Yamano, H. Niwa, T. Yoshida, and M. Yanagida.** 1993. Negative regulation of mitosis by the fission yeast protein phosphatase ppa2. Genes Dev. **6**:1059-1071.

19. **Koide, T., S. M. Salem-Izacc, S. L. Gomes, and R. Z. N. Vêncio.** 2006. SpotWhatR: a user-friendly microarray data analysis system. Genet. Mol. Res. **5:**93-107.

20. **Leaver, C. J., and J. S. Lovett.** 1974. An analysis of protein and RNA synthesis during encystment and outgrowth (germination) of *Blastocladiella* zoospores. Cell Differ. **3**: 165-192.

21. **Liu, T., Q. Zhang, L. Wang, L. Yu, W. Leng, J. Yang, L. Chen,J. Peng, L. Ma, J. Dong, X. Xu, Y. Xue, Y. Zhu, W. Zhang, L. Yang, W. Li, L. Sun, Z. Wan, G. Ding, F. Yu, K. Tu, Z. Qian, R. Li, Y. Shen, Y. Li and Q. Jin.** 2007. The use of global transcriptional analysis to reveal the biological and cellular events involved in distinct development phases of *Trichophyton rubrum* conidial germination. BMC Genomics. **8**:100.

22. **Livak, K. J., and T. D. Schmittgen.** 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. **25**:402-408.

23. **Lovett, J. S.** 1968. Reactivation of ribonucleic acid and protein synthesis during germination of *Blastocladiella* zoospores and the role of the ribosomal nuclear cap. J. Bacteriol. **96**:962-969.

24. **Maia, J. C. C., and E. P. Camargo.** 1974. cAMP phosphodiesterase activity during growth and differentiation in *Blastocladiella emersonii*. Cell Differ. **3**: 147-155.

25. **Marques, M. V., A. C. C. Borges, J. C. F. Oliveira, and S. L. Gomes.** 1992. Coordinate pretranslational control of cAMP-dependent protein kinase subunit expression during development in the water mold *Blastocladiella emersonii*. Dev.Biol. **149**:432-439.

26. **Mayer-Jaekel R. E., and B. A. Hemmings.** 1994. Protein phosphatase 2A--a 'ménage à trois'. Trends Cell Biol. **4**:287-291.

27. **Osherov, N., and G. S. May.** 2001. The molecular mechanisms of conidial germination. FEMS Microbiol. Lett. **199**:153-160.

28. **Ribichich, K. F., S. M. Salem-Izacc, R. C. Georg, R. Z. N. Vêncio, L. D. Navarro, and S. L. Gomes.** 2005. Gene discovery and expression profile analysis through sequencing of expressed sequence tags from different developmental stages of the chytridiomycete *Blastocladiella emersonii*. Eukaryot. Cell. **4**:455-464.

29. **Seong, K. Y., X. Zhao, J. R. Xu, U. Güldener, and H. C. Kistler.** 2008. Conidinal germination in the filamentous fungus *Fusarium graminearum*. Fungal Genet. Biol. **45**:389-399.

30. **Silva, A. M., J. C. Maia, and M. H. Juliani.** 1987. Changes in the pattern of protein synthesis during zoospore germination in *Blastocladiella emersonii*. J. Bacteriol. **169**:2069-2078.

31. **Silverman, P. M., and P. M. Epstein.** 1975. Cyclic nucleotide metabolism coupled to cytodifferentiation of *Blastocladiella emersonii*. Proc. Natl. Acad. Sci. U S A. **72**:442-446.

32. **Silverman, P. M., M. M. Huh, and L. Sun.** 1974. Protein synthesis during zoospore germination in the aquatic phycomycete *Blastocladiella emersonii*. Dev. Biol. **40**:59-70.

33. **Simão, R. C., and S. L. Gomes.** 2001. Structure, expression, and functional analysis of the gene coding for calmodulin in the chytridiomycete *Blastocladiella emersonii*. J. Bacteriol. **183**:2280–2288.

34. **Smith, J. D., and P. M. Silverman.** 1973. Lipid turnover during morphogenesis in the water mold *Blastocladiella emersonii*. Biochem. Biophys. Res. Commun. **54**:1191-1197.

35. **Soll, D. R., and D. R. Sonneborn.** 1969. Zoospore germination in the water mold. *Blastocladiella emersonii*. II. Influence of cellular and environmental variables on germination. Dev. Biol. **20**:218-35.

36. **Soll, D. R., R. Bromberg, and D. R. Sonneborn.** 1969. Zoospore germination in the water mold. *Blastocladiella emersonii*. I. Measurement of germination and sequence of subcellular morphological changes. Dev. Biol. **20**:183-217.

37. **Soll, D. R., and D. R. Sonneborn.** 1971. Zoospore germination in *Blastocladiella emersonii*: Cell differentiation without protein synthesis? Proc. Natl. Acad. Sci. U S A. **68**: 459-463.

38. **Soll, D. R., and D. R. Sonneborn**. 1971. Zoospore germination in Blastocladiella emersonii. III. Structural changes in relation to protein and RNA synthesis. J. Cell Sci. **9**:679-699.

39. **Stanbrough, M., and B. Magasanik.** 1995. Role of the GATA factors Gln3p and Nil1p of Saccharomyces cerevisiae in the expression of nitrogen-regulated genes. Proc. Natl Acad. Sci. U S A. **92**:9450-9454.

40. **Stanbrough, M., and B. Magasanik.** 1995. Transcriptional and posttranslational regulation of the general amino acid permease of *Saccharomyces cerevisiae*. J. Bacteriol. **177**:94-102

41. **Truesdell, L. C., and E. C. Cantino.** 1971. The induction and early events of germination in the zoospore of *Blastocladiella emersonii*. Curr. Top. Dev. Biol. **6**:1-44.

42. **Vale, M. R., and J. C. Maia.** 1976. Changes in cGMP phosphodiesterase levels during growth and differentiation in *Blastocladiella emersonii*. FEBS Lett. **70**:205-208.

43. **Vale, V. L., S. L. Gomes, J. C. C. Maia, and L. Mennuci.** 1976. Transient cyclic AMP accumulation in germinating zoospores of *Blastocladiella emersonii.* FEBS Lett. **67**:189-192.

44. **Van Brunt, J., and F. M. Harold.** 1980. Ionic control of germination of *Blastocladiella emersonii* zoospores. J. Bacteriol. **141**:735-44.

45. **Vandercammen, A., J. M. François, B. B. Torres, J. C. Maia, and H. G. Hers.** 1990. Fructose 2,6-biphosphate and carbohydrate metabolism during the life cycle of the aquatic fungus *Blastocladiella emersonii*. J. Gen. Microbiol. **136**:137-146.

46. **Vêncio, R. Z. N., and T. Koide.** 2005. HTself: self-self based statistical test for low replication microarray studies. DNA Res. **12:**211-214.

47. **Vêncio, R. Z. N., T. Koide, S. L. Gomes, and C. A. B. Pereira.** 2006. BayGO: Bayesian analysis of ontology term enrichment in microarray data. *BMC Bioinformatics.* **7:**86.

48. **Wendland, J.** 2001. Comparison of morphogenetic networks of filamentous fungi and yeast. Fungal Genet. Biol. **34**:63-82.

49. **Williams, R. J.** 1992. Calcium and calmodulin. Cell Calcium. **13**:355-362.

50. **Zahiri, A. R., M. R. Babu, and B. J. Saville.** 2005. Differential gene expression during teliospore germination in *Ustilago maydis*. Mol. Genet. Genomics. **273**:394-403.

FIGURE LEGENDS

FIGURE 1. Functional classification of the *B. emersonii* genes spotted on the microarrays. The number of *B. emersonii* genes classified in different biological processes according to the *Gene Ontology Consortium* (GO) are shown.

FIGURE 2. *K*-means clustering with 10 groups using the complete profiles of differentially expressed genes. The *y* axis shows the $\log_2$ expression ratio of the normalized values and the *x* axis shows the time after induction of germination in nutrient medium. The overrepresented gene categories for each cluster are also shown. A functional category was considered overrepresented if its statistical association with its presence in the cluster is significant ($P < 0.05$).

FIGURE 3. Expression levels of seven selected genes during germination in nutrient medium evaluated by real-time RT-PCR (open triangles) and microarray experiments (black squares). *M* is the $\log_2$ expression ratio of the normalized values. The results are the median values from independent biological samples.

FIGURE 4. Number of genes differentially expressed when germination induced by adenine (A) or potassium (K) is compared with germination in nutrient medium.

TABLES

TABLE 1. Competitive hybridizations.

A) Time course of germination in nutrient medium

Germination induced in nutrient medium DM3	
Reference RNA	Test RNA
ZSP (zoospores; time = 0 of germination)	G30 (30 min of germination in DM3)
ZSP (zoospores; time = 0 of germination)	G60 (60 min of germination in DM3)
ZSP (zoospores; time = 0 of germination)	G90 (90 min of germination in DM3)

B) Comparison of germination in nutrient medium with germination induced in the presence of either adenine or potassium.

Germination induced by either adenine or potassium	
Reference RNA	Test RNA
G30 (30 min of germination in DM3)	A30 (30 min of germination induced by adenine)
G30 (30 min of germination in DM3)	K30 (30 min of germination induced by
G60 (60 min of germination in DM3)	A60 (60 min of germination induced by adenine)
G60 (60 min of germination in DM3)	K60 (60 min of germination induced by

TABLE 2. Genes involved in cell signaling presenting the same expression profile irrespective of the inducer of germination

Clone ID	Best alignment (Swiss-Prot)	GO description
BeE60H30H04	O93887 G-protein beta subunit GPB1	G-protein coupled receptor protein signaling pathway
BeG120N11H04	Q05425 Guanine nucleotide-binding protein alpha-1 subunit	G-protein coupled receptor protein signaling pathway
BeE60N16D02[a]	Q54PH7 G-protein subunit alpha 8	G-protein coupled receptor protein signaling pathway
BeZSPN08H03	Q5KPS8 Heterotrimeric G protein alpha subunit B, putative	G-protein coupled receptor protein signaling pathway
BeE120N26D09	Q68EF8 Rapgefl1 protein (Fragment)	G-protein coupled receptor protein signaling pathway
BeE60H26A07	Q870G5 Guanine nucleotide-binding protein gamma subunit	G-protein coupled receptor protein signaling pathway
BeG60N08H03[a]	Q8J0B8 G protein alpha subunit	G-protein coupled receptor protein signaling pathway
BeE120N28E07[a]	Q9HFN1 G protein alpha subunit (Fragment)	G-protein coupled receptor protein signaling pathway
BeE120N18F01[a]	Q86WN6 Phosphodiesterase PDE9A13	cAMP/cGMP mediated signaling
BeE60N08A04	Q3ZLB7 ASP	cAMP-dependent protein kinase, regulator activity
BeE120N30B12	P31320 cAMP-dependent protein kinase regulatory subunit	cAMP-dependent protein kinase, regulator activity
BeZSPN16D09[a]	Q12741 cAMP-dependent protein kinase catalytic subunit	protein serine/threonine kinase activity
BeE60N11G06[a]	Q05116 Dual specificity mitogen-activated protein	protein serine/threonine kinase activity
BeE60N03C06[a]	Q9HDE1 Calcineurin B	Calmodulin stimulated protein

	regulatory subunit	phosphatase
BeG30N18A11	Q15269 Periodic tryptophan protein 2 homolog	signal transduction
BeE60C09B03	Q4KIT4 Methyl-accepting chemotaxis protein	signal transduction/chemotaxis
BeE120N04B04[a]	Q9Y4G8 Rap guanine nucleotide exchange factor 2	cAMP-mediated signaling/MAPKKK cascade/small GTPase mediated signal transduction
BeE60H31A02	Q4DZ75 Small GTP-binding protein Rab28, putative	small GTPase mediated signal transduction
BeE30N10H08	Q4P7T0 YPT1_NEUCR GTP-binding protein ypt1	small GTPase mediated signal transduction
BeE90N24C02	Q4PB75 Hypothetical protein	small GTPase mediated signal transduction
BeZSPN11G01	Q4PG14 Hypothetical protein	small GTPase mediated signal transduction
BeE120N32A08	Q4WGK7 Septin B	small GTPase mediated signal transduction
BeZSPN17H07	Q4WZP3 Cell division control protein Cdc25, putative	small GTPase mediated signal transduction
BeE30N13H07	Q5K7V1 GTPase, putative	small GTPase mediated signal transduction
BeE60H32H12	Q7Q7M3 ENSANGP00000020929	small GTPase mediated signal transduction
BeE90N21E01	Q7RVG3 RAS-related protein RAB1BV	small GTPase mediated signal transduction
BeG30N14G03	Q7T0S9 MGC69017 protein	small GTPase mediated signal transduction
BeE120N24H03	Q86F33 Clone ZZD1150 mRNA sequence	small GTPase mediated signal transduction

BeE120N26G04	Q86G47 Nucleotide exchange factor RasGEF Q	small GTPase mediated signal transduction
BeE60N03A04	Q8K2P9 Rabl2a protein	small GTPase mediated signal transduction
BeE60N07H08	Q8T367 Small G protein	small GTPase mediated signal transduction
BeZSPN15D02	Q9PW31 Rac GTPase	small GTPase mediated signal transduction
BeE30N04H05	Q9Y2Y0 Arf-like 2 binding protein BART1	small GTPase regulatory/interacting protein activity
BeE60C35C07	Q7WF10 Phosphate regulon sensor protein	two-component sensor molecule activity
BeE60C18E02	Q3KBH6 PAS/PAC Sensor Hybrid Histidine Kinase	two-component signal transduction system (phosphorelay)
BeE60C36F03	Q4KAJ4 Sensor histidine kinase/response regulator	two-component signal transduction system (phosphorelay)
BeE60C14D12	Q4ZP96 PAS	two-component signal transduction system (phosphorelay)

[a]*Transcripts downregulated during germination in nutrient medium.*

TABLE 3. Genes upregulated in cells germinating in the presence of adenine or potassium (30 min after induction) relative to germination in nutrient medium

Clone ID	Best alignment (Swiss-Prot)	GO description	Ratio A[a]	Ratio K[b]
BeE90N01D10	Q9Y823 Probable homocitrate synthase, mitocondrial	amino acid biosynthesis	1.83	1.87

BeE30N06E01	Q34DS8 Amino acid permease-associated region	amino acid transport	2.36	2.21
BeE120N01A01	Q3RIG0 Amino acid permease-associated region	amino acid transport	1.10	1.13
BeG30N13H09	Q3WZJ7 Amino acid permease-associated region	amino acid transport	1.97	1.90
BeE30N10F07	Q440D2 Amino acid permease-associated region precursor	amino acid transport	1.76	1.83
BeG60N01F09	Q4WZ19 High affinity methionine permease	amino acid transport	1.30	0.97
BeE120N09C08	Q8P9N1 Cationic amino acid transporter	amino acid transport	0.77	0.84
BeE60H21G09	Q8PLF9 Cationic amino acid transporter	amino acid transport	2.01	2.21
BeZSPN17H06	O06479 YfnA	amino acid transport	2.96	2.84
BeG30N15D05	Q2SCN2 Carbonic anhydrase	carbon utilization	0.694	1.81
BeE30N14D03	Q7ZUY3 Histone H2A.x	chromosome organization and biogenesis	1.62	1.14
BeE60H06B09	Q4PEF9 H2A_NEUCR Histone H2A	chromosome organization and biogenesis	1.83	1.25
BeZSPN03D06	Q9HDN1 Histone H3	chromosome organization and biogenesis	1.61	0.88
BeE30N02C10	Q4P7J7 H3_EMENI Histone H3	chromosome organization and biogenesis	2.13	1.31
BeZSPN11B02	Q2UFJ0 Histone H4	chromosome organization and biogenesis	2.01	1.24

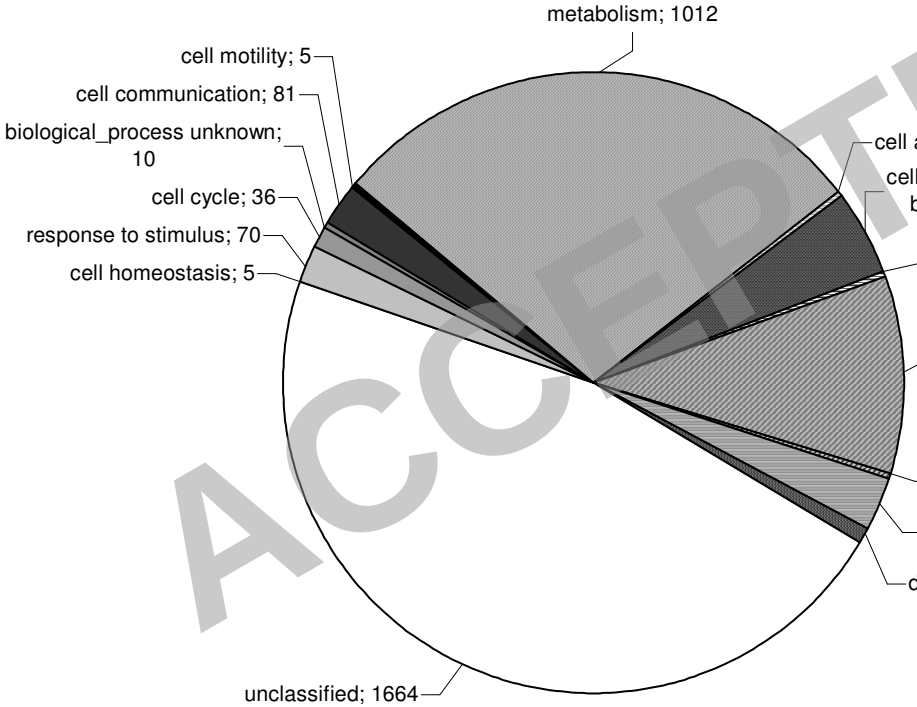
BeG90N18F10	Q8SZ87 RE13747p	mitosis	1.04	1.02
BeE60N03D06	Q4WJ09 Ketoreductase, putative	oxidation reduction	1.75	0.95
BeE60C17C02	P38356 Metal homeostatis protein BSD2	cation transport	1.72	1.15
BeZSPN09E08	Q7Z8B6 H(+)-ATPase	proton transport	0.778	0.631
BeZSPN10H02	Q7RW00 Hypothetical protein	electron transport	1.56	1.48
BeE60N09D01	Q2S1W1 Acyl-coenzyme A oxidase I, putative	oxidoreductase activity	1.43	1.12
BeZSPN14D10	O93787 Chs3	transferase activity	1.20	0.724
BeZSPN02F03	Q96338 AMP-binding protein	fatty acid metabolism	1.45	1.13
BeE60N19B01	Q95R88 SD01152p	fatty acid metabolism	2.00	1.01
BeG30N06H02	O74879 SPCC330.09 protein	rRNA processing	1.41	1.42
BeG30N09H07	Q5KN22 Ubiquitin-conjugating enzyme E2	ubiquitin cycle	0.84	0.858

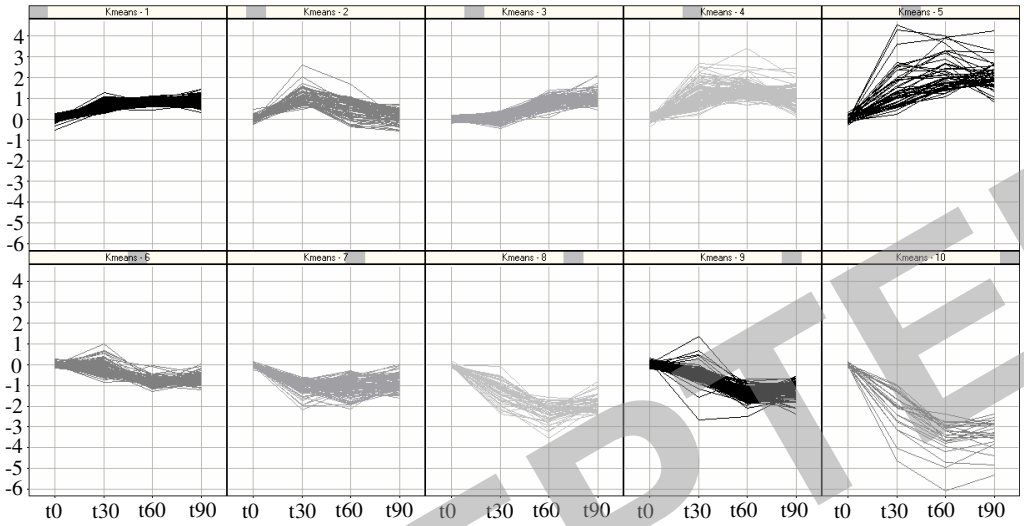
[a] *log_2 of the normalized expression ratios for cells germinating in adenine compared to germination in nutrient medium*

[b] *log_2 of the normalized expression ratios for cells germinating in potassium compared to germination in nutrient medium*

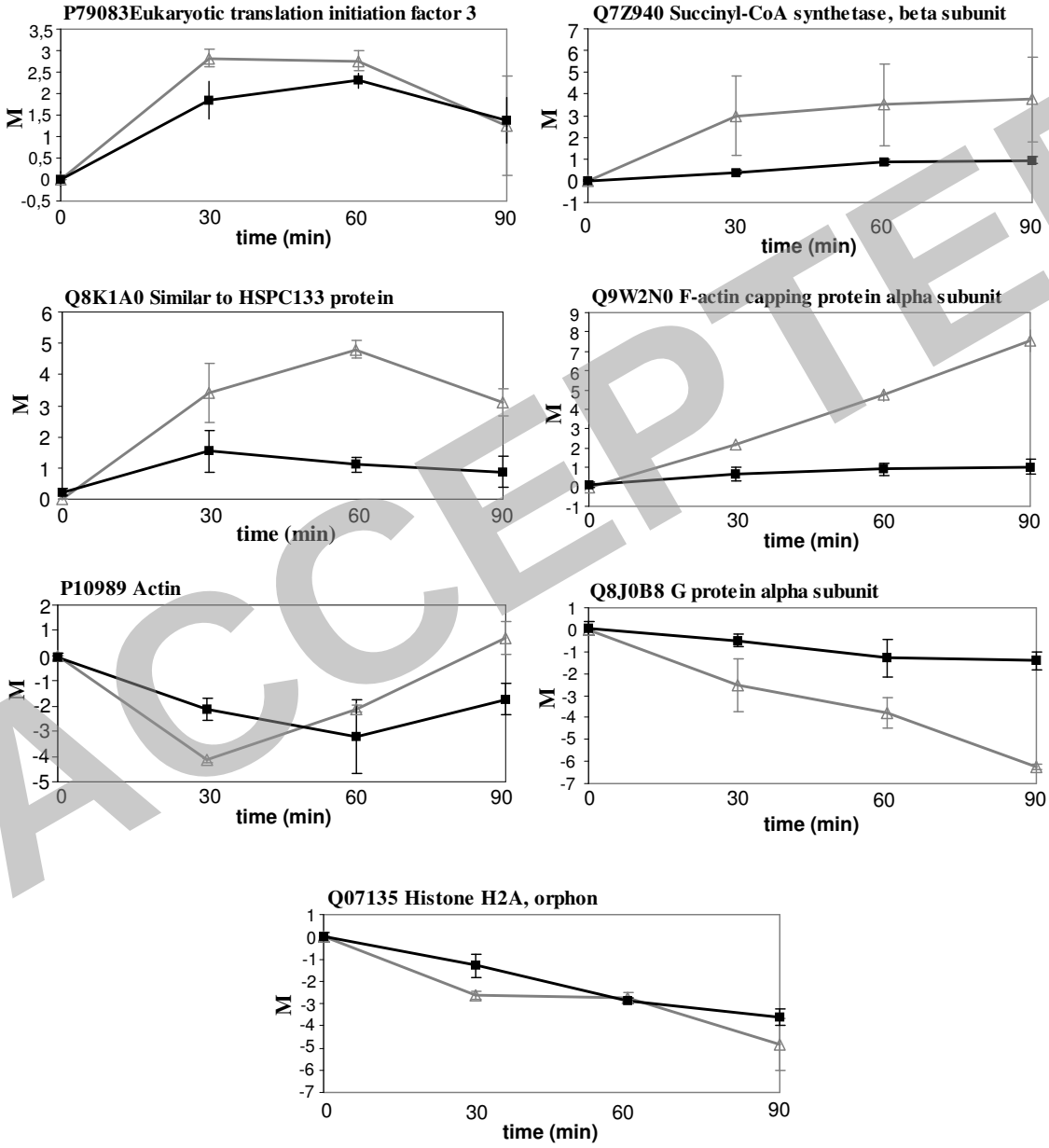
TABLE 4. Functional GO categories observed among the genes downregulated when zoospores germinate in inorganic salt solution in comparison with germination in the presence of nutrients.

30 min of germination	60 min of germination
Nucleosome assembly	Nucleosome assembly
Nucleotide biosynthesis	Regulation of transcription, DNA-dependent
Transcription	Transcription
rRNA processing	rRNA processing
Ribosome biogenesis	Ribosome biogenesis
Ribosome assembly	Translation initiation factor activity
Regulation of translational initiation	Protein biosynthesis
Translational termination	Protein targeting
Purine salvage	Protein-mitochondrial targeting
Arginine biosynthesis	Protein transport
Citrulline metabolism	Chaperone activity
Establishment of cell polarity	Transport
Cell motility	Pseudouridine synthesis
Cytokinesis	Small GTPase mediated signal transduction
Glycolysis	
Ubiquitin-dependent protein catabolism	
Cell cycle	



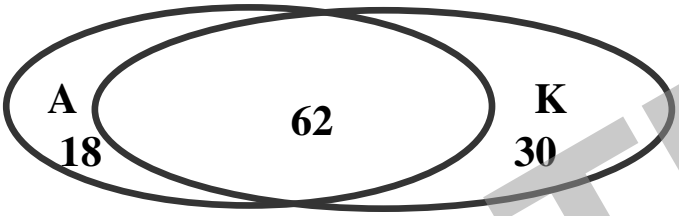


Group	*Overrepresented gene categories*
K1	protein biosynthesis, small GTPase mediated signal transduction, calcium ion transport, protein folding
K2	rRNA processing, transcription, ribosome biogenesis, purine nucleotide biosynthesis, cell adhesion, amino acid metabolism
K3	protein biosynthesis, glycolysis
K4	translation initiation factor activity, protein biosynthesis, carbohydrate transport, oligopeptide transport, cell cycle
K5	tricarboxylic acid cycle, glycolysis, fatty acid biosynthesis, anion transport, GTP biosynthesis, UTP biosynthesis, protein-nucleus import
K6	chitin synthase activity, proton transport
K7	signal transduction, actin binding activity
K8	ion channel activity
K9	amino acid transport, intracellular protein transport, protein serine/threonine kinase activity, calcium ion binding activity, signal transducer activity
K10	chromosome organization and biogenesis

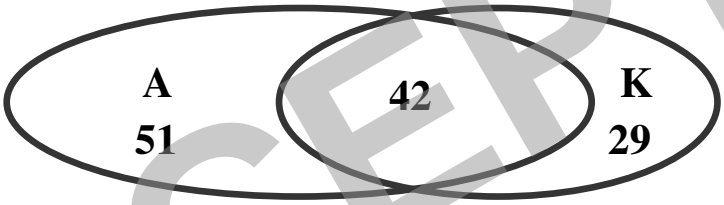


30 MIN OF GERMINATION

downregulated

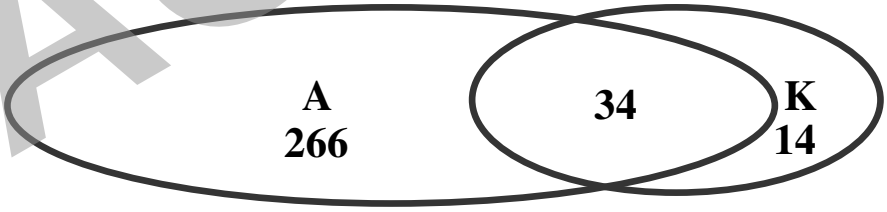


upregulated



60 MIN OF GERMINATION

downregulated



upregulated