Biomédica 2011;31:570-9

ARTÍCULO ORIGINAL

Kinetic analysis of gene expression during mycelium to yeast transition and yeast to mycelium germination in *Paracoccidioides brasiliensis*

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Introduction: Paracoccidioidomycosis is an endemic systemic mycosis caused by *Paracoccidioides brasiliensis*, a thermally dimorphic fungus that in tissues and cultures at 37 °C grows as a yeast while at lower temperatures (less than 24 °C) it becomes a mold; however the genes that rule these processes and their expression are poorly understood.
Objective: This research focused on the kinetic expression of certain genes in *P. brasiliensis* throughout the dimorphic process, one that involves the transition from the mycelium to yeast forms and the germination from the yeast to mycelium form.
Materials and methods: A real-time quantitative polymerase chain reaction (RT-qPCR) was optimized to measure the expression of ten genes connected with diverse cellular functions including cell synthesis and wall structure, oxidative stress response, heat shock response, metabolism, proteins' processing, solute transport across the cell membrane and signal transduction pathways at different time points during the mycelia to yeast transition, as well as in the yeast to mycelia germination processes.
Results: Genes involved in cell synthesis and wall structure, metabolism and signal transduction were differentially expressed and highly up-regulated during the yeast to mycelia germination process; on the other hand, genes involved in heat shock response, cell synthesis and wall structure were highly up-regulated during the mycelia to yeast transition process. The remaining genes were differentially regulated during both processes.
Conclusion: In this work the up-regulation of certain genes involved in the morphological changes occurring in *P. brasiliensis* yeast and mycelia forms were confirmed, indicating that these biological processes play an important role during the host-pathogen interactions, as well as in the fungus adaptation to environmental conditions

Key words: *Paracoccidioides*, paracoccidioidomycosis, yeasts, mycelium, germination, gene expression.

Análisis de la cinética de expresión de genes durante la transición de micelio a levadura y la germinación levadura a micelio en *Paracoccidioides brasiliensis*

Introducción. La paracoccidioidomicosis es una micosis sistémica causada por el hongo termodimorfo *Paracoccidioides brasiliensis*. En tejidos y cultivos a 37 °C crece como levadura, mientras que a temperaturas menores de 24 °C crece como un moho. Sin embargo, se conoce poco sobre los genes que regulan estos procesos.
Objetivo. Se evaluó la cinética de expresión de algunos genes en *P. brasiliensis* mediante el proceso de dimorfismo incluida la transición del micelio a levadura y de la germinación de levadura a micelio.
Materiales y métodos. Se optimizó una PCR cuantitativa en tiempo real (RT-qPCR) para medir la expresión de diez genes relacionados con diversas funciones celulares que incluyeron: síntesis de pared, respuesta al estrés oxidativo, respuesta al choque térmico, metabolismo, procesamiento de proteínas, trasporte de solutos a través de membranas y transducción de señales, todo ello a diferentes tiempos durante la transición de micelio a levadura, así como de la germinación de levadura a micelio.

Author contributions:
Orville Hernández contributed to both the design and development of laboratory assays, analysis of data and writing the manuscript.
Diana Tamayo and Isaura Torres contributed to development of fungal .growth, RNA extraction, cDNA synthesis and Real time PCR laboratory assays.
Angela Restrepo and Juan G. McEwen contributed to the writing and correction of the manuscript.
Ana María García contributed to the design and development of the project, data analysis and writing and correction of the manuscript.

Resultados. Se encontró que los genes relacionados con síntesis de pared, metabolismo y transducción de señales, se expresaban de manera diferencial y con regulación positiva durante la germinación levadura a micelio, mientras que algunos genes relacionados con respuesta a choque térmico y a síntesis de pared estaban sobreexpresados en la transición de micelio a levadura. Los genes restantes se regularon de manera diferencial en ambos procesos.
Conclusiones. En este trabajo se confirma la regulación positiva de algunos genes relacionados con los cambios morfológicos de las fases levadura y micelio en *P. brasiliensis*, procesos biológicos que juegan un papel de importancia durante la interacción huésped-parásito y durante la adaptación del hongo al ambiente, respectivamente.

Palabras clave: *Paracoccidioides*, paracoccidioidomicosis, levaduras, micelio, germinación, expresión génica.

Paracoccidioidomycosis is one of the most important endemic systemic mycosis in Latin America, affecting most countries within the area, especially Brazil, where the largest number of cases have been reported. Colombia, Venezuela, Ecuador and Bolivia follow in incidence albeit at an important distance (1,2). In paracoccidioidomycosis the primary infection site is represented by he lungs but dissemination to other organs occurs frequently (3). Two distinctive clinical forms are found, the chronic and the acute/subacute (4,5), with predominance of adult males as in females the inhibitory action of their hormones hinders the micelium to yeast transition process during the initial stages of the infection (6,7). The disease is caused by *Paracoccidioides brasiliensis*, a thermally dimorphic fungus that in tissues and cultures at 37 °C appears as round to oval yeast cells of variable size (6 to 40 µm) and in the environment or *in vitro* at temperatures lower than 24 °C, it changes its morphology to the mold form which is composed of thin, septated hyphae capable to producing asexual propagules, microconidia (<5 µm), when cultured under starvation conditions in minimal media (2). Conidia and mycelial fragments are considered the infectious particles. Once inhaled, they reach the alveoli where at the host's tissues temperature (37 °C) change from its saprophytic form to the parasitic multiple budding yeast form capable of producing disease (8). On the same way, the germination process from the yeast to the mycelium form is an important process during *P. brasiliensis* life cycle since this allows the fungus to grow under different environmental conditions allowing its return to the natural habitat to re-acquire in this way, its infectious capacity (9).

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Recibido: 02/05/11; aceptado:22/08/11

A similar process occurs in other dimorphic fungi such as *Histoplasma capsulatum* and *Blastomyces dermatitidis* (3,10).

Previous studies have reported the over-expression of genes involved in diverse cellular pathways during the mycelium to yeast transition in *P. brasiliensis*, including metabolism, signal transduction, energy, protein and fat synthesis, cellular transport, biogenesis of cellular components, cell wall and membrane remodeling and genes encoding putative virulence factors (11-13). Additionally, in a study on the conidium to yeast transition process, some genes were expressed after 48 hours on transition and shown to be involved in different biologic processes such as metabolism, cell wall synthesis and signal transduction: some of these were considered specific for this process (14). Other studies have focused on the morphological aspects during yeast to mycelium germination (15,16) and the conidium to mycelium germination (17), but the molecular mechanisms of these processes have been poorly explored. A single paper has been published in which an EST library was obtained after 96 hours during conidial germination that showed some hypothetical and unknown proteins involved in this process (18).

In the present study with *P. brasiliensis*, we analyzed the expression profile of 10 genes at different time-points during the first 96 h of the mycelium to yeast transition, as well as during the first 120 h of the yeast to mycelium germination, some of which had been previously described in relation with pathogenic and transition processes (11,12,14). All the selected genes were involved in a diverse range of cellular functions in *P. brasiliensis* and some of them were described as expressed during the conidium to yeast transition (14), with exception of *Kex2,* found by Venancio to be associated to the transition process as a serine-protease involved in protein fate, with an important role during pathogenesis (19-21), glucan

synthases α (*αGS)* and glucan synthases 1 (*GS1),* proteins of importance in fungal cell wall synthesis (22-25) and *AOX* as important protein controlling oxidative stress (26). In this study we showed that *HSP90* and *αGS* presented very high expression during the mycelium to yeast transition, *Prr1, GS* and *GDh* were over expressed during the yeast to mycelium germination and the other genes studied were differentially regulated in both processes. Altogether, the detailed analysis of the expression profile of this group of genes will bring forth relevant information on the dynamics of transition and germination processes as a whole, and help in selecting which pathways could be targeted for future studies intended to evaluate the pathogenic capacity of *P. brasiliensi*s.

Materials and methods

Fungal growth mycelium to yeast transition and yeast to mycelium germination

Paracoccidioides brasiliensis mycelial form (strain ATCC 60855) was regularly grown in the modified Synthetic McVeigh Morton (SMVM) medium plus thiamine 1 % (27). For morphological transition assays, cultures in their exponential growth and with viability above 97 % were used. Cell concentration and viability were determined using a Neubauer chamber and ethidium bromide-fluorescence staining procedures, respectively (28).

To induce the mycelium to yeast transition process, mycelial cultures were incubated under constant agitation in 500 ml Erlenmeyer flasks at 36.5 °C±0.5 with 250 ml of Brain Heart Infusion broth (Becton Dickinson and Company Sparks, MD 21152 USA) plus 1 % glucose (14). Twenty-five ml of this growth medium were used to obtain RNA samples and to evaluate microscopically the transition process prior to RNA extraction. To induce yeast to mycelia germination, yeast cultures grown under the same conditions were incubated at 19 °C±1 °C. Twenty-five ml of this growing medium were used to obtain RNA sample and to evaluate germination process microscopically prior to RNA extraction.

RNA extraction and cDNA synthesis

Total RNA was obtained by maceration of the cells with liquid nitrogen followed by the addition of TRIzol ® (Invitrogen, Carlsbad, CA, USA) at different time points of the mycelium to yeast transition (0, 1, 3, 6, 12, 24, 48, 72 and 96 hours), as well as of yeast to mycelium germination process (0, 1, 3, 6, 12, 24, 48, 72, 96 and 120 hours). Total RNA was treated with DNase I (Invitrogen, Carlsbad, CA, USA) and tested through conventional PCR using β-tubulin primers to confirm absence of chromosomal DNA contamination (13). cDNA was synthesized using 2 µg of total RNA with SuperScript III reverse transcriptase according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA).

Real time PCR assays

We measured the expression level of 10 genes involved in different *P. brasiliensis* cellular functions and described as being expressed during de conidium to yeast transition by Garcia in 2010, (14), with the exception of *Kex2* (21), *GS1, αGS* and *AOX.* All of them had previously been describe in the Broad Institute *P. brasiliensis* genomic data base (http://www.broadinstitute.org/annotation/genome/paracoccidioides_brasiliensis). The Broad Institute accession code and references for each gene are listed in table 1. RT-qPCR was performed using SuperScript™ III Platinum® Two-Step qRT-PCR Kit with SYBR® Green, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). The CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories Inc.) was used to measure the expression level. The variations in mRNA expression were calculated using $2^{\Delta\Delta CT}$ formula, where ΔΔCT is the difference between the target gene and β-tubulin (*β–Tub*) as housekeeping gene, in accordance with previous studies performed by several authors (13,29). Primers were designed using *P. brasiliensis* sequence data available at the Broad Institute Internet site (http://www.broadinstitute.org/annotation/genome). The primer sequences are listed in table 1. Three biological replicas were carried out for the evaluation of both the mycelium to yeast transition and the yeast to mycelium germination processes and three different qRT-PCR runs for each biological replica (Bio-Rad cat No 2239441) were determined. Data were analyzed with the SPSS statistics 17.0 with ANOVA. A *p* value less than or equal to 0.05 was considered statistically significant.

Results

As it concerns morphological changes, when the mycelium was incubated at 37 °C, its cell wall became thicker and after 3 hours small internal swellings began to be visualized. In the next 48 hours, these swellings increased in size until acquiring the intracellular vacuoles (lipid vesicles) that characterize *P. brasiliensis* yeast cells and after 96 hours, multiple budding cells were observed

Table 1. Gene name, Broad Institute accession code and real time PCR primers

Gene and Broad Institute accession code	Primers
Alpha-1,3-glucan synthase (*αGS*) *P. brasiliensis* Pb18: PADG_03169	Left: gac cgc tac gaa act cta tgg t Right: agc gga aga taa acc acc aa
Alternative oxidase (*AOX*) *P. brasiliensis* Pb18: PADG_03747	Left: ctt ggg agc aag agg tgc t Right: agg gct ggg aaa tat tct ttg
Dolichyl-phosphate-mannose-protein mannosyltransferase (*MT*) *P. brasiliensis* Pb18: PADG_06081	Left: cgg cat cga aga att ccc a Right: cgc tgt caa ccg gag cat aat
Glucan synthase-1 (*GS1 - βGS*) *P. brasiliensis* Pb03: PABG_05555	Left: ctg cct cgc atc tct cat t Right: aac tcc cta tcc aca ccg tct
Glycine Dehydrogenase (*GDh*) *P. brasiliensis* Pb18: PADG_00210	Left: tcc tcc gtt tta ttg cgt gg Right: gcc aat tgc agg cat tgt cta
Heat shock protein 90 (*HSP90*) *P. brasiliensis* Pb18: PADG_07715	Left: agg caa aca aaa ccc tca cca Right: aat tgc tta gtg cca gag cgg
Neuroendocrine convertase, kexin protease (*Kex2*) *P. brasiliensis* Pb18: PADG_01553	Left: tta ctt cgc tgt cca tct cga Right: tgt atg gtg gtc tgc caa ttc
Peptide Transport Protein *Ptr2*, (*Ptr*) Major Facilitator Superfamily *P. brasiliensis* Pb18: PADG_00993	Left: tca atg aac tgc ccg ctg t Right: gcg aaa aac ttg gca aga gg
Poly(Rc)-Binding Protein (BP) *P. brasiliensis* Pb18: PADG_03733	Left: tca acc aga tgc caa acg c Right: ggt tgc ttc tgt tgc agt gct
Transcription factor prr1 (*Prr1*) *P. brasiliensis* Pb18: PADG_04915	Left: gca gca tac gca cta gac ca Right: gca cta ggg ttc atc gta gca
Tubulin beta chain (*β-tub*) P brasiliensis Pb03: PABG_00486	Left: gtg gac cag gtg atc gat gt Right: acc ctg gag gca gtc aca

(see arrows in figure 1A). On the other side, the incubation of yeast cells at 18 °C stimulated the production of germ tubes from the first 6 hours on transition; these germ tubes became elongated and produced hyphae with loses of intracellular inclusions (see arrows in figure 1B).

RT-qPCR was successfully completed for all evaluated genes, determining the expression level of each one during the mycelium to yeast transition and yeast to mycelium germination processes.

Genes involved in response to stimuli are differentially up-regulated during morphological changes

Three genes were involved in the perception and response to stimuli, namely, a heat shock protein (*HSP90*), a signal transduction gene, a transcription factor (*Prr1*) and an oxidative stress response protein (*AOX*). Data analysis indicated that the *HSP90* gene expression was not altered during the yeast to mycelium germination time-course maintaining a level between 2 and 4 fold. However, this gene was strongly up-regulated during the mycelium to yeast transition process (more than 60 times fold) and reached its higher level during the first 3 hours but was sustained up to 48h on transition, with significant differences in comparison with the yeast to mycelium germination or mycelia and yeast forms (figure 2A). Whereas, the expression of *Prr-1* gene was between 1 and 2.5 times fold during the mycelium to yeast transition without significant alterations, since this gene had higher expression levels (almost 4 fold) all throughout the germination process with meaningful differences in comparison with yeast and mycelium cells (figure 2B). Also, the *AOX* gene expression was up-regulated during the first 12 hours of the mycelium to yeast transition process (6 fold). Meanwhile, during the yeast to mycelium germination and also in the mycelium form, lower values were observed (figure 2C).

Genes involved in protein modification and fate, played an important role during the morphological changes occurring in Paracoccidioides brasiliensis

The expression of two genes involved in protein fate *(Kex2 and Bp)* and one gene related with cell wall synthesis and structure *(MT)* were evaluated. *Kex2* gene expression levels reached their highest values in the middle of the mycelium to yeast transition process (24 h) and in the middle of the yeast to mycelium germination process (24, 48 and

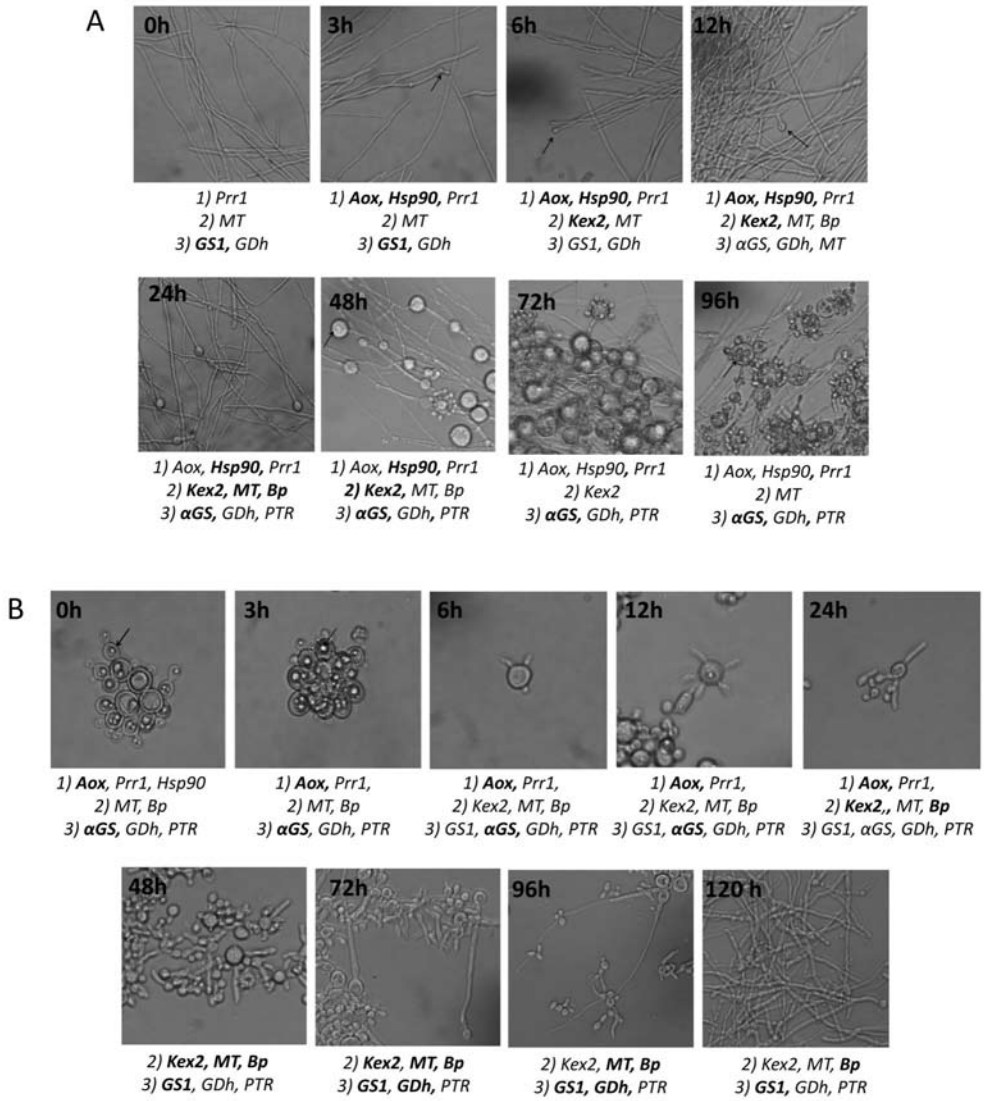

Figure 1. *Paracoccidioides brasiliensis* gene expression analysis during mycelium to yeast transition and yeast to mycelium germination in relation to morphological changes. **A)** Mycelium to yeast transition process. **B)** Yeast to mycelium germination process. Genes were grouped according to 4 cellular functions corresponding to: **1)** Perception and response to stimuli (oxidative stress, heath shock response and signal transduction). **2)** Protein fate-folding modification and destination. **3)** Metabolism, cellular transport and biogenesis of cellular components. Genes with remarkable over expression at the specific time points are indicated in bold.

72 h) resulting in a 2 - 3 fold increase. Nonetheless, its expression was lower in yeast cells, in the mycelium and also in the transition processes (figure 3A). The *MT* gene showed high expression levels (almost 3.5 fold) at 24 hours during the mycelium to yeast transition process while during the yeast to mycelium germination, such gene was up regulated at 48, 72 and 96 hours. Lower levels of expression were observed in mycelial and yeast cells (figure 3B). Also, high expression levels of the *Bp* gene were noticed at 24 hours of the myceliumto yeast transition (almost 4 fold) was observed; in contrast, during the yeast to mycelium germination, this gene was up regulated with its expression peak occurring at 72 h (more than 6 fold) and with significant differences in comparison with its expression in mycelial and yeast cells (figure 3C).

Transition and morphological changes stimulate expression of some genes involved in metabolism, cellular transport and biogenesis of cellular components

Four genes involved in metabolism (*GDh*), cellular transport - transport facilitation and transport

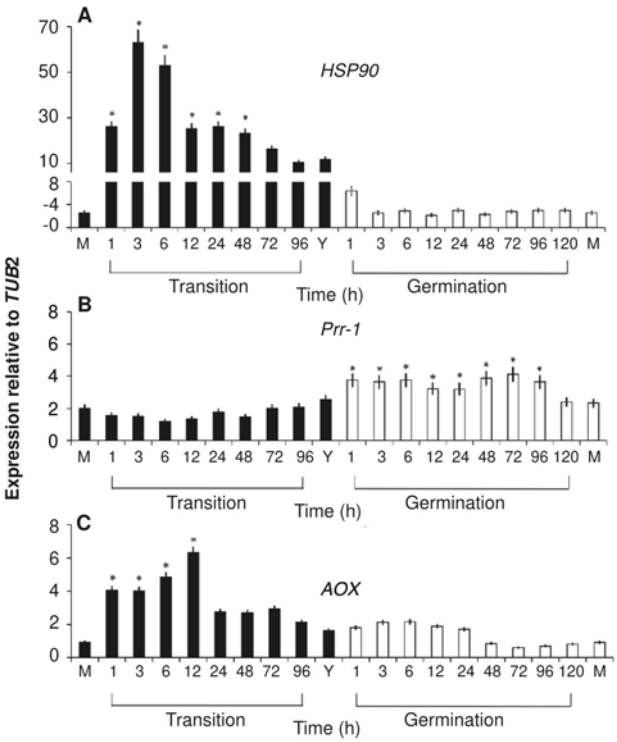

Figure 2. Expression of genes involved in response to stimuli during *Paracoccidioides brasiliensis* transition and germination process: qPCR analysis of relative gene expression using β-tubulin as housekeeping gene. A) *HSP90*, B) *Prr1*, C) *AOX*.
* Statistical significance differences p< 0.05 in comparison with mycelial and yeast cells.

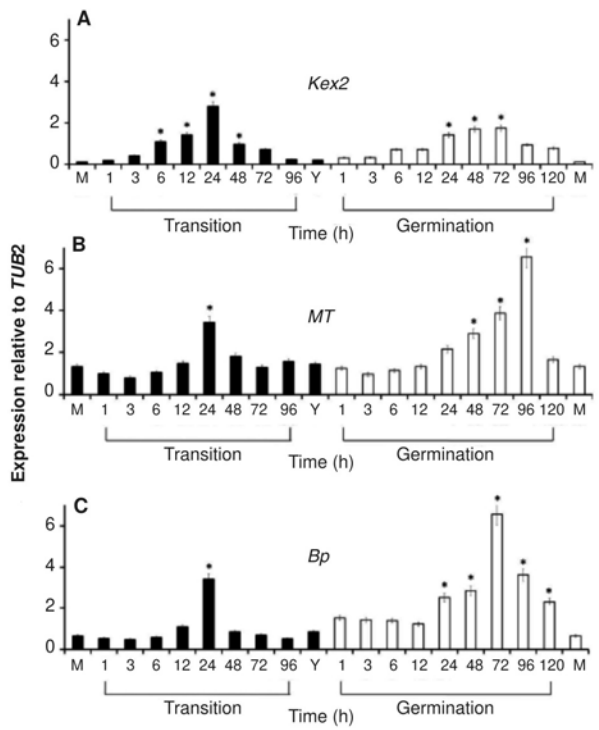

Figure 3. Expression of genes involved in protein fate-folding modification and destination during *Paracoccidoides brasiliensis* transition and germination process: qPCR analysis of relative gene expression using β-tubulin as housekeeping gene. A) *Kex2*, B) *MT*, C) *Bp*.
* Statistical significance differences *p*< 0.05 in comparison with mycelial and/or yeast cells.

routes (*Ptr*) and biogenesis of cellular components, such as cell wall synthesis (*αGS* and *GS1*, were also evaluated. While *GDh* was not altered during the mycelium to yeast transition neither in the mycelial or yeast forms, it was expressed at significant levels at 72 and 96 h during the yeast to mycelium germination process (6 and 7.5 times fold, respectively), with significance differences in comparison with the mycelium and yeast forms and the transition process (figure 4A). Also, we observed that the *Ptr* gene was up regulated after 24h of the mycelia to yeast transition process, and until 96 hours of the yeast to mycelium germination process, with statistically significant higher levels in the yeast form than in the mycelial one (figure 4B). The increase in the *αGS* gene expression during the mycelium to yeast transition correlated with a decrease in the expression levels of the *GS1* gene. On the contrary, an increase in the later *GS1* gene level expression at the same time as in the yeast to mycelium germination occurred and was correlated with a decrease in the expression levels of *αGS* with sustained high levels of *αGS* in yeast cells and high levels of *GS1* in the mycelial form (figure 4C and 4D).

Discussion

In *P. brasiliensis,* the transition process is generally considered as one of the most important steps during pathogenesis and adaptation of the fungus to its host (3), whereas the germination processes is a crucial step for fungal growth and adaptation to environmental conditions with continued value in its biological cycle (9). In both cases molecular regulation is still poorly understood and few studies had reported genes that could possibly be involved in both processes (12,14,18).

The relevance of a gene in a process could be revealed by a quantitative measurement of its expression, whereas higher expression levels could represent its importance in a particular process. This approach has been used before to evaluate genes found in the EST library of *P. brasiliensis* yeast cells (12). Consequently, measurement of gene expression is an initial step towards understanding gene role during a process. In our study new

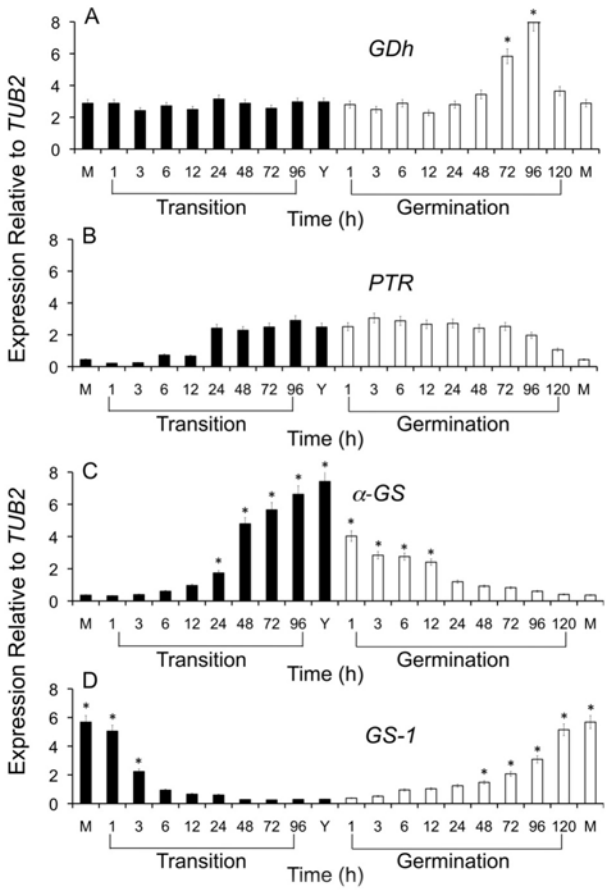

Figure 4. Expression of genes involved in metabolism, cellular transport and biogenesis of cellular components during *P. brasiliensis* transition and germination process: qPCR analysis of relative gene expression using β-tubulin as housekeeping gene. A) *GDh*, B) *PTR*, C) *αGS*, D) *GS1*.
* Statistical significance differences $p< 0.05$ in comparison with mycelia and yeast cells.

molecular outlines on the complex mycelium to yeast and yeast to mycelium morphogenetic processes have been revealed by evaluating the kinetic expression profile of some genes that could participate in *P. brasiliensis* dimorphic processes. Changes in the profile of gene expression during the transition and germination processes are correlated with morphological changes occurring in fungal cells, giving clue times at which individual gene expression switches on or off, leading to production of proteins that rule such morphological changes by acting as modifiers of both structure and fungal metabolism (13).

Three genes were found to enhance expression during the mycelium to yeast transition processes (*HSP90*, *AOX* and *αGS*). Temperature changes represent a kind of environmental stress as fungi and other eukaryotic and prokaryotic cells respond to this stress by inducing synthesis of heat-shock proteins, including *HSP90* (30,31). One of the most important features in *HSP90* is its ATPase activity which enables chaperone functions of this protein (32). Previous studies in *P. brasiliensis* have been reported on *HSP90* expression in the yeast and mycelial forms, (13,33) but this gene is preferentially expressed in the yeast form (34). In *Candida albicans, HSP90* rules the cellular pathway required for transition from yeast to filamentous growth, a crucial feature for virulence in this pathogen (35). In our study, the expression of this gene was strongly up-regulated at the beginning of the transition process, just at the time point when the first changes in the mycelia structure were visualized (figure 1). Similar data were reported by Nicola, *et al.* (34). This evidence indicates that this protein is essential for thermal adaptation of *P. brasiliensis* to new environment status resembling host conditions, especially after the first hours of contact and later on in connection with its adaptation as a yeast cell inside the host.

The up-regulation of the *AOX* gene throughout the mycelia transition could be attributed to the capacity of this gene to reduce the reactive oxygen species (36), elements that can alter the energy status of the yeast cells due to the non-phosphorylative nature of the alternative respiratory pathway (37) produced during a change in cellular metabolism in order to obtain the carbon and energy sources required for an effective transition process. This condition could be of particular relevance during the whole transition and even during the maintenance of the yeast cells *in vitro* or in tissues, where their own oxidative stress activity makes it necessary a turn on of mechanisms to compensate the morphological changes involved in becoming a yeast cell to ensure its survival in the host´s tissues.

Three genes were up regulated during the yeast to mycelium germination (*Prr-1*, *GDh* and *αGS*). *Prr-1* and its homologous in *Saccharomyces cerevisiae, Skn7*, are recognized for their role in the oxidative stress response (38,39) but their importance in dimorphism has not been reported before. This regulator gene is part of the Two Component Signal System that acts as a response regulator (40). Unless this gene could be expressed during both the mycelium to yeast transition and the yeast to mycelium germination processes, its constant up-regulation mainly during all the germination processes (4 times), making of it a candidate for the initial effectors in the signal transduction pathway in charge of inducing the yeast to mycelia germination.

Glycine dehydrogenase (*GDh*), present in the yeast EST library published by Goldman, *et al.* (13), plays an important role in producing ATP (41). We observed a constant over expression of the corresponding *GDh* gene during morphological changes and mainly at the end of the germination process, indicating that it as a key element in the cell cycle and possibly also by playing an important role in formation and maintenance of *P. brasiliensis* hyphae.

In dimorphic fungi, *αGS* and *GS1*, are important proteins in cell wall structure, *αGS* in yeast cells and *GS1* in mycelial cells (25). During morphological changes occurring in *P. brasiliensis* cell wall remodeling, a change in the glucan structure occurs going from the beta to the alpha structure during transition while in the yeast cell, changes assist in the evasion from the host immune responses. On the contrary, a change of alpha to beta structure occurs during the germination process (25). These changes cause an alteration in cell shape originated by the exchange of glucans at each stage. We observed a rise in *GS1* expression throughout the entire yeast to mycelium germination and of *αGS* during the mycelium to yeast transition, in accordance with required cell wall structural changes required for mycelium or yeast cellular architecture, respectively.

The three remaining genes, *Kex2* involved in processing of proteins, *MT* important for posttranscriptional manosilation of proteins essential to cell synthesis and wall structure, and involved in protein fate-folding modification and destination; and *Ptr*, related to cellular transport, transport facilitation and transport routes (42-44), were over expressed in both processes, mycelium to yeast transition and yeast to mycelium germination while the expression of these genes was low in yeast and mycelium forms. This expression could be correlated with the high production of proteins required for morphological changes, and the later adaptation to new environmental conditions. The over expression of these genes stimulate changes in the structural and functional profiles of proteins. These proteins could be correlated with changes in cell walls and in the activation of metabolic pathways needed fpr adaptation to new environmental conditions such as temperature changes involved in thermal dimorphism, among others (20,45,46).

The up-regulation of certain genes involved in the morphological changes corresponding to the mycelial and yeast forms were noticed. All these biological processes should play a crucial role in transition development as this is closely related to host-pathogen interactions and also to germination, a process that is fundamental in environmental adaptation. These results establish a foundation for the understanding of the dimorphic process, and for the characterization of molecular mechanisms that will contribute to understanding the role of some genes in the morphological changes and establishment of a pathogenic process. These genes could be functionally evaluated using knockdown technology (already established for *P. brasiliensis*) (47) to recognize it importance as virulence and adaptation factors.

Conflicts of interest

The authors declare not have conflict of interest in the present work.

Acknowledgement

This work was supported by COLCIENCIAS, Colombia (project no. 2213-343-19183), the Corporación para Investigaciones Biológicas and the Instituto de Biología of the Universidad de Antioquia. The National Doctoral Program of COLCIENCIAS 2008 supported Orville Hernández. We thank Fernando Rodrigues from the School of Health Sciences, University of Minho, Braga, Portugal.

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