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Gene expression modulation by paraquat-induced oxidative stress conditions in *Paracoccidioides brasiliensis*

Marcus Vinícius de Oliveira [a], Ana Claudia de Freitas Oliveira [a], Cláudio S. Shida [b], Regina Costa de Oliveira [a], Luiz R. Nunes [c,*]

[a] Núcleo Integrado de Biotecnologia, Universidade de Mogi das Cruzes (UMC), Av. Dr. Cândido Xavier de Almeida Souza, 200, Mogi das Cruzes, SP, CEP 08780-911, Brazil
[b] Instituto de Ciência e Tecnologia Universidade Federal de São Paulo, Rua Talim, 330, São José dos Campos, SP, CEP 12231-280, Brazil
[c] Centro de Ciências Naturais e Humanidades, Universidade Federal do ABC, Rua Santa Adélia, 166, Santo André, SP, CEP 09210-170, Brazil

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ABSTRACT

Paracoccidioides brasiliensis is a thermodimorphic fungus associated with paracoccidioidomycosis (PCM), the most common systemic mycosis in Latin America. The infection is initiated by inhalation of environmentally dispersed conidia produced by the saprophytic phase of the fungus. In the lungs, *P. brasiliensis* assumes the parasitic yeast form and must cope with the adverse conditions imposed by cells of the host immune system, which includes a harsh environment, highly concentrated in reactive oxygen species (ROS). In this work, we used the ROS-generating agent paraquat to experimentally simulate oxidative stress conditions in order to evaluate the stress-induced modulation of gene expression in cultured *P. brasiliensis* yeast cells, using a microarray hybridization approach. The large-scale evaluation inherent to microarray-based analyses identified 2070 genes differentially transcribed in response to paraquat exposure, allowing an integrated visualization of the major metabolic changes that constitute the systemic defense mechanism used by the fungus to overcome the deleterious effects of ROS. These include overexpression of detoxifying agents, as well as of molecular scavengers and genes involved in maintenance of the intracellular redox potential. Particularly noteworthy was to verify that the oxidative stress resistance mechanism of *P. brasiliensis* also involves coordinated overexpression of a series of genes responsible for chitin-biosynthesis, suggesting that this pathway may constitute a specific regulon. Further analyses aiming at confirming and understanding the mechanisms that control such regulon may provide interesting new targets for chemotherapeutic approaches against *P. brasiliensis* and other pathogenic fungi.

1. Introduction

Paracoccidioides brasiliensis is the causative agent of paracoccidioidomycosis (PCM), the most common systemic mycosis in Latin America (San-Blas and Niño-Vega, 2001). The infection is initiated by inhalation of environmentally dispersed conidia produced by the saprophytic phase of the fungus (Restrepo et al., 2001). In the lungs, the fungus assumes the parasitic yeast form and must overcome the microbial killing mechanisms of the alveolar macrophages, which include a harsh environment, highly concentrated in reactive oxygen species (ROS) generated by the phagocyte NADPH oxidase system. This oxidative burst is not a simple challenge to the fungus, since these species are highly reactive and able to damage cellular constituents, such as DNA, lipids and proteins (Jamieson, 1998; Missal et al., 2004).

Oxidative stress may be experimentally simulated by the addition of ROS-generating agents to the culture medium, and these substances constitute useful tools for *in vitro* investigation regarding the antioxidant defense mechanisms of several microorganisms, being widely used in yeast studies (Bai et al., 2001; Marisco et al., 2011). One of these agents is paraquat, a well-known redox-cycling drug that transfers electrons to molecular oxygen during its metabolism, resulting in superoxide formation (Herrero et al., 2008; Morano et al., 2011). Paraquat has been widely used as an herbicide and it has been shown that the exposure of different types of fungal cells to paraquat triggers a range of harmful effects, which derive, directly and indirectly, from the oxidative stress induced by this substance. In *Saccharomyces cerevisiae*, paraquat exposure affects *in vitro* cell growth and yeast responses against superoxide stress seem to be dependent on enhanced copper-zinc Superoxide Dismutase (Cu/Zn-*sod*) expression and activity, since *S. cerevisiae* yeast cells deleted for *sod1* are hypersensitive to paraquat (Culotta, 2000; Hansson and Häggström, 1986; Jamieson, 1998). Comparable results are observed in filamentous fungi, including *Aspergillus niger* and *Fusarium*

* Corresponding author. Fax: +55 (11) 4996 3166.
E-mail address: Luiz.Nunes@ufabc.edu.br (L.R. Nunes).

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oxysporum, when exposed to the same ROS-generating agent. In such species, paraquat exposure affects *in vitro* mycelial growth and the viability of fungal spores. As seen in *S. cerevisiae*, cellular responses of filamentous fungi against superoxide stress also include enhanced *sod* expression and activity, as well as a small induction in the catalase activity (Angelova et al., 2005; Moradas-Ferreira et al., 1996).

Regardless the origin of oxidative stress, fungi undergo changes in gene expression that rapidly ensure molecular responses to protect and/or repair their cellular components from the ROS-induced oxidative damage (Estruch, 2000). Previous results obtained by our group allowed the identification of several genes that encode enzymes related to antioxidant defense in *P. brasiliensis*, including superoxide dismutases and peroxidases, as well as small molecules such as glutaredoxins, thioredoxins and a peroxiredoxin (Goldman et al., 2003; Nunes et al., 2005). These mechanisms are activated by the pathogen under oxidative conditions, in an effort to maintain cell viability (Dantas et al., 2008; Ruiz et al., 2011; Tavares et al., 2007).

In addition to the antioxidant defense systems, another cellular element that is crucial to protect pathogenic microorganisms against the harmful effects of stress conditions is the cell wall. The fungal cell wall establishes a direct contact with external environments and, therefore, constitutes a primary pathogen defense against the oxidative attack of ROS released by the phagocytes of the host immune system in the course of infection (Fuchs and Mylonakis, 2009). Indeed, when fungal cells are exposed to stress conditions a massive deposition of chitin in the cell wall is initiated, leading to its strengthening, which is an essential step to avoid the toxic effects of stress (Fuchs and Mylonakis, 2009). Up to this date, however, the exact mechanisms that control such increase in chitin content are not completely understood (Walker et al., 2008). Although the cell wall composition varies among fungal species, it is known that up to 90% of its structure is formed by polysaccharides, especially β-glucans (glucose polymers) and chitin (N-acetylglucosamine polymer) (Latgé, 2007). The chitin content in fungal cell wall varies from 2% in some yeast, up to 61% in some mycelial fungi. However, even in organisms in which chitin is present in small quantities, it is essential for cell viability (Ruiz-Herrera and San-Blas, 2003). In *P. brasiliensis*, the chitin content in cell wall varies according to the fungal morphology ranging from 13% in hyphae to 43% in pathogenic yeast (San-Blas and San-Blas, 1984).

In this work we used a microarray hybridization approach to examine gene expression of *P. brasiliensis* yeast cells exposed to *in vitro* oxidative stress conditions induced by paraquat. This experiment identified 2,070 genes whose expressions displayed statistically significant modulation in response to this type of oxidative stress. Some of these genes – particularly those directly involved in oxidative stress responses, had their expression profiles confirmed by Real-Time qPCR and helped us to ascertain the reliability of the microarray hybridization data.

Taken together, detailed evaluation of this dataset allowed unequivocal identification and functional designation to many *P. brasiliensis* genes that had been previously identified solely on the basis of large-scale genomic comparisons. Our data also provided a thorough and integrated visualization of the cellular mechanisms employed by the microorganism to overcome the damaging conditions imposed by paraquat-induced oxidative stress, including overexpression of detoxifying agents, as well as molecules that participate in scavenging of ROS and maintenance of intracellular redox potential. Particularly noteworthy, however, was to verify that practically all enzymes involved in chitin biosynthesis displayed increased expression profiles in response to paraquat, which correlated with a significant increase in the cell wall's chitin content. These results point to the possibility that the genes from the chitin-biosynthesis pathway may be collectively governed by common regulatory mechanisms, a phenomenon not yet demonstrated in *P. brasiliensis* and any other fungi. Given the importance and uniqueness of chitin biosynthesis to the development and survival of fungal cells, a more comprehensive study of the mechanisms that govern the induction of this pathway may aid in the development of novel chemotherapeutic approaches against pathogenic fungi.

2. Materials and methods

2.1. Strains, culture media and growth conditions

We used *P. brasiliensis* isolate 18, (provided by R. Puccia, UNIFESP, São Paulo, Brazil and F. G. Nóbrega, UNESP, São Paulo, Brazil) throughout the work. Fungal cells were cultivated in YPD medium (1% yeast extract, 2% casein peptone, 2% glucose, pH 6.5) at 36 °C (yeast phase); liquid cultures were shaken at 120 rpm in a rotatory shaker.

2.2. Conditions of drug exposure for gene expression assays

To determinate adequate concentrations of drug exposure, paraquat was added to *P. brasiliensis* yeast cultures (OD_{600} = 0.2) at final concentrations ranging from 0.5 to 20 mM. Cultures were then incubated in the conditions described above for 7 days and fungal growth was monitored on a daily basis, through OD_{600} measurements. For RNA isolation, 300 mL of a culture grown until an OD_{600} = 2.15 – late phase of exponential growth – was divided into three equal parts and two of them were treated with paraquat for final concentrations of 0.5 and 5.0 mM, respectively, while the third part remained untreated (unexposed control). Cultures were incubated in the same conditions for an additional 5 h period, as suggested by Angelova et al. (2005) and Kwon et al. (2006). Following the paraquat exposure period, yeast cells were harvested by filtration and immediately frozen in liquid nitrogen.

2.3. RNA isolation and cDNA labeling

Yeast cells were disrupted with glass beads and by grinding in liquid nitrogen (Goldman et al., 2003), and immediately mixed with Trizol (Invitrogen) for RNA extraction following the supplier's recommendations. After extraction, the RNA samples were purified with aid of an RNeasy kit (QIAGEN) and labeled by incorporation of Cy3- or Cy5-dCTP (Monte and Somerville, 2002).

2.4. Microarray hybridization and analysis

Hybridizations were carried out as previously described (Nunes et al., 2005), using RNA samples from two independent biological replicas. Thus, each treatment condition was analyzed with four independent hybridizations (two pairs of hybridizations, with dye-swaps within each pair) and a total of eight intensity readings were generated for each element in the microarray (4 for the 0.5 mM concentration and 4 for the 5 mM concentration). Images were analyzed with the TIGR Spotfinder program (v.3.1). All spots with median values lower than the median local background plus 2 standard deviations (SD) have been flagged and excluded from further analyses. The results from each hybridization were submitted to a series of mathematical transformations with the aid of the software TIGR MIDAS v.2.19. These included filtering out all spots, whose integrated intensities were below 10,000 a/d units, normalization between the two channels with the aid of the Lowess algorithm and SD regularization of the Cy5/Cy3 ratios across all sectors (blocks) of the array. Finally, replicated slides were submitted to a

dye swap consistency checking, and the results from each individual experiment were loaded into the software TIGR Multi-Experiment Viewer (TMEV), v.3.1. Experiments were then normalized and genes that displayed statistically significant modulation were identified by a one-way ANOVA, considering $p < 0.01$ as a cut-off value. For details regarding the use of the TIGR microarray software suite (TM4), see Saeed et al. (2003). Statistically significant modulated genes were then submitted to functional categorization against the Gene Ontology (GO) database (www.geneontology.org) using the Blast2GO software (www.blast2go.org). Raw and normalized data from all microarray hybridizations, as well as the microarray complete annotation file have been submitted, in MIAME-compliant format, to NCBÍs Gene Expression Omnibus (GEO) and can be assessed through Series number GSE41148.

2.5. RT-PCR and real-time qPCR reactions

The RT-PCR reactions were performed with the same RNA samples used in microarray experiments. Reverse transcription reactions were performed starting from 3 μg of total RNA derived from each experimental condition and 2 μg of oligo (dT) 15 primer (100 pmol/μL). Then, 50 ng of each resulting cDNA were used along with SYBR Green PCR Master mix (2×) in Real-time qPCR reactions. Amplification assays were performed using an ABI Prism 7500 Sequence Detection System (Applied Biosystem, USA). The thermocycling conditions comprised an initial step at 50 °C for 2 min, followed by 10 min at 95 °C, and 40 cycles at 95 °C for 15 sec and 60 °C for 1 min. The sequence BQ501668, which encodes a mitochondrial intermembrane space protein (YMR002w) has been used as an endogenous control for experimental normalization, since the microarray hybridization experiments showed that this gene is constitutively expressed under the experimental conditions evaluated. Primers were synthesized through the Applied Biosystems Assay-by-Design service and all reactions were prepared essentially as recommended by the manufacturer.

2.6. Preparation of alkali insoluble material (AIM)

The cell wall chitin content was measured from yeast cells exposed to paraquat for a 5-h period, in the same experimental conditions employed for RNA isolation (see Section 2.2 in Material and Methods). Measurements were performed according to the protocol established by Zamani et al. (2008). Briefly, after the treatment period, *P. brasiliensis* yeast cells were harvested by filtration, washed with distilled water and dried at 50 °C. Then, dry samples were treated with 0.5 M NaOH (30 mL/g of cells) at 90 °C overnight. The alkali insoluble material (AIM), a chitin-concentrated material, was separated by centrifugation (10 min, 4000 g), washed with distilled water and dried at 50 °C, weighed, and stored. All experiments were performed in triplicate and results are presented as averages.

2.7. GlcNAc measurement

To 10 mg of each AIM sample was added 0.3 mL of 72% (v/v) sulfuric acid and the suspensions were mixed periodically for 90 min at room temperature. Then, 8.4 mL of water was added to each tube, which were closed tightly and placed in an autoclave at 121 °C during 1 h for a diluted sulfuric acid hydrolysis. With the solutions still hot (around 100 °C), two samples of each 0.5 mL (named A and B) were taken from each tube. After cooling to room temperature, 0.5 mL of 1 M $NaNO_2$ or 0.5 mL of water were added to samples A and B, respectively. All tubes were closed, mixed, and left for 6 h at room temperature. They were then opened and left overnight under the hood. At this point, chitin was converted to anhydromannose, which was quantified by the colorimetric method presented by Plassard et al. (1982) with minor modifications. Briefly, 0.5 mL of ammonium sulfamate (12 wt%) was added to each set of samples A and B to inactivate the nitrous acid formed and the samples were mixed for 4 min. Then, 0.5 mL of 0.5% MBTH (3-methyl- 2-benzothiozolone-hydrazone-hydrochloride) was added, and the tubes were kept during 1 h without mixing. This step was followed by addition of 0.5 mL of 0.5% $FeCl_3$ and mixing. After 1 h, the sets were diluted 100 times with water, and the absorbance of solution A was measured at 650 nm against solution B (without $NaNO_2$). A solution of pure GlcN hydrochloride in 2.48% (v/v) sulfuric acid (8.4 mL of water mixed with 0.3 mL of 72% sulfuric acid) was used as a standard, and the concentration of GlcN in samples was measured spectrophotometrically.

3. Results

3.1. Global transcriptional response of P. brasiliensis yeast cells exposed to paraquat

To verify the effect of paraquat on growth of the pathogenic yeast form of *P. brasiliensis*, cultures were established, as described in Section 2.2 and paraquat was added to each culture, at concentrations of 0.5 mM, 5 mM, 10 mM and 20 mM. As observed in Fig. 1, paraquat was able to affect cellular growth in a dose-dependent manner, although concentrations above 10 mM completely inhibited cell growth. Thus, we chose to evaluate the effect of paraquat on *P. brasiliensis* gene expression using 0.5 and 5 mM concentrations, which are likely to represent two very distinct conditions: while the 0.5 mM concentration appears to represent a sublethal dose, in which the relatively moderate oxidative stress barely affects growth rates, the 5 mM concentration introduces a severe growth inhibition. Although still tolerable by the fungus, this second condition is likely to induce a much more intense stress condition, requiring broader physiological adaptations to the new environmental conditions.

Evaluation of the fungal transcriptome profile in response to exposure to the abovementioned concentrations of paraquat was performed with the aid of previously described *P. brasiliensis* microarrays, which carry sequences representing 4692 genes from this fungus (Nunes et al., 2005). Although the complete *P. brasiliensis* genome is currently known to consist of ~8700 genes (Desjardins et al., 2011), experiments using these arrays still allow a significant visualization of the celÍs integrated metabolism, since they have been shown to carry genes involved in practically all cellular processes, such as protein synthesis, growth and developmental control, genome structuring, oxidative stress responses, cell wall metabolism and others (Nunes et al., 2005).

Microarray hybridizations were carried out with dye-swapped replicas, using RNA obtained from cells exposed to 0.5 and 5.0 mM paraquat, in an attempt to avoid compromising the overall cellular viability, as observed when cultures were treated with 10 and 20 mM concentrations. Moreover, cells harvested for RNA extractions destined to microarray hybridizations were treated at a lower paraquat/cell ratio (see Section 2.2), increasing the likelihood that the observed changes in gene expression relate to *P. brasiliensis* cells successfully adapting to the new environmental conditions, rather than to the result of non-specific metabolic changes associated with toxicity-induced cell death. Data resulting from microarray hybridizations were normalized and mathematically transformed, as described in Section 2.4, and the final statistical analysis of the dataset revealed a total of 2070 genes with differential expression in at least one experimental point of drug exposure. The entire list of differentially expressed genes, as well as their individualized functional categorizations and respective expression ratios can be found in Supplementary Table 1 and through NCBÍs Gene Expression Omnibus (GEO) series number

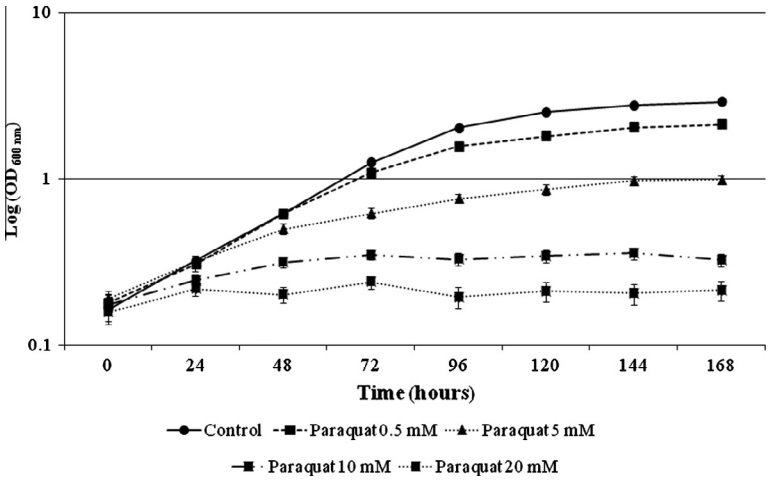

Fig. 1. *P. brasiliensis* growth patterns in YPD media in the absence (untreated control) and presence of 0.5, 5, 10 and 20 mM paraquat. Cultures were incubated in an orbital shaker at 36 °C and 120 rpm. The growth of cultures was daily monitored through OD_{600} readings. Measurements were performed in triplicate and graphic shows the average values and their respective standard deviations.

GSE41148 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE41148). This series also provides access to the raw data from all microarray hibridizations and a thorough description of the experimental and analytical procedures used in this work, in accordance to the guidelines established by the MIAME (Minimal Information About Microarray Experiments) standards (Brazma et al., 2001). Fig. 2 shows a summarized description of such genes, as well as their distribution in the main categories of the Gene Ontology (GO) database.

As observed in Fig. 2, modulated genes account to ~44% of the elements present in the array and include representatives from a broad range of GO functional categories. Thus, paraquat-induced oxidative stress response in this fungus appears to involve major changes in its transcriptome, which include up- and down-regulation of not only genes already known to be involved in such responses, as well as novel genes, whose direct correlation with the process are still unknown. In order to access the overall reliability of these data, we have used real-time qPCR to confirm gene expression variation of a few key genes, known to be involved in a series of direct and indirect fungal responses against oxidative stress (see below). These data are shown in Table 1, which clearly demonstrates the existence of intense correlation between the average ratio expression values using both microarray hybridization and qPCR approaches in distinct stress-resistance mechanisms.

As an example, we confirmed the expression modulation of genes that act in direct detoxification of different types of ROS into fully reduced water molecules, as is the case of the Cu,Zn-dependent superoxide dismutases (*sod* - BQ496515) and glutathione peroxidases (BQ490900) (Herrero et al., 2008; Missal et al., 2004). We also confirmed up-regulation of genes encoding enzymes involved in redox homeostasis, such as thiol antioxidants (BQ503375/BQ499382) and peroxiredoxins (BQ498804), (which belong to a family of thiol-specific antioxidant enzymes involved in cell defense against the reduction of cellular hydroperoxide). We also verified transcriptional modulation of genes whose products are involved with the formation of free radical scavengers, such as 4-hydroxyphenylpyruvate dioxygenase (*4-hppd*), which leads to the production of homogentisic acid (an intermediary of the tyrosine catabolism) that can act as a scavenger of ROS molecules and may lead to the formation of melanin in certain pathogenic fungi (Keller et al., 2011; Schmaler-Ripcke et al., 2009).

Changes have also been confirmed in the expression levels of enzymes that are involved in other metabolic pathways, but have been shown to play indirect roles in protecting cells against oxidative stresses (Grant, 2008). As an example, we confirmed the down-regulation in expression of glyceraldehyde-3-phosphate dehydrogenase (BQ491108), a classic glycolytic enzyme that also controls oxidative-stress induced cell death by nuclear translocation and cellular aggregation processes (Nakajima et al., 2009), as well as the up-regulation of carnitine acetyltransferase (BQ504239), part of the carnitine shuttle, which transfers peroxisomal activated acetyl-residues to the mitochondria of yeast cells and has been demonstrated to protect cells from oxidative and organic acid stresses in *S. cerevisiae* (Franken et al., 2008). Other genes within this category include cysteine dioxygenase (BQ497804), involved in the biosynthesis of taurine – an aminoacid that has been shown to have potent anti-oxidant properties under various pathophysiological conditions (Chen et al., 2012; Honjoh et al., 2010) and two genes that encode enzymes involved in the biosynthesis of chitin, the major glycan component of fungal cell wall (glucosamine-fructose-6-phosphate aminotransferase - BQ498822 and chitin synthase III – DR164480).

3.2. Chitin synthesis in response to oxidative stress

The thickness and chitin content of fungal cell walls seem to play an important role in establishing a first line of defense against external sources of ROS in pathogenic fungi. However, the exact molecular mechanisms that underline the process by which fungi modulate their cell wall chitin content are yet poorly understood (Liu et al., 2010). In this sense, our attention was immediately drawn to the fact that our microarray analysis evidenced up-regulation of at least 5 genes encoding enzymes that synthesize metabolic intermediates related to chitin synthesis (see Supplementary Table 1). These include the genes that encode glucosamine-fructose-6-phosphate aminotransferase (BQ498822), which catalyzes the first step in this pathway (i.e.: conversion of fructose-6-phosphate into glucosamine-6-phosphate, or GlcN-6-P); UDP-N-acetylglucosamine pyrophosphorylase (DR164606) which catalyses the synthesis of UDP-GlcNAc from GlcNAc-1-P and UTP, as well as three chitin synthases (BQ491651, BQ493657 e BQ497792).

These findings were a bit surprising, since the control of complex biosynthetic routes, such as chitin synthesis, often rely on modulation of only a few key enzymes of the pathway (and even then, such modulation may be exerted by post-transcriptional control mechanisms). In this case, the microarray hybridization assays

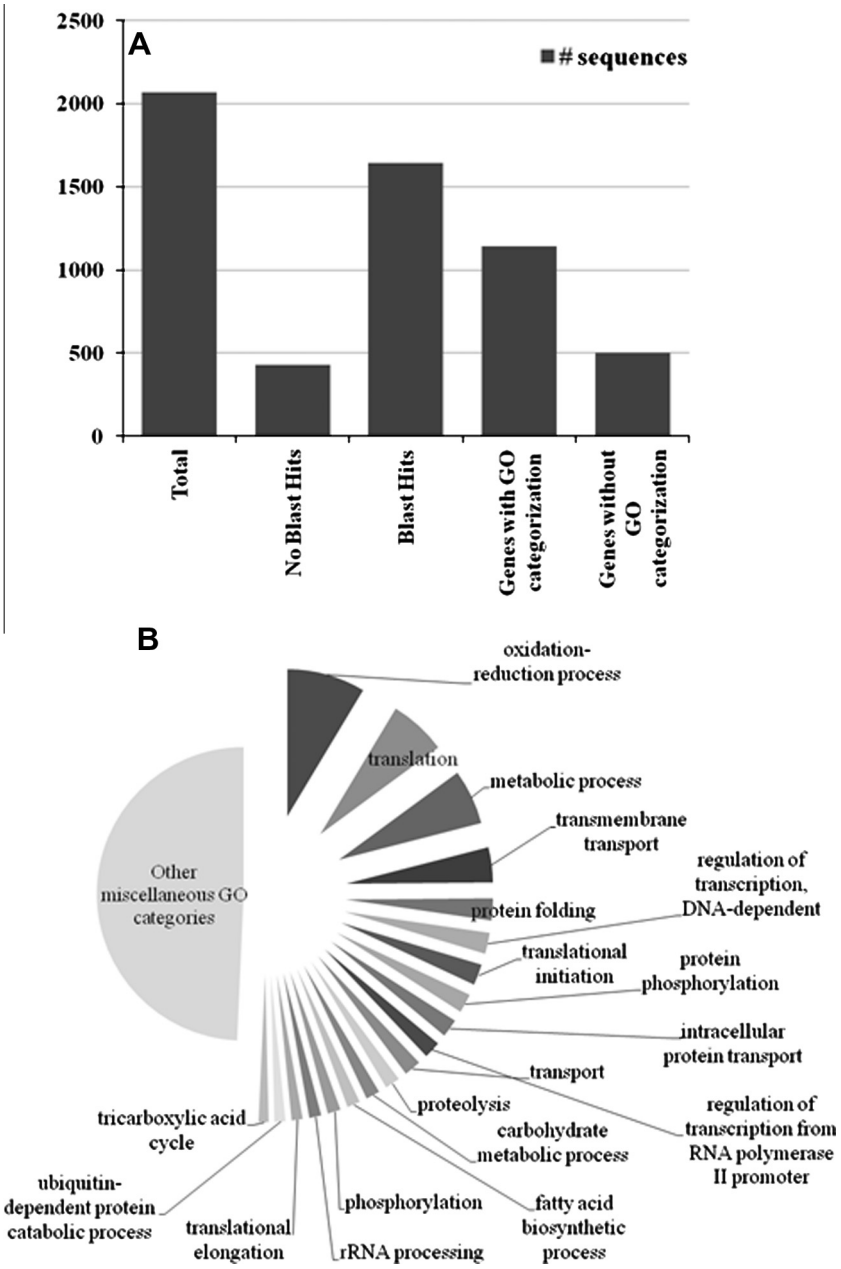

Fig. 2. GO categorization of the differentially expressed genes of *P. brasiliensis* in response to paraquat exposure. (A) Summary of the functional analysis of all 2070 genes found to be up-regulated during paraquat-induced oxidative stress in *P. brasiliensis*. Sequences of the modulated genes have been used to search the nr (non-redundant) Genebank database using BLAST, confirming the identification of putative functions for 1642 *P. brasiliensis* genes. These sequences were distributed in functional categories of the Gene Ontology (GO) database using BLAST2GO, as described in Materials and Methods. These analyses resulted in the identification of 1143 genes, distributed throughout many different GO categories, and 499 genes with no specific GO categorization. (B) Functional distribution of the 1143 genes identified by BLAST2GO into different GO Biological Process (P) categories. Only gene groups that contained more that 10 modulated elements are individually shown in this chart. The other GO categories, carrying <10 elements, have been grouped as miscellaneous GO categories. Detailed identification of the genes distributed across all GO classes (Biological Process; Molecular Function and Cellular Component) can be found in Supplementary Table 1.

seemed to suggest that chitin synthesis in *P. brasiliensis* might be controlled by transcriptional up-regulation of several genes involved in this anabolic route. This possibility encouraged us to conduct a more detailed analysis of the expression profiles regarding genes involved in chitin biosynthesis. Thus, six genes related to four steps along this pathway were selected for the real-time qPCR assays. The results, shown in Table 2, indicate that, in fact, practically all chitin-biosynthetic genes have their expression up-regulated in response to oxidative stress. Moreover, exposure to paraquat resulted on modulation in a dose-dependent manner (Table 2). The only exception in this analysis was chitin synthase III (DR164480), which only displayed an enhanced expression ratio at the 5 mM paraquat concentration. However, fungi have been shown to carry different isomorphs of this gene and our analysis confirmed intense overexpression of BQ493332 (another *P. brasiliensis* chitin synthase) in response to both paraqu at concentrations, indicating that practically the entire chitin biosynthesis pathway seems to have its gene expression up-regulated in both mild and more intense oxidative stress conditions. Taken together, these results show that the fungal exposure to paraquat not only promoted the overexpression of chitin synthase genes but also the up-regulation of the enzymes involved in the synthesis of metabolic precursors of chitin, probably in a cell effort to ensure cell wall integrity in response to the oxidative stress.

Table 1
Comparison of gene expression values obtained with microarray hybridization and Real-time qPCR.

Gene product	Value from array/Real-time qPCR[a]	
	Paraquat 0.5 mM	Paraquat 5 mM
Glutathioneperoxidase (BQ490900)	0.11/0.51	1.08/0.10
Glyceraldehyde 3-phosphate dehydrogenase (BQ491108)	−2.88/−0.31	−0.22/−0.86
4-Hydroxyphenylpyruvate dioxygenase (BQ499298)	−0.45/−1.83	2.12/1.52
Cu, Zn superoxidedismutase (BQ496515)	0.06/0.13	1.91/1.40
Carnitine acetyltransferase (BQ504239)	0.91/1.71	1.57/1.60
Peroxiredoxin V protein (BQ498804)	0.08/0.55	1.86/1.41
Putative thiol specific antioxidant protein Tsa1 (BQ499382)	−3.26/−1.59	0.90/1.37
Cysteine dioxygenase (BQ497804)	2.41/2.52	0.91/1.55
Glucosamine fructose 6 phosphate aminotransferase (BQ498822)	0.54/0.85	0.47/0.82
Chitin synthase III (DR164480)	−0.07/−0.06	0.72/1.02

[a] The values are shown as the $\log_2$ ratios between the values obtained at the indicated experimental condition and the reference value (untreated control). The numbers in parenthesis indicate the Genebank accession number for each gene present in our microarray.

In order to verify whether the up-regulation of genes involved in chitin biosynthesis correlate with an actual increase in cell wall chitin levels, we quantified the chitin content of yeast cells exposed to the same experimental conditions employed in the gene expression assays. The measurements were performed according the protocol established by Zamani et al. (2008) and, as observed in Fig. 3, chitin content in *P. brasiliensis* yeast cells was significantly increased after exposure to both paraquat concentrations, reaching approximately 0.37 g/g of cells (0.5 mM paraquat) and 0.35 g/g cells (5 mM paraquat). These values represent a 50–60% increase in chitin content, when compared to control *P. brasiliensis* cells, grown with no exposure to this ROS-generating agent. Surprisingly, however, the increase in the cell wall's chitin content did not occur in a dose-dependent manner, suggesting that other control mechanisms are likely to act, together with the transcriptional up-regulation of the chitin biosynthesis pathway, in determining cell wall structure and chitin content in response to oxidative stress.

Table 2
Transcriptional modulation of the chitin-biosynthesis pathway genes obtained with Real-time qPCR.

Gene product (gene)	Value real-time qPCR[a,b,c]	
	Paraquat 0.5 mM	Paraquat 5 mM
Glucose 6-phosphate isomerase (BQ496641)	0.401065	0.742461
Glucosamine-fructose-6-phosphate aminotransferase (BQ498822)	0.541104	0.466739
Phosphoacetylglucosamine mutase (BQ493599)	0.016354	0.52707
UDP-N-acetylglucosamine pyrophosphorilase (DR164606)	0.182057	0.396686
Chitin synthase D (BQ493332)	0.997006	2.060045
Chitin synthase III (DR164480)	−0.07194	0.724926

[a] The values are shown as the $\log_2$ ratios between the values obtained at the indicated experimental condition and the reference value (untreated control). The numbers in parenthesis indicate the Genebank accession number for each gene present in our microarray.
[b] Positive and negative values represent up- and down-regulated genes, respectively.
[c] Experiments were performed in triplicate.

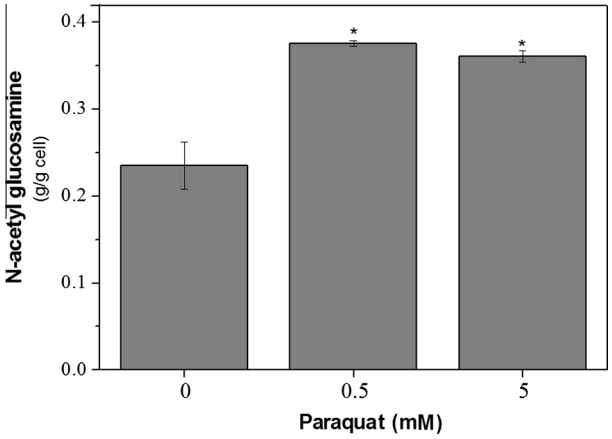

Fig. 3. Chitin content in *P. brasiliensis* cell wall in response to paraquat exposure. The chitin content was measured from cell wall of yeasts grown in the absence or presence (0.5 mM and 5 mM) of paraquat. Cell wall chitin assays were performed five times on three biologically independent samples. Asterisks indicate significant differences from unexposed cells grown under the same conditions.

4. Discussion

P. brasiliensis is a pathogenic fungus that invades the human body primarily through the respiratory tract. In the lungs, the pathogen is phagocytized by alveolar macrophages and exposed to a set of toxic elements, including ROS, produced as a part of the host defense mechanisms against invading microorganisms (Jamieson, 1998; Missal et al., 2004). Thus, pathogens have their own set of defense mechanisms that act in a coordinated manner to circumvent the host-induced environmental stresses and optimize their chances of survival under such adverse conditions. The microarray hybridization analysis shown in the current study has allowed us to evaluate, for the first time, the integrated and complex interplay among elements involved in many different metabolic traits that constitutes the antioxidant defense system in the pathogenic fungus *P. brasiliensis*.

As already stated in the literature, oxidant-exposed yeast cells must maintain their intracellular redox potential, while free-radical scavengers and detoxifying enzymes act to reduce the amount of intracellular ROS (Shenton and Grant, 2003; Krüger et al., 2011; Ralser et al., 2009). In this sense, we were able to confirm overexpression of genes encoding the most relevant components of the fungus's antioxidant defense mechanisms, such as superoxide dismutases (SOD), which catalyze dismutation of superoxide anion ($O_2^{\cdot-}$) to H_2O_2 and O_2 and glutathione peroxidase (GPX) that is involved in the decomposition of H_2O_2 to H_2O (Jamieson, 1998; Missal et al., 2004; Ruiz et al., 2011; Tavares et al., 2007). In eukaryotic cells, superoxide dismutases probably constitute the major antioxidant enzymes involved in detoxification of ROS, which are generated either in the mitochondrial respiratory chain or under stress conditions (Halliwell and Gutteridge, 1991; Liu et al., 2010). Fungi have two SOD isomorphs that differ in regard to metal cofactor and intracellular location: copper/zinc (Cu, Zn-SOD), which is located in the cytosol and in small quantities in the mitochondrial intermembrane space, and manganese (Mn-SOD) that is concentrated in the mitochondrial matrix (Fréalle et al., 2005; Fridovich, 1995). Several studies have shown that overexpression of both enzymes is a primary response of fungi undergoing oxidative stress, particularly that induced by the superoxide anion (Emri et al., 1999; Gasch et al., 2000; Jeong et al., 2001). Moreover, studies with *S. cerevisiae*, *Cryptococcus neoformans* and *Candida albicans* revealed that *sod1* and *sod2* null mutants were more sensitive than wild-type to superoxide anion generators, such as paraquat (Gralla and Valentine, 1991; Jeong

et al., 2001; Liu et al., 1992; Longo et al., 1996). As mentioned above, the present work confirmed that *P. brasiliensis* yeast cells, when exposed to paraquat, displayed overexpression of genes encoding Cu, Zn-SOD (BQ496515) and Mn-SOD (BQ499157). Similar results regarding the expression profile of Cu, Zn-SOD were also observed in *P. brasiliensis* yeast cells upon internalization by murine macrophages, reinforcing the importance of this enzyme in ROS detoxification under oxidative stress conditions faced by this pathogen (Tavares et al., 2007).

However, the up-regulation of SODs – although undoubtedly a crucial event to prevent cell damages caused by superoxide anion – does not ensure complete cell protection against the deleterious effects of oxidative stress. To effectively protect their cellular components, the pathogen must also trigger mechanisms to reduce peroxide to water, thus avoiding or decreasing the hydroxyl radical formation. In this sense, our results have also revealed the up-regulation in the expression of a specific glutathione peroxidase (GPX) (BQ490900). GPXs are enzymes that catalyze the reduction of both H_2O_2 and hydroperoxides using glutathione (GSH) as a reductant, and, together with catalase, form the major defense system against H_2O_2 in most cells (Ekholm and Björkman, 1997; Galiazzo et al., 1987; Halliwell and Gutteridge, 1991; Herrero et al., 2008; Jamieson, 1998; Missal et al., 2004). Thus, the overexpression of GPX does not constitute an unexpected finding; but an essential phenomenon to complete the process of superoxide detoxification.

The enzyme 4-hydroxyphenylpyruvate dioxygenase (4-HPPD) is also likely to play an important role among the main ROS-detoxifying mechanisms in *P. brasiliensis*. Although originally involved in the catabolism of phenylalanine and tyrosine, 4-HPPD has already been described as the product of *mel*A, a gene directly involved with the synthesis of pyomelanin, a melanin-like pigment typically found in bacteria, which is formed through the oxidative-dependent polymerization of homogentisic acid, the end-product of 4-HPPD (Moran, 2005). Thus, accumulation of homogentisic acid, as a result of increased 4-HPPD expression might represent a mechanism to provide the cell with an efficient scavenger molecule that will, ultimately, result in the formation of melanin, as part of the detoxification mechanisms against oxidative conditions. Melanins are thought to play a protective role in the establishment of pathogenicity by several fungi (Langfelder et al., 2003) and, in most species, a specific type of melanin, called eumelanin, is formed by oxidation of diphenolic compounds, like L-3,4-dihydroxyphenylalanine (L-DOPA), which are polymerized by a copper-dependent diphenol oxidase from the laccase family (Gómez et al., 2001). Recently, however, the existence of pyomelanin, as well as its importance as a pathogenicity factor has been acknowledged in pathogenic fungi, such as *Aspergillus fumigatus* (Keller et al., 2011; Schmaler-Ripcke et al., 2009). Moreover, the involvement of 4-HPPD in the oxidative stress response of *P. brasiliensis* is particularly relevant, since this enzyme has been shown to be of paramount importance during the mycelium-to-yeast differentiation in this fungus – an information confirmed by the use of nitisinone, a specific inhibitor of 4-HPPD activity, which was able to inhibit growth and differentiation of the pathogenic yeast phase of the fungus *in vitro* (Nunes et al., 2005). Thus, the results described herein reinforce the possibility that nitisinone, as well as some derivatives of this drug, might be used to develop new chemotherapeutic approaches against paracoccidioidomycosis (Nunes et al., 2005).

However, in spite of its fundamental importance in preventing cell damage during oxidative stress conditions, the up-regulation of genes encoding enzymes and proteins involved in detoxification and scavenging of oxidants is not the only strategy used by pathogenic fungi to withstand oxidative environments. Adaptive responses to oxidative stress conditions often involve changes in fungal gene expression that affect enzymes related to other metabolic pathways, producing a highly integrated and sufficiently robust defense system that enables the fungus to endure the ROS attack. (Grant, 2008). Among such mechanisms, we have been able to verify increased expression of genes encoding subunits of the mitochondrial respiratory complexes. The mitochondrial involvement in the yeast response to oxidative stress is not clearly understood, since mitochondrial respiratory chain constitutes the main source of ROS in aerobically growing cells (Cadenas, 1989; Landolfo et al., 2008). Nonetheless, high respiration rates have been shown to promote resistance against oxidative stress in yeast cells exposed to oxidants, including H_2O_2, diamide and menadione, among others (Grant et al., 1997; Grüning et al., 2011). However, under such conditions, respiratory complexes become susceptible to damage caused by ROS, which results in impairment of electron flow through electron transport chain and, consequently, partial inhibition of mitochondrial respiration (Cortés-Rojo et al., 2007).

Our results showed that the exposure of yeast cells to paraquat resulted in up-regulation of genes encoding proteins of the mitochondrial respiratory complexes I, II, III and IV from *P. brasiliensis*, which might indicate a cellular attempt to ensure not only the structural and functional integrity of the electron transport chain, but also to maintain the oxidative stress resistance that increased rates of mitochondrial respiration provide.

Finally, the involvement of a cell wall strengthening mechanism, mediated by chitin accumulation should be mentioned as an important part of the *P. brasiliensis* oxidative stress response. Several studies have recently highlighted this mechanism as an important adaptation to the adversities encountered by pathogenic fungi – both in the environment or within the vertebrate host (Fuchs and Mylonakis, 2009; Herrero et al., 2008; Lenardon et al., 2010; Walker et al., 2008). Because of its enormous tensile strength, chitin significantly contributes to the integrity of the fungal cell wall and, in pathogenic fungi, such as *P. brasiliensis*, the polymer also plays an important role when the fungus is exposed to cell wall stress conditions, in which wall damage may impair cell viability (Bulik et al., 2003; Walker et al., 2008). Under these circumstances, increase of chitin synthesis ensures strengthening of the cell wall, which can be critical for cell survival (García-Rodriguez et al., 2000; Walker et al., 2008). This phenomenon is particularly important during host invasion, when fungal cells must face the temperature-induced transition to the yeast form, while simultaneously interact with ROS-rich environments created by immune cells, in response to infection (Bowman and Free, 2006; Lenardon et al., 2010; Nunes et al., 2005; Ruiz et al., 2011). Although the cell wall does not constitute the main element in the fungal defense against damages from oxidative stress conditions, its importance cannot be neglected, since it is the outermost structure of the cell and has a dramatic influence in the pathogeńs tolerance to harmful environments (Fuchs and Mylonakis, 2009; García-Rodriguez et al., 2000).

The induction of chitin synthase genes under *in vitro* oxidative stress conditions was recently described in the mycelial phase of *P. brasiliensis*, suggesting that this is also an important phenomenon in the mycelium adaptation to harsh environments, such as the macrophages and phagosomes present in the hosťs tissues during the initial phase of infection (Niño-Vega et al., 2009). It has been recently documented that *P. brasiliensis* (isolate 18) carries a total of seven chitin synthases and two chitinase genes, which are, ultimately, responsible for chitin synthesis and degradation in the cell wall. Our microarray carries representative sequences of all these gens, but only the three chitin synthases mentioned above displayed statistically significant gene expression modulation under paraquat-induced oxidative stress. However, our results showed that such conditions lead increased expression of not only chitin synthases; other genes, involved in four out of the five steps that lead to chitin biosynthesis from glucose are also up-regulated:

(i) glucose-6-phosphate isomerase, that catalyses the conversion of glucose-6-phosphate to fructose-6-phosphate; (ii) glucosamine-fructose-6-phosphate aminotransferase, which convert fructose-6-phosphate to GlcN-6-phosphate; (iii) phosphoacetylglucosamine mutase, which catalyses the conversion of the GlcNAc-6-P to GlcNAc-1-P; (iv) UDP-N-acetylglucosamine pyrophosphorylase, that catalyses the synthesis of UDP-GlcNAc from GlcNAc-1-P and UTP. Chitin is then synthesized by chitin synthases using UDP-GlcNAc as a substrate. For all these enzymes, 0.5 mM, paraquat induced less marked effects on gene expression up-regulation than those obtained with exposure to 5 mM, indicating a dose-dependent response, in which the magnitude of the changes in gene expression is proportional to the intensity of the stress. Measurements of cellular chitin levels, however, showed different results. In fact, as shown in Fig. 3, the increases in chitin levels in cells exposed to 5 mM paraquat are roughly the same in cells exposed to a 0.5 mM concentration. This finding suggests that the increase in chitin content in response to paraquat exposure is a phenomenon regulated at both transcriptional and post-transcriptional levels and, apparently, the lowest concentration of paraquat is already sufficient to trigger maximal effects on polymer production.

In summary, we demonstrated that *P. brasiliensis* yeast cells combine several mechanisms in the assembly of a highly integrated and sufficiently robust defense system against oxidative stress imposed by the ROS-generating agent paraquat. The fungal response involved not only up-regulation of genes that act to reduce the amount of intracellular ROS, such as free-radical scavengers and detoxifying enzymes, but also included the overexpression of genes associated with other metabolic pathways, such as the chitin biosynthetic pathway. The overexpression profile shared by practically all chitin-biosynthetic genes suggests that they might have their expression levels controlled by a common factor or stimulus, maybe constituting a specific genetic regulon, not yet characterized in *P. brasiliensis*, or in any other fungi. Although our data clearly suggests that other cellular processes are likely to act in determining cell wall structure and chitin content in response to oxidative stress, a better characterization of this putative chitin-biosynthesis regulon, as well as the other mechanisms that control chitin deposition on the cell wall may provide interesting new targets for chemotherapeutic approaches against *P. brasiliensis* and other pathogenic fungi.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fgb.2013.05.004.

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