

RNAi technology targeting PbGP43 and PbP27 in *Paracoccidioides brasiliensis*

Isaura Torres Gómez^{1,2}, Orville Hernandez Ruiz^{1,3}, Jose F. Muñoz^{1,2}, Ana María García¹, Angela Restrepo¹, Juan G. McEwen^{1,4}

¹Unidad de Biología Celular y Molecular, Corporación para Investigaciones Biológicas (CIB), Medellín, Colombia

²Instituto de Biología, Universidad de Antioquia, Medellín, Colombia

³Grupo de Investigación en Biociencias, Facultad de Ciencias de la Salud, Institucion Universitaria Colegio Mayor de Antioquia, Medellín, Colombia

⁴Facultad de Medicina, Universidad de Antioquia, Medellín, Colombia

Email: isaurap10@gmail.com

Received 7 May 2013; revised 7 June 2013; accepted 30 June 2013

Copyright © 2013 Isaura Torres Gómez *et al.* This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

ABSTRACT

Efficient technologies for gene silencing would be important to carry out functional analysis with *P. brasiliensis* genes, as well as for a better understanding of the biology and pathogenesis of this pathogenic fungus. Due to the fact that homologous recombination is unusual in *P. brasiliensis*, the development of knock-out isolates is currently non-feasible. The goal of this work was to assess RNA interference (RNAi) technology as an alternative tool for gene silencing previously employed successfully in *H. capsulatum*. For this purpose, we built different inverted repeat transgenic hairpin constructs to down-regulate the PbGP43 and PbP27 genes known to codify for two fungal immunogenic proteins that elicit a strong immune response during experimental paracoccidioidomycosis. Using the RNAi strategy, a reduction in the mRNA levels of the PbGP43 and PbP27 genes was observed during the first 20 days after selection; however, in the transformed yeast cells, the gene silencing status proved non-stable through the assay. We demonstrated that electrotransformation was suitable to transform *P. brasiliensis* yeast cells and integrate the hairpin constructions; nonetheless, gene silencing was not stable along the experimental time. A detailed analysis of the underlying molecular RNAi machinery may provide further insights into the intracellular mechanism that governs this reverse genetic tool.

Keywords: *Paracoccidioides brasiliensis*; Interference RNA; Gene Silencing; PbGP43; PbP27; Gene Expression

1. INTRODUCTION

RNA interference (RNAi), a natural mechanism conserved all along evolution, has been implicated in gene silencing in eukaryotic systems [1]. In addition, RNAi participates in the regulation of genetic expression mediated by certain classes of small endogenous RNAs such as micro RNA (miRNA), which acts by using double stranded RNA (dsRNA) homologous to the target sequence [2].

Due to the fact that generation of knockout isolates is time consuming and requires sequential positive and negative selection steps, in order to enrich the desired recombination event, RNAi technology has rapidly become one of the key methods in functional genomics studies, and is used to block gene expression and create potential phenotypes capable of yielding clues concerning the function of these genes [2]. This technology has been successfully used to obtain gene disruption in dimorphic fungi, e.g. *Histoplasma capsulatum* [3] and *Blastomyces dermatitidis* [4].

Paracoccidioides brasiliensis, a thermally dimorphic fungus, is the etiological agent of paracoccidioidomycosis (PCM), an important systemic, endemic mycosis in Central and South America [5]. In this fungus, gene disruption methods are even more laborious and currently seem unfeasible. This could be due to the presence of dominant illegitimate recombinant events (by non-homologous end-joining) overriding homologous recombination [6]. In *P. brasiliensis*, RNAi could be employed as an alternative method to transcriptional gene silencing mediated by sequence-specific mRNA depletion. The aim of this work was to determine if RNAi strategy was a proficient tool to down regulate both PbGP43 (a 43 kDa gly-

coprotein) [7] and PbP27 (a 27-kDa protein) [8] gene expression, two *P. brasiliensis* immunogenic antigens with as yet unknown biological functions.

In this work, by means of bioinformatics analysis, we demonstrated the presence of genes involved in the RNAi route in the *Paracoccidioides* spp. genome. Our results indicated that RNAi strategy appears to be an effective method to integrate the RNAi hairpin constructions in the genome of this fungus; however, the gene silencing status was not stable along the time in the isolates evaluated.

2. MATERIALS AND METHODS

2.1. Strains and Culture Conditions

P. brasiliensis Pb339, a strain that produces high quantities of extracellular antigens, especially gp43 [9-11], and p27 [8,12] during its parasitic phase was used in this study. Yeast cell cultures and growth curves were performed in BHI media supplemented with 1% glucose (Beckton Dickinson and Company, Sparks, MD) at 37°C with aeration in a mechanical shaker and were routinely collected during the early exponential phase (72 - 96 h). *Escherichia coli* DH5 α grown at 36°C in Luria Bertani (LB) culture medium supplemented with appropriate antibiotics, was used for cloning and plasmids propagation assays [13].

2.2. RNAi Orthologs in *Paracoccidioides* spp. Genome

We performed a search in the *Paracoccidioides* spp. Ge-

nome references strains Pb18, Pb03 (*P. brasiliensis*) and Pb01 (*Paracoccidioides lutzii*)

(http://www.Broadinstitute.org/annotation/genome/paracoccidioides_brasiliensis) of the putative homologous proteins involved in *Neurospora crassa* RNA silencing [14] using BLAST tools. Furthermore, we look for the key domains involved in RNAi using profile hidden Markov models (HMMs) with HMMER scan [15].

2.3. Molecular Cloning and Silencing Cassette Construction

The RNAi plasmid pCR99 (Figure 1(a)) (provided by Chad Rappleye, Washington University in St. Louis, Missouri USA) was used to construct the silencing cassette targeting *P. brasiliensis* PbGP43 and PbP27 (Figure 1(b)). *H. capsulatum* CBP1 promoter (889 bp) was used to initiate transcription of RNAi targets, and the 733 bp intergenic region downstream of the CATB gene was used as a transcriptional termination signal (T-catB). An 87 bp loop and either the approximate length of the double-stranded were used in target region in the RNA hairpin. Primers were designed using *P. brasiliensis* strain; sequence data available from

<http://www.broadinstitute.org/annotation/genome>). For PbGP43 Pb18: PADG_07615 includes exon 1 (E1) and exon 2 (E2); and for PbP27 Pb18: PADG_08402 (supplementary Table S1). To construct the PbGP43E1RNAi cassette, a PbGP43 457-bp fragment was amplified from genomic DNA and cloned in the opposite orientation into pCR99 *Asc*I-*Xho*I and *Age*I-*Xba*I cloning sites; PbGP43-

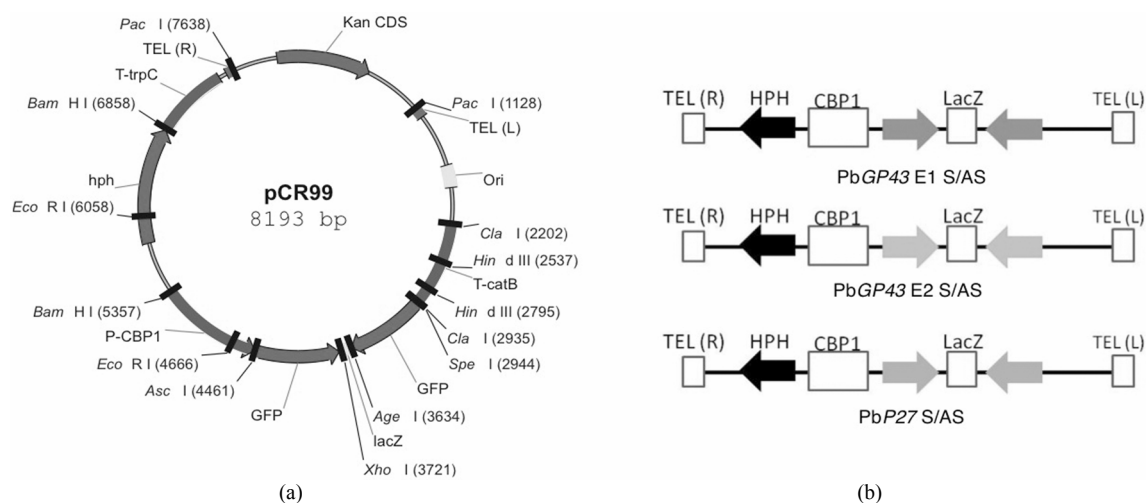


Figure 1. Hairpin RNAi constructions aimed at triggering gene silencing of PbGP43 and PbP27 in *P. brasiliensis*. A. Map of the telomeric plasmid pCR99 used to trigger RNAi in *P. brasiliensis*. *H. capsulatum* telomeres (TEL) were described previously [16]. The CBP1 promoter (889 bp) was used to initiate transcription of RNAi targets and the 733 bp intergenic region downstream of the CatB gene was used for transcriptional termination signals (T). This plasmid was used to clone individually the inverted copies of target genes for PbGP43 exon 1 (PbGP43E1) and exon 2 (PbGP43E2) and PbP27, separated by a lacZ fragment to produce the RNA hairpin. Kan: kanamycin resistance; hph: hygromycin resistance cassette. B. RNA hairpin constructs targeting PbGP43E1 (457 bp), PbGP43E2 (788 bp) and PbP27 (500 pb).

E2RNAi and PbP27RNAi cassettes were designed, amplified and constructed using the same strategy employed to PbGP43E1RNAi cassette (**Figure 1(b)**). The primers are described in supplementary **Table S1**, the sizes of the target sequences were 788-pb and 500-pb respectively. All PCR products were amplified using the Platinum high-fidelity TaqDNA polymerase (Invitrogen, Carlsbad, CA, USA).

2.4. *P. brasiliensis* Transformations and Screening

The Pb339 strain was electrotransformed with *PmeI*-linearized plasmid according to the protocol previously described [16]. Briefly, *P. brasiliensis* yeast cells were grown in BHI batch cultures to their exponential growth phase with shaking at 36°C, washed once with 10% mannitol, sterilized by filtration as electroporation solution. Yeast cells were electrotransformed with 2 µg of *PmeI*-digested pCR99 constructions (PbGP43E1RNAi, PbGP43E2 RNAi and PbP27 RNAi) and an empty vector (PbEV) as a control, in a Gene Pulser Electroporator (Bio-Rad, Hercules, CA), using the following conditions: capacitance of 25 µF, resistance of 600 Ω and set voltage of 0.75 kV [16,17]. Following transformation, cells were spread onto selective BHI media supplemented with 100 µg/ml of hygromycin B (Sigma, Aldrich, MO, USA). Selection plates were monitored for colony forming ability at 37°C for 15 to 20 days. The phenotypic stability of *P. brasiliensis* transformants yeast cells was determined by analyzing the stability of hygromycin B resistance [18]. Sixty individual colonies were selected and subcultured in selective medium, (solid BHI containing 150 µg/ml hygromycin B) each 5 days at 37°C for three consecutive times and then subcultured in liquid BHI containing 150 µg/ml hygromycin B three times, RNA extraction was then done and used in the RT-qPCR assay.

2.5. Molecular Detection of the Hygromycin Resistance Gene (*HPH*)

Genomic DNAs from PbWt, PbEV, PbGP43E1RNAi, PbGP43E2 RNAi and PbP27 RNAi transformants yeast cells were isolated using the glass beads protocol described by Van Burik (1998) [19]. In order to confirm the presence of the hygromycin B resistance cassette, PCR analysis was carried out to detect an *hph* 1000-bp amplification product using primers *hphF* (5'-AACTACC-GCGACGTCTGTCTCGA-3') and *hphR* (5'-CTACACAGCCATCGGTCCAGA-3'). PCR amplification included 30 cycles of 1 min at 94°C, for denaturation, 1 min at 68°C for annealing, and 1.5 min at 72°C for extension. The reaction products were analyzed in 1% agarose gel and visualized with ethidium bromide under UV light.

2.6. RNA Extraction, cDNA Synthesis and Real-Time RT-qPCR Analysis

P. brasiliensis yeast cells were grown in liquid BHI supplemented with glucose 1% and hygromycin B 150 µg/ml at 37°C and harvested after 5 days of growth. RNA was obtained using the TRIzol[®] reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Total RNA was treated with DNaseI (Invitrogen, Carlsbad, CA, USA) and tested for chromosomal DNA contamination using conventional PCR for the β -tubulin gene [20]. cDNA was synthesized using 1 µg of total RNA and Superscript III reverse transcriptase according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Real-time PCR (RT-qPCR) was performed using Maxima[®] SYBR Green/Fluorescein qPCR Master Mix, according to the manufacturer's instructions (Fermentas Maryland, USA). The CFX96 Real-Time PCR Detection System (Bio-Rad, Headquarters Hercules, California, USA) was used to evaluate PbGP43 or PbP27 gene expression; β -tubulin was selected as house keeping gene [20]. Melting curve analysis was done after the amplification phase to eliminate the possibility of nonspecific amplification or primer dimer formation. Folding changes in mRNA expression were calculated using the $2^{-\Delta\Delta CT}$ formula, where $\Delta\Delta CT$ is the difference between target and β -tubulin genes [21]. Each experiment was carried out in triplicate and the expression level was measured three times.

3. RESULTS

In *P. brasiliensis* Electrotransformed Yeast Cells, PbGP43 and PbP27 Gene Silencing Achieved by Using the RNAi System, Was Effective but Not Stable during Time of the Experiments

Three different RNAi constructions were designed from exon 1 and exon 2 from PbGP43 (PbGP43E1 RNAi, PbGP43E2 RNAi) and one from PbP27 (PbP27 RNAi), and transformed individually in PbWt yeast cells. Fourteen transformants with reduced gene expression levels were obtained by electrotransformation with the constructions PbGP43E1-RNAi (n: 6), PbGP43E2-RNAi (n: 5) and PbP27-RNAi (n: 3) and selected after phenotypic and mitotic stability tests (**Table 1**). The presence of the RNAi cassette in the knock down strains and the yeast cells transformed with PbEV was demonstrated by *hph* gene amplification. This gene was not observed in PbWt yeast cells (**Figure 2**).

In all yeast transformants cells, mitotic stability remained stable *in vitro* cultures with continuous subcultures in selective medium containing hygromycin B; in parallel, all transformants were cultured several times in non-selective medium; after this, all of them remained

Table 1. Genetic transformation of *P. brasiliensis* by electroporation using hairpin RNAi constructions.

Construct (2 µg)	Colony number after phenotypic stability test [*]	Number of stable transformants after mitotic stability test ^{**}	Mitotic stability (%)
PbGP43E1 RNAi	20	6	30
PbGP43E2 RNAi	31	5	16
PbP27 RNAi	33	3	9

^{*}Number of transformants obtained using 2 µg of *PmeI*-linearized plasmid DNA; ^{**}Mitotic stability of putative transformants was analyzed after 5 subcultures on non-selective medium followed by plating on hygromycin B (150 µg/ml).

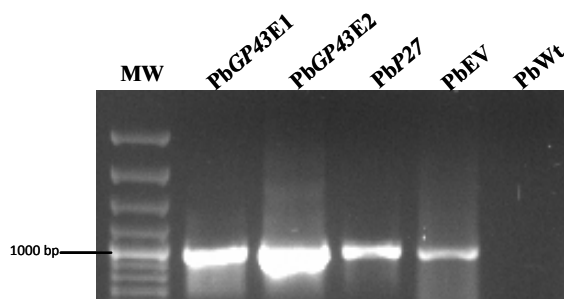


Figure 2. Molecular analysis of the integration of the *hph* resistance cassette into *P. brasiliensis* putative transformants. Wild type host strain, PbWt harboring the empty vector (PbEV). Three putative transformants from the RNAi hairpin constructions selected randomly were subjected to PCR using the phosphotransferase gene (*hph*) specific primers, *hph*-F and *hph*-R, in order to amplify a 1000 bp internal fragment of the *hph* gene MW: DNA molecular size marker.

their capacity to grow in the hygromycin B-containing medium (Table 1).

After 20 days of growth in culture media, a decrease in gene expression level ranging from 64% to 84% was observed in PbGP43E1 RNAi isolates (Figure 3(a)), and from 42% to 71% in the PbGP43E2 RNAi isolates (Figure 3(b)). In an equal manner, in the yeast cells transformants that had been obtained using the PbP27RNAi construction, a decrease in PbP27 gene expression level ranging from 39% to 79%, was observed in comparison with the expression levels in PbWt and PbEV (Figure 3(c)). However, after 45 and 60 days of growth in the selective culture media, an increase in PbGP43 gene expression level in both PbGP43E1 RNAi and in PbGP43E2 RNAi transformants was observed. Similarly, an increase of PbP27 gene expression was observed in PbP27RNAi yeast cells, indicating that in *P. brasiliensis* electrotransformed yeast cells silencing had not been stable during the course of time.

BLAST searches using published data and available genomes corresponding to *P. brasiliensis* Pb18, Pb03 and, Pb01, as well as *P. lutzii* [22] (www.broadinstitute.org) were done. We identified all genes that participated in the RNA silencing related with predicted function such RNA-directed RNA polymerases (*qde-1*, *sad-1*, *rrp-3*), Argo-

naut-like (*qde-2*, *Sms-2*), Dicer-like (*dcl-2*, *Sms-2*) and RecQ helicase-like (*qde-3*, *RecQ-2*) (Table 2). Furthermore, we found that the key protein domain of the RNAi system in the proteins detected in the BLAST search, indicating that the RNAi system could be used to down regulate specific genes in *P. brasiliensis* (Supplementary Figure S1).

4. DISCUSSION

Efficient technologies to achieve gene silencing could help to undertake functional analysis of *P. brasiliensis* genes and increase the understanding of the biology and virulence attributes of this fungus, overcoming many of the difficulties associated with traditional gene disruption. RNAi does not depend on the homologous recombination machinery, making this strategy an attractive alternative to gene silencing [17].

In this study, we found that in *Paracoccidioides* spp, there were homologous proteins involved in the RNA silencing process suggesting the presence of the components required for RNAi gene silencing thus supporting the hypothesis that in this fungus such event should occur under natural conditions.

Our results show that the electroporation is an efficient tool to introduce the linearized construction of RNAi in *P. brasiliensis* yeast cells genome, as confirmed by the presence of the *hph* gene into the transformed cells. However, the reduced mitotic stability observed in the transformant isolates would indicate that the fungus tends to lose the *hph* fragment during continuous fungal growth under non-selective pressure. As previously reported, the efficiency of silencing depends upon several factors including the length and structure of the hairpin constructions. Rappleye *et al.*, (2004) [17] have shown, that a greater silencing effect was observed with a shorter loop and a longer target gene sequence; these conditions were used in our experiments but our results were different.

Currently, a gene silencing strategy based on antisense-RNA (asRNA) has been efficiently employed in *P. brasiliensis* to induce the knockdown of PbCDC42, PbHAD32, PbAOX, and PbHSP90 [23-26]. Additionally, based in our previous results using the antisense RNA strategy targeting PbGP43 and PbP27 (unpublished), and

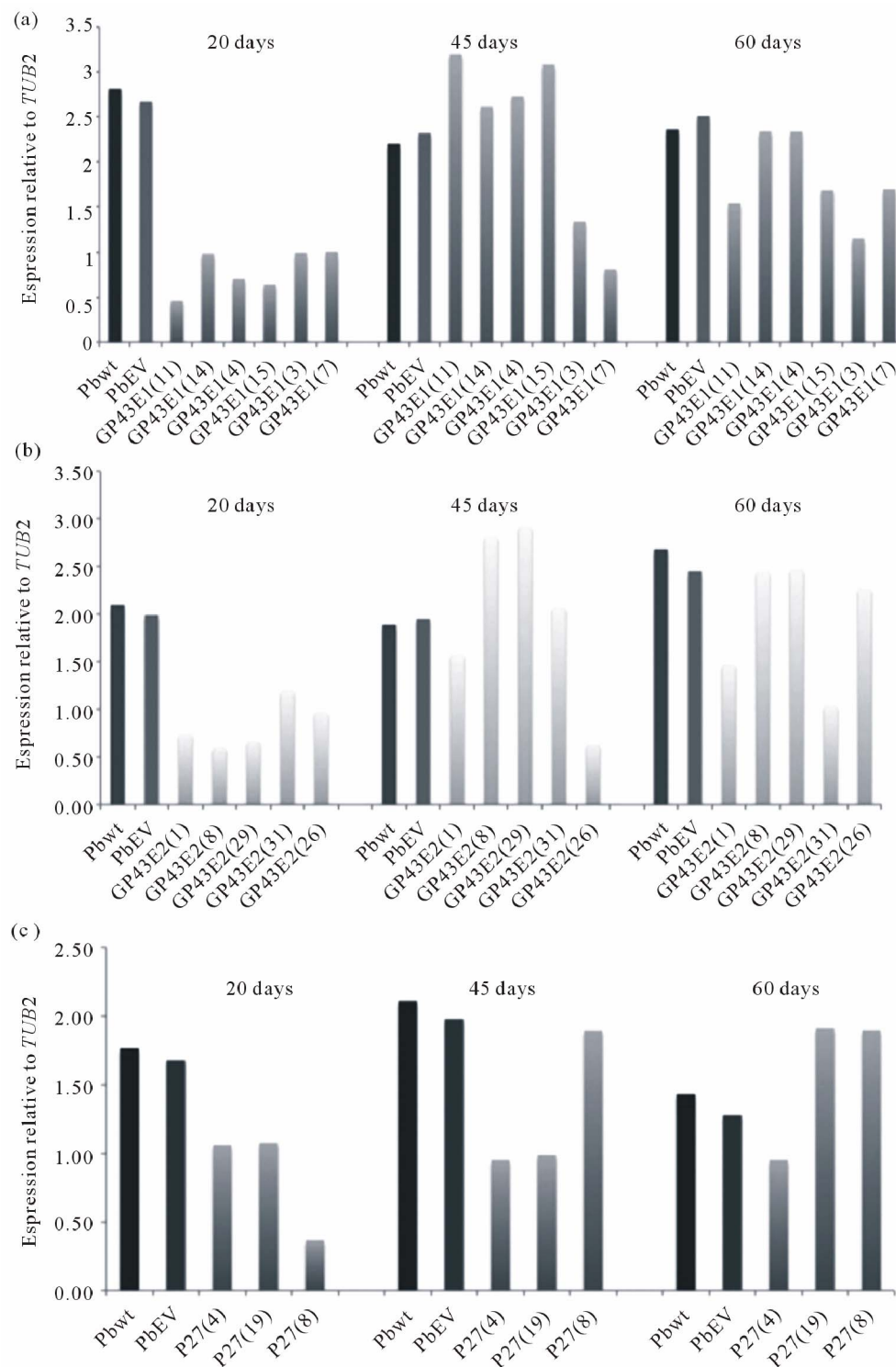


Figure 3. Gene expression levels of PbGP43 and PbP27 by RT-qPCR. (a) PbGP43 gene expression in the PbWt, PbEV and the PbGP43E1 transformant yeast cells, selected after phenotypic and mitotic stability. (b) PbGP43E2 RNAi gene expression in the PbWt, PbEV and PbGP43E2 RNAi transformants and (c) PbP27 gene expression in PbWt, PbEV and PbP27 RNAi transformants. All evaluations were performed in the yeast cells after subculture for 20, 40 and 60 days. Low expression was observed during the 20-day evaluation but not in those corresponding to the 45 and 60 days evaluations. Gene expression levels obtained by RT-qPCR were normalized to the level of expression of the internal control gene TUB2 [20].

Table 2. Presence of related RNA silencing genes in *Paracoccidioides* SP. Accession numbers of homologous genes founded in *P. brasiliensis* (Pb18 and Pb03) and *P. lutzii* (Pb01) genomes, using BLASTX with predicted protein involved in the RNA machinery in *N. crassa* [14].

Predicted protein	<i>Paracoccidioides</i>			
	<i>Neurospora crassa</i>	Pb18	Pb03	Pb01
RNA-directed RNA polymerases	qde-1 (NCU07534.1)	PADG_07439.1	PABG_03975.1	PAAG_04185.1
	sad-1 (NCU02178.1)	PADG_03312.1	PABG_00852.1	PAAG_03813.1
	rrp-3 (NCU08435.1)	PADG_06286.1	PABG_06875.1	PAAG_03539.1
Argonaute-like, related to translation initiation factors	qde-2 (NCU04730.1)	PADG_03108.1	PABG_00673.1	PAAG_03231.1
Dicer-like, related to SFII-RNase III	Sms-2 (NCU09434.1)	PADG_00716.1	PABG_02302.1	PAAG_02052.1
Ribonucleases of the carpal factory	dcl-2 (NCU06766.1)	PADG_07189.1	PABG_05105.1	PAAG_00072.1
RecQ helicase-like, related to Bloom's and Werner syndrome helicases	Sms-3 (NCU08270.1)	PADG_05567.1	PABG_04917.1	PAAG_02421.1
	qde-3 (NCU08598.1)	PADG_03738.1	PABG_01669.1	PAAG_01087.1
	RecQ-2 (NCU03337.1)73	PADG_08306.1	PABG_07570.1:	PAAG_07608.1

the results obtained in this work using RNA interference strategy, we conclude that the non-stability in gene silencing is due to strategy and transformation methodology employed to down regulate specific gene. However, it will be important to explore an alternative gene silencing strategy such as RNAi, in order to diminish the time required for obtaining transformants with the desired phenotype, and achieve higher transformation efficiency. For the above reasons, and taking into account that RNAi machinery is present in the *Paracoccidioides* spp genomes, and that the strategy has been employed efficiently in *H. capsulatum*, we conclude that RNAi is an easier and faster tool than asRNA for gene silencing and has all potentialities for the study of many functional genes in *P. brasiliensis*. However further studies need to be conducted for the synthesis of new and more stable constructions based on RNAi technology. Presently, use of the transformation system mediated by *A. tumefaciens*, which could show best results in *P. brasiliensis* gene silencing [23-26].

This is the first report using electrotransformation of hairpin construction based on RNAi technology targeting two important *P. brasiliensis* antigens (gp43 and p27). A detailed analysis of the underlying molecular RNAi machinery may provide further insight into the intracellular mechanism that governs this reverse genetic tool. Present results provide an initial framework for further studies of virulence factors in fungi such as *P. brasiliensis*, in which the genetic manipulation represents a challenge.

5. ACKNOWLEDGEMENTS

This work was supported by Colciencias project No 221340820447. Support was also obtained from the Corporación para Investigaciones Biológicas (CIB) and the Universidad of Antioquia through the fund

“Sostenibilidad 2010-2011”. COLCIENCIAS National Doctoral Program supported Isaura Torres.

REFERENCES

- [1] Meister, G. and Tuschl, T. (2004) Mechanisms of gene silencing by double-stranded RNA. *Nature*, **431**, 343-349. doi:10.1038/nature02873
- [2] Appasani, K. (2005) RNA interference technology: From basic science to drug development. Cambridge University Press, New York, 1-13.
- [3] Sebghati, T.S., Engle, J.T. and Goldman, W.E. (2000) Intracellular parasitism by *Histoplasma capsulatum*: Fungal virulence and calcium dependence. *Science*, **290**, 1368-1372. doi:10.1126/science.290.5495.1368
- [4] Brandhorst, T.T., Wuthrich, M., Warner, T. and Klein, B. (1999) Targeted gene disruption reveals an adhesion indispensable for pathogenicity of *Blastomyces dermatitidis*. *The Journal of Experimental Medicine*, **189**, 1207-1216. doi:10.1084/jem.189.8.1207
- [5] Brummer, E., Castaneda, E. and Restrepo, A. (1993) Paracoccidioidomycosis: An update. *Clinical Microbiology Reviews*, **6**, 89-117.
- [6] Sturme, M.H., Puccia, R., Goldman, G.H. and Rodrigues, F. (2011) Molecular biology of the dimorphic fungi *Paracoccidioides* spp. *Fungal Biol Reviews*, **25**, 89-97. doi:10.1016/j.fbr.2011.04.002
- [7] Puccia, R., Schenkman, S., Gorin, P.A. and Travassos, L.R. (1986) Exocellular components of *Paracoccidioides brasiliensis*: Identification of a specific antigen. *Infection and Immunity*, **53**, 199-206.
- [8] McEwen, J.G., Ortiz, B.L., Garcia, A.M., Florez, A.M., Botero, S. and Restrepo, A. (1996) Molecular cloning, nucleotide sequencing, and characterization of a 27-kDa antigenic protein from *Paracoccidioides brasiliensis*. *Fungal Genetics and Biology*, **20**, 125-131.

[doi:10.1006/fgbi.1996.0027](https://doi.org/10.1006/fgbi.1996.0027)

- [9] De Camargo, Z., Unterkircher, C., Campoy, S.P. and Travassos, L.R. (1988) Production of *Paracoccidioides brasiliensis* exoantigens for immunodiffusion tests. *Journal of Clinical Microbiology*, **26**, 2147-2151.
- [10] Camargo, Z.P., Berzaghi, R., Amaral, C.C. and Silva, S.H. (2003) Simplified method for producing *Paracoccidioides brasiliensis* exoantigens for use in immunodiffusion tests. *Medical Mycology*, **41**, 539-542. [doi:10.1080/13693780310001615358](https://doi.org/10.1080/13693780310001615358)
- [11] Puccia, R. and Travassos, L.R. (1991) The 43-kDa glycoprotein from the human pathogen *Paracoccidioides brasiliensis* and its deglycosylated form: Excretion and susceptibility to proteolysis. *Archives of Biochem Biophys*, **289**, 298-302. [doi:10.1016/0003-9861\(91\)90475-X](https://doi.org/10.1016/0003-9861(91)90475-X)
- [12] Garcia Blanco, S., Munoz, J.F., Torres, I., Diez Posada, S., Gomez, B.L., McEwen, J.G., Restrepo, S. and Garcia, A.M. (2011) Differential Pbp27 expression in the yeast and mycelial forms of the *Paracoccidioides brasiliensis* species complex. *Fungal Genetics and Biology*, **48**, 1087-1095. [doi:10.1016/j.fgb.2011.09.001](https://doi.org/10.1016/j.fgb.2011.09.001)
- [13] Sambrook, J. and Russell, D.W. (2001) Molecular cloning: A laboratory manual. Harbor Laboratory Press, New York, Cold Spring.
- [14] Galagan, J.E., Calvo, S.E., Borkovich, K.A., Selker, E.U., Read, N.D., Jaffe, D., FitzHugh, W., Ma, L.J., Smirnov, S., Purcell, S., Rehman, B., Elkins, T., Engels, R., Wang, S., Nielsen, C.B., Butler, J., Endrizzi, M., Qui, D., Iankiev, P., Bell-Pedersen, D., Nelson, M.A., Werner-Washburne, M., Selitrennikoff, C.P., Kinsey, J.A., Braun, E.L., Zelter, A., Schulte, U., Kothe, G.O., Jedd, G., Mewes, W., Staben, C., Marcotte, E., Greenberg, D., Roy, A., Foley, K., Naylor, J., Stange-Thomann, N., Barrett, R., Gnerre, S., Kamal, M., Kamvysselis, M., Mauceli, E., Bielke, C., Rudd, S., Frishman, D., Krystofova, S., Rasnussen, C., Metzner, R.L., Perkins, D.D., Kroken, S., Cogoni, C., Macino, G., Catcheside, D., Li, W., Pratt, R.J., Osmani, S.A., DeSouza, C.P., Glass, L., Orbach, M.J., Berglund, J.A., Voelker, R., Yarden, O., Plamann, M., Seiler, S., Dunlap, J., Radford, A., Aramayo, R., Natvig, D.O., Alex, L.A., Mannhaupt, G., Ebbole, D.J., Freitag, M., Paulsen, I., Sachs, M.S., Lander, E.S., Nusbaum, C. and Birren, B. (2003) The genome sequence of the filamentous fungus *Neurospora crassa*. *Nature*, **422**, 859-868. [doi:10.1038/nature01554](https://doi.org/10.1038/nature01554)
- [15] Finn, R.D., Clements, J. and Eddy, S.R. (2011) HMMER web server: Interactive sequence similarity searching. *Nucleic Acids Research*, **39**, W29-W37. [doi:10.1093/nar/gkr367](https://doi.org/10.1093/nar/gkr367)
- [16] Woods, J.P. and Goldman, W.E. (1993) Autonomous replication of foreign DNA in *Histoplasma capsulatum*: Role of native telomeric sequences. *Journal of Bacteriology*, **175**, 636-641.
- [17] Rappleye, C.A., Engle, J.T., and Goldman W.E. (2004) RNA interference in *Histoplasma capsulatum* demonstrates a role for alpha-(1,3)-glucan in virulence. *Molecular Microbiology*, **53**, 153-165. [doi:10.1111/j.1365-2958.2004.04131.x](https://doi.org/10.1111/j.1365-2958.2004.04131.x)
- [18] Zhang, Y., Li, G., He, D., Yu, B., Yokoyama, K. and Wang L. (2011) Efficient insertional mutagenesis system for the dimorphic pathogenic fungus *Sporothrix schenckii* using *Agrobacterium tumefaciens*. *Journal of Microbiological Methods*, **84**, 418-422. [doi:10.1016/j.mimet.2011.01.017](https://doi.org/10.1016/j.mimet.2011.01.017)
- [19] vanBurik, J.A., Schreckhise, R.W., White, T.C., Bowden, R.A. and Myerson D. (1998) Comparison of six extraction techniques for isolation of DNA from filamentous fungi. *Medical Mycology*, **36**, 299-303. [doi:10.1080/02681219880000471](https://doi.org/10.1080/02681219880000471)
- [20] Goldman, G.H., dos Reis Marques, E., Duarte Ribeiro, D.C., de Souza Bernardes, L.A., Quiapin, A.C., Vitorelli, P.M., Savoldi, M., Semighini, C.P., de Oliveira, R.C., Nunes, L.R., Travassos, L.R., Puccia, R., Batista, W.L., Ferreira, L.E., Moreira, J.C., Bogossian, A.P., Tekaia, F., Nobrega, M.P., Nobrega, F.G. and Goldman M.H. (2003) Expressed sequence tag analysis of the human pathogen *Paracoccidioides brasiliensis* yeast phase: Identification of putative homologues of *Candida albicans* virulence and pathogenicity genes. *Eukaryotical Cell*, **2**, 34-48. [doi:10.1128/EC.2.1.34-48.2003](https://doi.org/10.1128/EC.2.1.34-48.2003)
- [21] Livak, K.J. and Schmittgen T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*, **25**, 402-408. [doi:10.1006/meth.2001.1262](https://doi.org/10.1006/meth.2001.1262)
- [22] Desjardins, C.A., Champion, M.D., Holder, J.W., Muszewska, A., Goldberg, J., Bailao, A.M., Brigido, M.M., Ferreira, M.E., Garcia, A.M., Grynberg, M., Gujja, S., Heiman, D.I., Henn, M.R., Kodira, C.D., Leon-Narvaez, H., Longo, L.V., Ma, L.J., Malavazi, I., Matsuo, A.L., Morais, F.V., Pereira, M., Rodriguez-Brito, S., Sakthikumar, S., Salem-Izacc, S.M., Sykes, S.M., Teixeira, M.M., Vallejo, M.C., Walter, M.E., Yandava, C., Young, S., Zeng, Q., Zucker, J., Felipe, M.S., Goldman, G.H., Haas, B.J., McEwen, J.G., Nino-Vega, G., Puccia, R., San-Blas, G., Soares, C.M., Birren, B.W. and Cuomo C.A. (2011) Comparative genomic analysis of human fungal pathogens causing paracoccidioidomycosis. *PLoS Genet*, **7**, e1002345. [doi:10.1371/journal.pgen.1002345](https://doi.org/10.1371/journal.pgen.1002345)
- [23] Almeida, A.J., Cunha, C., Carmona, J.A., Sampaio-Marques, B., Carvalho, A., Malavazi, I., Steensma, H.Y., Johnson, D.I., Leao, C., Logarinho, E., Goldman, G.H., Castro, A.G., Ludovico, P. and Rodrigues, F. (2009) Cdc42p controls yeast-cell shape and virulence of *Paracoccidioides brasiliensis*. *Fungal Genetics and Biology*, **46**, 919-926. [doi:10.1016/j.fgb.2009.08.004](https://doi.org/10.1016/j.fgb.2009.08.004)
- [24] Hernandez, O., Almeida, A.J., Gonzalez, A., Garcia, A.M., Tamayo, D., Cano, L.E. Restrepo, A. and McEwen, J.G. (2010) A 32-kilodalton hydrolase plays an important role in *Paracoccidioides brasiliensis* adherence to host cells and influences pathogenicity. *Infection and Immunity*, **78**, 5280- 5286. [doi:10.1128/IAI.00692-10](https://doi.org/10.1128/IAI.00692-10)
- [25] Ruiz, O.H., Gonzalez, A., Almeida, A.J., Tamayo, D., Garcia, A.M., Restrepo, A. and McEwen, J.G. (2011) Alternative oxidase mediates pathogen resistance in *Paracoccidioides brasiliensis* infection. *PLOS Neglected Tropical Diseases*, **5**, e1353. [doi:10.1371/journal.pntd.0001353](https://doi.org/10.1371/journal.pntd.0001353)
- [26] Tamayo, D., Munoz, J.F., Torres, I., Almeida, A.J., Restrepo, A., McEwen, J.G. and Hernandez, O. (2013) Involvement of the 90 kDa heat shock protein during adaptation

tion of *Paracoccidioides brasiliensis* to different environmental conditions. *Fungal Genetics and Biology*, **51**,

34-41. doi:10.1016/j.fgb.2012.11.005

APPENDIX

Supplementary Digital Content S1

Table S1. Primers used to construct the RNAi cassettes targeting *P. brasiliensis* PbGP43 and PbP27.

Target gene (hairpin size)	Primer name	Primer Sequence 5'-----3'
PbGP43 E1 (457-bp)	PbGP43E1-AscI-F	AGGCGCGCCTTTAGTTCTCTCAACCTGGC
	PbGP43E1-XhoI-R	GTCTCGAG CACCATGGAGATCGATGACG
	PbGP43E1-XbaI-F	CGTCTAGATTTAGTTCTCTCAACCTGGC
	PbGP43E1-AgeI-R	GTACCGGTCACCATGGAGATCGATGACG
PbGP43 E2 (788-bp)	PbGP43E2-AscI-F	AGGCGCGCCTCCCGGGTTCTCAAAAACGGC
	PbGP43E2-XhoI-R	GTCTCGAGTCACCTGCATCCACCATACT
	PbGP43E2-XbaI-F	CGTCTAGATCCCGGGTTCTCAAAAACGGC
	PbGP43E2-AgeI-R	GTACCGGTTACCTGCATCCACCATACT
PbP27 (500-pb)	PbP27-AscI-F	AGGCGCGCCTCCGACGAGCTGAAAACTG
	PbP27-XhoI-R	GTCTCGAGACAGCGTGCATTTGATCTT
	PbP27-XbaI-F	CGTCTAGATCCGACGAGCTGAAAACTG
	PbP27-AgeI-R	GTACCGGTATGATATCACGAATATCGGT

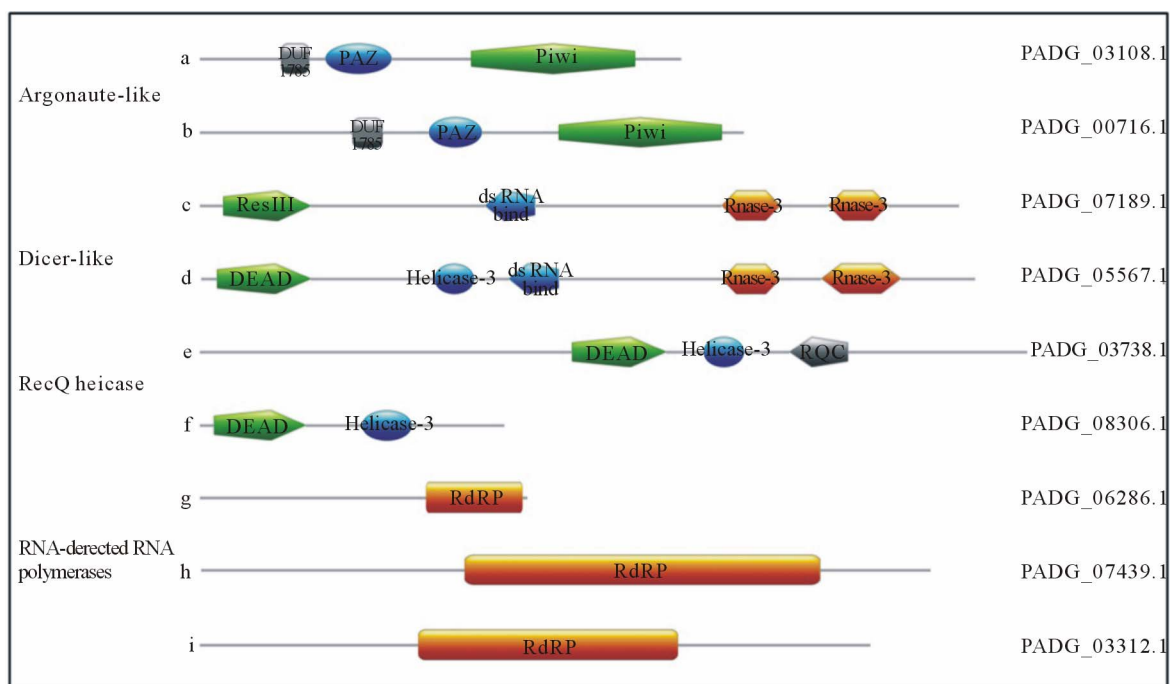


Figure S1. RNAi silencing in *Paracoccidioides* spp.: Key protein domains. Nine genes identified in the *P. brasiliensis* genome (strain Pb18) using BLAST showing the presence of protein domains involved in the RNAi silencing. Argonaute-like proteins (a,b) showing the PIWI (green) and PAZ (blue) domains. Dicer-like proteins (c,d) presenting the Ribonuclease III (orange) and double stranded RNA binding (blue pentagon) domains. Helicase conserved C terminal domain (blue circle); in d, e and f, DEAD/DEAH box helicase domain (green pentagon); in the Helicase-like protein (e,f) and the RNA dependent RNA polymerase domains (orange) in g, h, and i.