Comparison of transcription of multiple genes during mycelia transition to yeast cells of *Paracoccidioides brasiliensis* reveals insights to fungal differentiation and pathogenesis

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Received: 12 July 2007 / Accepted: 15 October 2007
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Abstract The ascomycete *Paracoccidioides brasiliensis* is a human pathogen with a broad distribution in Latin America. The infection process of *P. brasiliensis* is initiated by aerially dispersed mycelia propagules, which differentiate into the yeast parasitic phase in human lungs. Therefore, the transition to yeast is an initial and fundamental step in the infective process. In order to identify and characterize genes involved in *P. brasiliensis* transition to yeast, which could be potentially associated to early fungal adaptation to the host, expressed sequence tags (ESTs) were examined from a cDNA library, prepared from mycelia ongoing differentiation to yeast cells. In this study, it is presented a screen for a set of genes related to protein synthesis and to protein folding/modification/destination expressed during morphogenesis from mycelium to yeast. Our analysis revealed 43 genes that are

induced during the early transition process, when compared to mycelia. In addition, eight novel genes related to those processes were described in the *P. brasiliensis* transition cDNA library. The types of induced and novel genes in the transition cDNA library highlight some metabolic aspects, such as putative increase in protein synthesis, in protein glycosylation, and in the control of protein folding that seem to be relevant to the fungal transition to the parasitic phase.

Keywords Dimorphic transition · Induced transcripts · *Paracoccidioides brasiliensis* · Protein synthesis · Protein folding/modification/destination

Introduction

Paracoccidioides brasiliensis is a human pathogen with a broad distribution in Latin America. The fungus is thermally dimorphic. In the soil, the fungus grows as saprobic mycelium and upon elevation of the temperature to that of the mammalian body, the fungus adopts a yeast-like phase [1]. A human host through inhalation acquires the fungal pathogen. The disease, paracoccidioidomycosis, is characterized by a chronic granulomatous inflammation, and patients might present a broad spectrum of clinical manifestations ranging from a localized and benign disease to a progressive and potentially lethal systemic mycosis.

Electronic supplementary material The online version of this article (doi:[10.1007/s11046-007-9078-8](https://doi.org/10.1007/s11046-007-9078-8)) contains supplementary material, which is available to authorized users.

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The severe nature of the disease and occurrence of sequelae, frequently causing pulmonary dysfunction or other disabilities, render it a pathogen of considerable medical importance [2].

P. brasiliensis can successfully establish and cause disease, highlighting the need to a better understanding of the molecular mechanisms of pathogenesis. Pathogenicity can be related to the factors associated to the transition from the saprophytic phase to the yeast parasitic phase, since fungal strains that are unable to differentiate into yeast cells are not virulent [3]. In this way, the characterization of genes/proteins related to the differentiation to the parasitic phase may bring insights to the fungal pathogenesis.

The morphological transition in *P. brasiliensis* is governed predominantly by the temperature and is preceded by several molecular changes. Some biochemical processes related to the dimorphic transition had been elucidated. In this way, the dimorphic transition involves alterations in the cell wall composition and in the structure of carbohydrates polymers [4]. Hyper expression of the enzymes of the sulfur metabolism in the yeast phase during the fungal transition to yeast reinforces previous findings that this metabolic pathway could be important for this differentiation process [5, 6]. Transcriptional analysis of genes highly expressed during the mycelia to yeast conversion identified the product 4-hydroxyphenyl pyruvate dioxygenase (4-HPPD), predicted to function in the catabolism of aromatic amino acids. Inhibition of 4-HPPD by specific compounds impairs the in vitro differentiation of mycelium to the yeast phase [7]. Also, data indicate that *P. brasiliensis* transition from mycelium to yeast is controlled by changing cAMP levels, with the onset of transition correlating with a transient increase in cAMP, suggesting activation of the cAMP-signaling pathway [8, 9].

A number of proteins and genes had been described as potentially associated to the fungal transition and putatively to the host invasion and host fungal survival. Proteomics-based discovery approaches have successfully identified potential candidates to the dimorphic process. Proteomic analysis from mycelium ongoing differentiation to yeast cells was performed allowing the characterization of proteins that could be relevant to the fungal differentiation. In this sense, the proteins HSP70, HSP60, glyceraldehyde-3-phosphate dehydrogenase, catalase P and actin

were accumulated during the transition from mycelium to yeast [10–15].

A great amount of transcriptional data has been obtained from *P. brasiliensis* [16–21]. Approaches used in this fungus to identify phase-specific genes and or genes important for the dimorphic process, included microarray hybridization approaches [7, 16] and in silico EST subtraction [16]. Transcriptional profiling of microarrays built with ESTs of *P. brasiliensis* has identified 328 genes that are differentially expressed upon the phase transition [16]. In addition, constructed microarrays based on yeast-phase genes and hybridized to RNAs isolated from fungal cells at time points during the switch to the yeast phase enabled the identification of transcripts potentially associated to the fungal morphogenesis [7].

In a previous work, we have tested the concept that novel genes involved in *P. brasiliensis* phase transition could be described by applying a transcriptome analysis of cells undergoing mycelium to yeast transition. We reported the in silico analyses and comparison of ESTs from mycelium undergoing the early transition to yeast with mycelium differentiated cells. According to our data, the developmental program of *P. brasiliensis* is characterized by significant differential positive modulation of transcripts related to cellular processes, predominantly to the cell wall/membrane synthesis/remodeling, suggesting their importance in dimorphism [21].

In this study, in order to advance our understanding on the molecular mechanisms of dimorphic transition and of the initial steps of the fungal adaptation to the host, we sought to examine the profile of transcripts related to protein synthesis/processing/regulation/degradation in the ESTs generated from the cDNA library of mycelium undergoing transition to yeast cells. Using comparative sequence analyses, we could identify sequences, which were absent in the *P. brasiliensis* yeast and mycelium transcriptome and in public databases, as well as sequences induced during the early fungal transition. Through these approaches, it was found: (1) 54 possible homologues, including 18 induced/novel homologues of genes previously described as related to protein synthesis; and (2) 44 possible homologues, including 25 induced/novel homologues to genes related to protein folding/modification/destination. Those novel/induced genes provide ideal candidates

for further studies directed at understanding fungal morphogenesis and its regulation.

Materials and methods

RNA extraction and preparation of the cDNA library

The cDNA library was constructed, as previously reported [21]. Briefly, *P. brasiliensis*, isolate Pb01 (ATCC-MYA-826), was grown in Fava-Netto's medium [1% (w/v) peptone; 0.5% (w/v) yeast extract; 0.3% (w/v) proteose peptone; 0.5% (w/v) beef extract; 0.5% (w/v) NaCl; 4% (w/v) agar, pH 7.2], at 22°C, as mycelium. The differentiation was performed in liquid medium (Fava-Netto's medium) by changing the culture temperature from 22 to 36°C for the mycelium to yeast transition, as we previously described [13]. The cells were previously grown in liquid medium for 18 h before changing the incubation temperature, which was maintained for 22 h.

Total RNA was purified from *P. brasiliensis* mycelium in transition to yeast cells using TRIZOL (GIBCO™, Invitrogen, Carlsbad, CA). The mRNA was purified by using the Poly (A) Quick^R mRNA isolation kit (Stratagene, La Jolla, CA). The cDNA library was constructed in the unidirectional pCMV.SPORT 6 (Invitrogen) according to the manufacturer's instructions, exploiting the *NotI* and *SalI* restriction sites. The cDNA library was not normalized, i.e., no attempt was made to reduce the redundancy of highly expressed transcripts.

EST processing pipeline and annotation

The nucleotide sequences were uploaded to a relational database (MySQL) on a Linux (Fedora Core 3) platform, and processed using a modified version of the PHOREST tool [22]. The sequences generated during dimorphic transition of *P. brasiliensis* [21] were compared to sequences generated from yeast and mycelium [16]. Transcripts classification was performed by using the MIPS categorization (<http://www.mips.gsf.de/>). Similarities with E -values $\leq 10^{-4}$ were considered significant.

In silico determination of induced genes in the mycelium to yeast transition

In order to assign a differential expression character, the contigs formed with mycelium and the transition ESTs were statistically evaluated using the Audic and Claverie's method [23]. It were considered induced genes in the transition library those that were not previously described in the mycelium transcriptome database (<http://www.dna.biomol.unb.br/Pb>), and those more expressed as determined with a 99% confidence rate. A web site (<http://www.igs.cnrs-mrs.fr/Winflat/winflat.cgi>) was used to compute the probability of differential regulation. The *P. brasiliensis* transcriptome database at (<http://www.dna.biomol.unb.br/Pb>) and public databases (<http://www.ncbi.nlm.nih.gov>) were used to identify novel transcripts, by using the BLAST program [24], as described [21].

Results and discussion

cDNA library sequence annotation

The cDNA library was constructed in a non-normalized primary library without amplification, so the clone abundance presents the relative mRNA population. The quality of the cDNA library was checked by evaluating the presence of well-characterized transcripts in the MIPS category, such as, those encoding for energy and metabolism. The results of computational homology search of the genes related to protein synthesis/folding/modification/destination obtained from the *P. brasiliensis* mycelium undergoing differentiation to yeast cells are shown in the supplementary material, Tables 1 and 2, respectively. A total of 200 ESTs (27.3% of the total transition transcriptome) showed significant similarity to sequences related to protein synthesis/folding/modification/destination (E -value $\leq 10^{-4}$) based on BLAST searches. A total of 184 ESTs (25.12% of the total transition transcriptome) gave significant hits to ESTs present in the *P. brasiliensis* transcriptome database or in the GenBank database. In addition, 16 ESTs (2.18% of the total transition transcriptome) represented novel genes of *P. brasiliensis* regarding to the above processes.

A broad view of the nature of the adaptations made by *P. brasiliensis* concerning to protein synthesis during early transition to yeast was obtained by classifying the ESTs into 6 groups of functionally related genes (Table 1, supplementary material). Among the transcripts classified in the transition cDNA library and related to protein synthesis, it was found predominantly ESTs coding for ribosomal proteins, comprehending 39 unigenes.

Regarding to protein folding/modification/destination, the ESTs were classified into 6 groups of functionally related genes, as demonstrated in Table 2, supplementary material. Most of the ESTs represent unigenes related to the MIPS classification of posttranslational modification of amino acids (18 unigenes), followed by transcripts related to protein modification by ubiquitination (8 unigenes), proteasomal degradation (8 unigenes), protein processing (6 unigenes), and protein folding (3 unigenes).

Description of the ESTs related to protein synthesis and fate in the transition transcriptome

As shown in Fig. 1a, the ESTs related to protein synthesis were mainly represented as following: a

Fig. 1 Distribution of ESTs from *P. brasiliensis* mycelia ongoing transition to yeast according to their cDNA products. (a) Protein synthesis; (b) protein fate. The classification was based on *E*-value and performed according to the functional categories developed on the MIPS functional annotation scheme. The percentage of ESTs classification is indicated

total of 40.68% of the annotated ESTs corresponded to the ribosomal proteins of the ribosome large subunit; 24.58% in that category were related to the ribosomal proteins of the ribosome small subunit; 15.24% of the transcripts corresponded to homologues encoding translational initiation factors; 9.32% corresponded to ESTs related to the translation elongation machinery. Other ESTs were related to aminoacyl tRNA synthetases (1.69%), ribosome biogenesis (7.64%) and translation termination (0.85%). The Fig. 1b catalogues the ESTs related to protein fate according to the MIPS categories. Most of the transcripts were related to posttranslational modifications of proteins (38.27%) and protein ubiquitination (27.16%). Transcripts related to protein processing and proteasomal degradation of proteins represented 14.81 and 11.12 %, respectively. Classes with lower number of transcripts comprehended those related to protein folding (6.17%) and protein targeting (2.47%).

High abundant ESTs related to protein synthesis and fate in the transition transcriptome

Table 1 shows the 10 most abundant ESTs related to protein synthesis and folding/modification/destination in the transition transcriptome. The minimum number of ESTs that made up these most highly redundant contigs was 5. Eight out of the ten most abundant ESTs were identified as induced sequences according to the Audic and Claverie's method and one EST represented a novel transcript. Included among the most abundant transcripts were ESTs encoding for proteins related to ribosome assembly/biogenesis [25–27] and translation [28–30]. Moreover, a transcript encoding a 14 kDa mitochondrial ribosomal protein (*mrps14*) was detected as a novel transcript. In the *P. brasiliensis* mitochondrial genome, the *mrps14* gene was not found [31], suggesting, as described in *Arabidopsis thaliana* [32] its possible transference to the nucleus.

Also, among the highly redundant transcripts, it was detected homologues of proteins related to the acceleration of the protein folding and ubiquitination in many organisms [33, 34]. Transcript encoding proteins related to stress conditions, such as the homologue of the L-isoaspartate O-methyltransferase (*pcmt*) that specifically recognizes and methylates

Table 1 The most abundant transcripts related to protein synthesis and protein fate expressed during transition from mycelium to yeast

MIPS category	Gene name	Gene product	Best hit/accession number	E-value	Enzyme commission	Redundancy in the transition cDNA library	Function in organisms
<i>Protein synthesis</i>							
	<i>ubi/crp-6</i>	Ubiquitin fused to S27a protein ^a	<i>Aspergillus nidulans</i> / XP_409009	2e-60	–	7	Required for ribosome biogenesis serving to aid in the assembly of S27a into the ribosome in <i>Saccharomyces cerevisiae</i> [25] Not available
	<i>mrps14</i>	14 kDa mitochondrial ribosomal protein ^c	<i>Aspergillus nidulans</i> / XP_408748	4e-46	–	7	
	<i>rps5</i>	40S ribosomal protein S5 ^a	<i>Aspergillus nidulans</i> / XP_404980	8e-22	–	7	Required for the folding of 16S ribosomal RNA and translational fidelity in <i>E. coli</i> [26]
	<i>rpl20</i>	60S ribosomal protein L20 ^a	<i>Magnaporthe grisea</i> / XP_361110	3e-16	–	6	Required for ribosome assembly in <i>E. coli</i> [27].
	<i>sui1</i>	Translation initiation factor eIF1 subunit Sui1 ^a	<i>Gibberella zeae</i> / XP_389056	2e-36	–	5	Required for the recognition of the AUG codon during translation initiation and for activation of the nonsense-mediated mRNA decay pathway in <i>S. cerevisiae</i> [28, 29]
	<i>tef1</i>	Translational elongation factor EF-1 alpha	<i>Aspergillus nidulans</i> / XP_405299	4e-31	–	8	Essential for the delivery of aminoacyl-tRNAs in eukaryotes [30]
<i>Protein fate</i>							
	<i>ppi4</i>	Peptidyl-prolyl cis-trans isomerase-like 4 (Cyclophilin RRM) ^a	<i>Coccidioides immitis</i> / EAS29016	1e-46	5.2.1.8	5	Required for acceleration of proteins folding in organisms [33]
	<i>ubc-6</i>	Ubiquitin conjugating enzyme E2 ^a	<i>Gibberella zeae</i> / XP_388490	1e-29	6.3.2.19	7	Catalysis the covalent attachment of ubiquitin to proteolytic substrates in organisms [34]

Table 1 continued

MIPS category	Gene name	Gene product	Best hit/accession number	E-value	Enzyme commission	Redundancy in the transition cDNA library	Function in organisms
	<i>pcmt</i>	Protein-L-isoaspartate (D-aspartate) O-methyltransferase ^a	<i>Aspergillus nidulans</i> /XP_407601	5e-55	2.1.1.77	5	Required for metabolism of isoaspartyl residues preventing protein damage under physiological conditions in organisms [35]
	<i>pep</i>	Aspartyl proteinase ^a	<i>Paracoccidioides brasiliensis</i> /AAP32823	3e-72	3.4.23.24	7	Required for protein processing and for degradation of peptides. Promotes preferential cleavage in hydrophobic amino acids of proteins in eukaryotes [36]

^a Transcripts induced in the transition library in comparison to the mycelium transcriptome according to the Audic and Claverie's method

^b Novel genes detected in *P. brasiliensis*

isoaspartyl residues in a variety of proteins [35], thus preventing the accumulation of deamidated proteins under stressing conditions, was high abundant in the morphological transition. Regarding to protein processing, the transcript encoding aspartyl proteinase (*pep*) was detected. Aspartic proteases are widely distributed in all domains of life and are related to cleavage of peptides in regions of hydrophobic amino acids under acidic conditions. The *S. cerevisiae* vacuolar Pep4p homologue to the *P. brasiliensis* *pep* product has been described as required for the turnover of damaged molecules during stress conditions [36].

Induced genes related to protein synthesis identified by in silico EST subtraction

We attempted to determine the putative function of the set of 119 phrap unisequences by searching for homologues in the GenBank non-redundant protein database using BLAST X and by comparing the ESTs in the transition library to those present in the mycelium transcriptome database. The Fig. 2 presents the percentage of induced and novel genes related to protein synthesis in comparison to the total number of ESTs in the transition transcriptome, as described [21]. As observed, from the 119 ESTs related to protein synthesis (10.66% of the total), 3.97% were induced in the transition library and 0.9% was described as novel genes in *P. brasiliensis*.

The comparative analysis of all the induced ESTs related to protein synthesis in the transition library is available; Table 2 summarizes the results of such comparison. In *P. brasiliensis*, induced transcripts, putatively playing role in ribosomal biogenesis and maturation were detected during dimorphic transition, such as 60S ribosome subunit biogenesis protein (*nip7*), GTP-binding GTP1/OBG (*ygr210*) family protein and ubiquitin fused to S27a protein (*ubi/crp-6*). Proteins composing the small and large ribosomal subunits, as well as translational initiation factors, from both cytoplasmic and mitochondrial ribosomes were over expressed in the transition library; some represent novel genes (Table 2). Similar results were described suggesting that the fungal transition is likely to involve intense synthesis of new ribosome particles, affecting the rate of protein

Fig. 2 Prevalence distribution of ESTs from *P. brasiliensis* mycelia ongoing transition to yeast. The percentage of ESTs related to protein synthesis and fate in the total ESTs are represented along with the percentage of over expressed and novel genes of *P. brasiliensis* in the transition library (<http://192.168.0.5/phorestwww>)

synthesis [7]. In addition, some of the transcripts encode for ribosomal proteins whose orthologues are differentially regulated in organisms. In this sense, the *rps26* product which has no homologue among prokaryotic ribosomal proteins [37] is differentially expressed during environmental stress in plants [38]. Also, the developmental program of organisms seems to include the differential expression of ribosomal proteins; ribosomal protein *rpl5* product was specifically identified in schizonts and was undetectable in oocysts in the organism *Eimeria tenella* [39], suggesting its regulation under different life-cycle stages.

Induced genes related to protein fate identified by in silico EST subtraction: ESTs relevant to protein processing:

We also attempted to determine the putative function of the set of 81 phrap unsequences by searching for homologues in the GenBank non-redundant protein database using BLAST X and by comparing the ESTs in the transition library to those present in the mycelium transcriptome database. The classification of induced genes was designed as described. The Fig. 2 presents the percentage of induced and novel genes related to protein folding/modification/destination in comparison to the total number of ESTs in the transition transcriptome as described [21]. As observed, from the 81 ESTs (7.32% of the transition transcriptome), 48 (4.43% of the total transition transcriptome) were induced in the transition library and 6 (0.54%) were described as novel genes in *P. brasiliensis*.

The comparative analysis of the ESTs related to protein folding/modification/destination is available; Table 3 summarizes the results of such comparison. A cyclophilin seven suppressor 1 (*cns1*) (HSP90 chaperone complex component) was detected. The Hsp90 complex is one of the most abundant and highly conserved chaperone preventing the aggregation of proteins in a folding-competent state and is essential for cell viability in *S. cerevisiae* [40]. A tailless complex polypeptide 1 chaperonin, subunit epsilon (*tcp-1*) was also detected. The *tcp-1* is localized in the cytosol of higher eukaryotes and is similar to prokaryotes GroEL. The *tcp-1* product has been related to protein folding in *S. cerevisiae* playing role in cell development and cytoskeletal organization [41]. The two ORFs encoding homologues to the above proteins, presumably reflect the heat shock condition experienced by mycelia in transition to yeast cells.

Glycosyltransferases play vital roles in the biological function of native proteins, as well as, in the biosynthesis of numerous molecules within fungi, including cell wall components and its induced expression putatively reflect the cell wall remodeling that occurs during *P. brasiliensis* morphological transition [4, 21]. The novel/induced genes encode glycosyltransferases that could be related to galactosylation of N- and O-glycans, as described in *S. cerevisiae* [42]. Mannosyltransferases (*och1* and

Table 2 Novel and over expressed transcripts related to protein synthesis detected during dimorphic transition in *P. brasiliensis*

MIPS category	Gene name	Gene product	Best hit/accession number	<i>E</i> -value	Enzyme commission	Redundancy ^d	
						M	T
<i>Protein synthesis</i>							
<i>Ribosome biogenesis</i>							
	<i>nip7</i>	60S ribosome subunit biogenesis protein NIP7 ^b	<i>Aspergillus fumigatus</i> /AAM08680	3e−14	−	−	1
	<i>ygr210</i>	GTP-binding GTP1/OBG family protein ^b	<i>Aspergillus nidulans</i> /XP_404829	1e−70	−	−	1
	<i>ubi/crp−6</i>	Ubiquitin fused to S27a protein ^a	<i>Aspergillus nidulans</i> /XP_409009	2e−60	−	7	7
<i>Ribosomal proteins</i>							
<i>Small subunit</i>							
	<i>mrps14</i>	14 kDa mitochondrial ribosomal protein ^c	<i>Aspergillus nidulans</i> /XP_408748	4e−46	−	−	7
	<i>rps13</i>	40S ribosomal protein S13 ^b	<i>Neurospora crassa</i> /EAA34807	2e−37	−	−	1
	<i>rps26</i>	40S ribosomal protein S26 ^b	<i>Neurospora crassa</i> /CAA39162	3e−52	−	−	1
	<i>rps5</i>	40S ribosomal protein S5 ^a	<i>Aspergillus nidulans</i> /XP_404980	8e−22	−	8	7
	<i>mrps19</i>	Mitochondrial ribosomal protein S19 ^b	<i>Aspergillus nidulans</i> /XP_404292	5e−19	−	−	1
<i>Large subunit</i>							
	<i>rpl20</i>	60S ribosomal protein L20 ^a	<i>Magnaporthe grisea</i> /XP_361110	3e−16	−	3	6
	<i>rpl27</i>	60S ribosomal protein L27 ^a	<i>Aspergillus nidulans</i> /XP_408359	4e−63	−	1	3
	<i>rpl5</i>	60S ribosomal protein L2 ^b	<i>Coccidioides immitis</i> /EAS30555	9e−54	−	−	1
	<i>rpl3</i>	60S ribosomal protein L3 ^a	<i>Aspergillus fumigatus</i> /AAM43909	5e−85	−	1	2
	<i>rpl43</i>	60S ribosomal protein L43B ^b	<i>Ustilago maydis</i> /XP_400133	1e−30	−	−	1
<i>Translation initiation</i>							
	<i>eif3</i>	Translation initiation factor 3 subunit 2 ^c	<i>Aspergillus nidulans</i> /XP_660601	6e−80	−	−	3
	<i>eif−5A</i>	Translation initiation factor eIF−5A ^a	<i>Neurospora crassa</i> /P38672	6e−06	−	4	4
	−	Translational machinery component protein ^b	<i>Aspergillus nidulans</i> /XP_405417	1e−19	−	−	1
	<i>sui1</i>	Translation initiation factor eIF1 subunit Sui1 ^a	<i>Gibberella zeae</i> /XP_389056	2e−36	−	2	5
<i>Aminoacyl-tRNA synthetase</i>							
	<i>ils1</i>	Isoleucyl-tRNA synthetase ^b	<i>Aspergillus nidulans</i> /XP_407499	1e−52	6.1.1.5	−	2

^a Transcripts induced in the transition library in comparison to the mycelium transcriptome according to the Audic and Claverie's method

^b Transcripts non detected in the mycelia transcriptome (<http://www.dna.biomol.unb.br/Pb>)

^c Novel genes detected in *P. brasiliensis*

^d M: Redundancy in *P. brasiliensis* mycelia transcriptome (<http://www.dna.biomol.unb.br/Pb>); T: Redundancy in *P. brasiliensis* transition library (<http://192.168.0.5/phorestwww/>)

Table 3 Novel and over expressed transcripts related to protein fate detected during dimorphic transition in *P. brasiliensis*

MIPS category	Gene name	Gene product	Best hit/accession number	<i>E</i> -value	Enzyme commission	Redundancy ^c	
						M	T
<i>Protein fate</i>							
<i>Protein folding</i>							
	<i>cns1</i>	Cyclophilin seven suppressor 1 (HSP90 chaperone complex component) ^b	<i>Aspergillus nidulans</i> /XP_409575	8e−12	–	–	2
	<i>tcp−1</i>	Tailless complex polypeptide 1 chaperonin, subunit epsilon ^b	<i>Schizosaccharomyces pombe</i> /EAA65069	6e−16	–	–	2
<i>Posttranslational modification of amino acids</i>							
	<i>gma12</i>	Alpha−1, 2-galactosyltransferase ^c	<i>Aspergillus nidulans</i> /XP_406106	3e−14	2.4.1.-	–	1
	<i>mmt1</i>	Alpha−1, 2-mannosyltransferase ^a	<i>Neurospora crassa</i> /CAC18268	1e−29	2.4.1.131	3	3
	<i>och1</i>	Mannosyltransferase ^b	<i>Paracoccidioides brasiliensis</i> /AAK54761	3e−70	2.4.1.130	–	1
	<i>swp1</i>	Oligosaccharyltransferase subunit ribophorin II ^d	<i>Coccidioides immitis</i> /EAS29547	9e−37	2.4.1.119	–	1
	<i>rabggt</i>	Rab geranylgeranyl transferase ^c	<i>Aspergillus nidulans</i> /XP_412816	8e−13	2.5.1.60	–	1
	<i>cypb</i>	Peptidyl prolyl cis−trans isomerase ^b	<i>Neurospora crassa</i> /CAD21421	8e−39	5.2.1.8	–	1
	<i>ppil1</i>	Peptidyl-prolyl cis−trans isomerase-like 4 (Cyclophilin RRM) ^a	<i>Coccidioides immitis</i> /EAS29016	1e−46	5.2.1.8	1	5
	<i>pcmt</i>	Protein-L-isoaspartate (D-aspartate) O-methyltransferase ^a	<i>Aspergillus nidulans</i> /XP_407601	5e−55	2.1.1.77	4	5
	<i>gmd1</i>	Guanosine diphosphatase ^c	<i>Aspergillus nidulans</i> /XP_405219	2e−15	3.6.1.42	–	1
<i>Proteasomal degradation</i>							
	<i>rpt6</i>	26S proteasome regulatory subunit protein ^b	<i>Aspergillus nidulans</i> /XP_411125	4e−23	–	–	1
	<i>rpn12</i>	26s proteasome regulatory subunit rpn12 ^b	<i>Aspergillus nidulans</i> /XP_407156	5e−30	–	–	1
	<i>rpn5</i> ; <i>rpne</i>	26S proteasome regulatory subunit Non-ATPase ^c	<i>Aspergillus nidulans</i> /XP_408912	2e−68	–	–	1
	<i>csn5</i>	COP9 signalosome complex subunit 5 ^a	<i>Aspergillus nidulans</i> /XP_406266	1e−35	–	1	2
<i>Modification by ubiquitination</i>							
	<i>ubp1</i>	Ubiquitin-specific protease (C19) ^b	<i>Aspergillus nidulans</i> /XP_412211	7e−08	3.1.2.15	–	3
	<i>ubc−6</i>	Ubiquitin conjugating enzyme E2 ^a	<i>Gibberella zeae</i> /XP_388490	1e−29	6.3.2.19	6	7
	<i>ubq/rpl40</i>	Ubiquitin fusion protein ^a	<i>Schizosaccharomyces pombe</i> /NP_593923	8e−67	–	3	3

Table 3 continued

MIPS category	Gene name	Gene product	Best hit/accession number	E-value	Enzyme commission	Redundancy ^c	
						M	T
	<i>ubp1; otub1</i>	Ubiquitin thiolesterase otubain like protein ^c	<i>Aspergillus nidulans</i> /EAA60354	1e–28	3.4.-	–	1
	<i>rfln167</i>	Ring (really interesting new gene) type zinc finger (C3HC4) protein (E3 complex) ^b	<i>Schizosaccharomyces pombe</i> /CAB08748	5e–10	–	–	1
	<i>fbl7</i>	F-box/LRR-repeat protein 7 (E3 complex) ^b	<i>Aspergillus nidulans</i> /XP_408647	8e–28	–	–	3
<i>Protein Processing</i>							
	<i>pep</i>	Aspartyl proteinase ^a	<i>Paracoccidioides brasiliensis</i> /AAP32823	3e–72	3.4.23.24	3	7
	<i>lon</i>	Lon protease ^b	<i>Pseudomonas fluorescens</i> /AF250140_1	1e–05	3.4.21.53	–	1
	<i>lap</i>	Peptidase M28 domain protein ^c	<i>Coccidioides immitis</i> /EAS33583	1e–22	3.4.11.15	–	1
	<i>mde10</i>	Zinc metalloprotease (M12) ^b	<i>Neurospora crassa</i> /CAD21161	3e–47	3.4.24.-	–	1

^a Transcripts induced in the transition library in comparison to the mycelium transcriptome according to the Audic and Claverie's method

^b Transcripts non detected in the mycelia transcriptome (<http://www.dna.biomol.unb.br/Pb>)

^c Novel genes detected in *P. brasiliensis*

^d Genes not described previously in *P. brasiliensis* isolate Pb01, but present in public databases

^e M: Redundancy in *P. brasiliensis* mycelia transcriptome (<http://www.dna.biomol.unb.br/Pb>); T: Redundancy in *P. brasiliensis* transition library (<http://192.168.0.5/phorestwww/>)

mnt1) could be putatively related to the O-linked mannosylation of proteins, as observed in *C. albicans*. *C. albicans* mutants to either *mnt1* or *och1* showed hypersensitivity to cell wall perturbing agents, suggesting the proteins role in the cell wall maintenance [43, 44]. Moreover, a novel transcript encoding to guanosine diphosphatase (*gmd1*) was detected during the dimorphic transition, whose product is known to regulate mannosylation of N- and O-linked oligosaccharides in Golgi complex [45].

Peptidyl-prolyl cis/trans isomerases (cyclophilins) catalyze cis/trans isomerization of a prolyl bond and this isomerization is a time limiting step in folding of certain proteins [46]. Transcripts encoding to two-peptidyl prolyl cis–trans isomerases (*cypb* and *ppil*) were induced in *P. brasiliensis* during the transition from mycelium to yeast. Aside from their roles in cellular biochemistry, cyclophilins of microorganisms are particularly interesting since those proteins are found to have a key role in pathogenicity [47]. The *P. brasiliensis* CypB

deduced amino acid sequence presents four conserved amino acids: Arg, Phe, Trp and His (RFWH motif, data not shown) described as involved in peptidyl-prolyl cis–trans isomerase activity and related to the activity of the protein in the folding process as described [48].

Protein processing MIPS category is represented by four unigenes induced in *P. brasiliensis* transition library; some presents orthologues with function in stress response and differentiation. The aspartic protease (*pep*) with seven ESTs, was also included with the most abundant transcripts (see Table 1). The *pep* product belongs to family A1 of aspartic protease, related to pepsin and synthesized as a propeptide with signal peptide. This peptidase family is related to stress response in *S. cerevisiae* [36]. The deduced Lon protease (*lon*) shows homology with family S16, class 001 in MEROPS database (<http://www.merops.sanger.ac.uk>) and is induced in the transition transcriptome sharing identity with its counterparts in bacteria. The *lon* product was first identified in *E. coli*

Table 4 Homologues for protein synthesis and fate ESTs putatively related to fungal differentiation/virulence or stress tolerance

Gene product	Described role	Redundancy ^d		Reference
		M	T	
Cyclophilin seven suppressor 1 (<i>cms1</i>) ^b	Promotes increase in heat shock response in <i>Saccharomyces cerevisiae</i> .	–	2	[40]
Alpha-1, 2-mannosyltransferase (<i>mnt1</i>) ^a	Required for adhesion and virulence in <i>Candida albicans</i>	3	3	[43]
Mannosyltransferase (<i>och1p</i>) ^b	Required for cell wall integrity and virulence in <i>Candida albicans</i>	–	1	[44]
Zinc metalloprotease (<i>mde10</i>) ^b	Required for spore development in <i>Schizosaccharomyces pombe</i>	–	1	[52]
GTP-binding GTP1/OBG family protein (<i>ygr210</i>) ^b	Involved in regulation of differentiation in <i>Streptomyces coelicolor</i> .	–	1	[53]
Peptidyl prolyl cis–trans isomerase (<i>cypb</i>) ^b	Induced in heat shock response in <i>Aspergillus nidulans</i> .	–	1	[54]
Peptidyl-prolyl cis–trans isomerase-like 4 (<i>ppil1</i>) ^a	Related to thermoresistance in <i>Paramecium sp</i>	1	5	[55]
Peptidyl-prolyl cis/trans isomerase (<i>ess1</i>)	Required for <i>Cryptococcus neoformans</i> virulence	6	1	[56]
Peptidyl-prolyl cis–trans isomerase (<i>mip</i>)	Required for <i>Legionella pneumophila</i> survival into macrophages	2	2	[57]
Protein-L-isoaspartate (D-aspartate) O-methyltransferase (<i>pcmt</i>) ^{a,c}	Promotes increase in heat shock survival in <i>Escherichia coli</i> .	4	5	[58]
Ubiquitin conjugating enzyme E2 (<i>ubc6</i>) ^a	Promotes enhanced in growth of <i>Saccharomyces cerevisiae</i> at high temperature.	6	7	[59]
Aspartyl proteinase ^{a,c} (<i>pep</i>)	Secreted by <i>Aspergillus fumigatus</i> during invasion of the host lung.	3	7	[60]
Lon protease (<i>lon</i>) ^b	Required for cellular morphology and virulence in <i>Agrobacterium tumefaciens</i>	–	1	[61]

^a Transcripts induced in the transition library in comparison to the mycelium transcriptome according to the Audic and Claverie's method

^b Transcripts non detected in the mycelia transcriptome (<http://www.dna.biomol.unb.br/Pb>)

^c Also over expressed in yeast cells recovered from liver of infected mice (Costa et al. unpublished)

^d M: Redundancy in *P. brasiliensis* mycelia transcriptome (<http://www.dna.biomol.unb.br/Pb>); T: Redundancy in *P. brasiliensis* transition library (<http://192.168.0.5/phorestwww/>)

and its homologues were further discovered in many organisms sometimes in multiple copies, playing essential roles in protein quality control by destroying unfolded proteins [49]. In *P. brasiliensis*, a gene homologue encoding for a Lon protein of the S16 family, class 002 in MEROPS database (<http://www.merops.sanger.ac.uk>), was described previously [50], suggesting more than one Lon species in the fungal pathogen. A metalloprotease M28 domain protein (*lap*) was found as a novel gene in *P. brasiliensis*, encoding for a leucyl aminopeptidase. In *Thichoderma harzianum*, the M28 peptidase is induced during

nitrogen starvation suggesting its importance in the amino acid acquisition [51]. Other metalloprotease induced in *P. brasiliensis* dimorphic transition is the zinc metalloprotease belonging to M12 family (*mde10*), whose members were described in fungi [52].

Putative differentiation, virulence and stress tolerance factors

Factors putatively related to the differentiation process, fungal virulence and stress tolerance were

selected on basis with homology to other microorganisms in which defined functions are available. With these criteria, we classified some transcripts as shown in Table 4. The *cns1* product is an essential component of the HSP90 complex, which is induced in heat shock response [40]. Mannosyltransferases (*mnt1* and *och1*) orthologues are required for cell wall integrity/virulence and adhesion/virulence, respectively, in *C. albicans* [43, 44]. In *S. pombe*, the *mde10* product is essential for development of spore envelopes [52] evidencing its importance during differentiation process in the cell. The GTP-binding GTP1/OBG family product (*ygr210*) related to ribosome biogenesis has been described as a regulator of differentiation in *Streptomyces coelicolor*, playing a role in the onset of aerial mycelium formation and sporulation [53]. In *Aspergillus nidulans*, CypB is induced in response to heat shock indicating a possible role of this protein during growth in stress environments [54]. *P. tetraurelia* KIN241 homologue to peptidyl-prolyl cis–trans isomerase-like 4 (*ppil1*) is related to the organism thermoresistance [55]. A parvulin type Ess1 of *Cryptococcus neoformans* homologue to *P. brasiliensis* *ppil1* product is required for virulence, since Ess1 depleted strains are unable to cause experimental infection [56]. The Mip protein (macrophage infectivity potentiator) of *Legionella pneumophila* is a cyclophilin FKBP-type homolog which is related to bacterial virulence in intracellular infection in guinea pig [57]. *Escherichia coli* transformants over expressing L-isoadipate (D-aspartate) O-methyltransferase presented increase in the heat shock survival rates [58]. Yeast strains over expressing ubiquitin conjugating enzyme E2 are more tolerant to various stresses conditions, such as high temperature [59]. The *A. fumigatus* aspartyl protease (*pep*) is highly secreted during fungal invasion of host lung [60]. The Lon protease of *Agrobacterium tumefaciens* is required for normal growth, cellular morphology and full virulence [61].

Concluding remarks

Molecular strategies relying on ESTs has proved to be an efficient approach to identify genes expressed under a variety of conditions. This study presents a screen for genes related to protein synthesis/folding/modification/destination expressed during mycelium

to yeast differentiation of *P. brasiliensis* through EST analysis. By analysis of the induced and or novel genes it was possible to infer some metabolic adaptations of *P. brasiliensis* during early dimorphic transition that could include the increased control in the ribosome biogenesis and translation fidelity, increase in protein glycosylation and in the control of protein folding. In addition, the amino acids capture from the medium could be favored during the transition to the parasitic phase.

Acknowledgments This work at Universidade Federal de Goiás was supported by grants from CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico- 505658/2004-6). Juliana A. Parente and Clayton L. Borges are PhD fellows from CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) and CNPq, respectively.

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