The PAC-3 transcription factor critically regulates phenotype-associated genes in *Neurospora crassa*

Maira Pompeu Martins¹, Nilce Maria Martinez-Rossi¹, Pablo Rodrigo Sanches¹ and Antonio Rossi¹

¹Universidade de São Paulo, Faculdade de Medicina de Ribeirão Preto, Departamento de Genética, Ribeirão Preto, SP, Brazil

Abstract

Transcription factors play an important role in fungal environmental adaptive process by promoting adjustment to challenging stimuli via gene modulation and activation of signaling networks. The transcription factor encoded by the *pac-3/rim101/pacC* gene is involved in pH regulation and is associated with a wide variety of cellular functions. The deletion of *pac-3* affects fungal development. In *Neurospora crassa*, the Δ*pac-3* strain presents diminished aerial growth and reduced conidiation. However, the PAC-3-regulated genes associated with this altered phenotype have not been elucidated. In this study, we used RNA-seq to analyze the phenotypic plasticity induced after *pac-3* deletion in the filamentous fungus *N. crassa* cultivated in media supplemented with sufficient or limited inorganic phosphate. Genes related to morphology, hyphal development, and conidiation were of particular interest in this study. Our results suggest a *pac-3* dependency in gene regulation in a Pi-dependent manner. Furthermore, our analysis suggested that the fungus attempts to overcome the deletion effects in a Δ*pac-3* mutant through a complex combined regulatory mechanism. Finally, the modulatory responses observed in the Δ*pac-3* strain, a double mutant generated based on the Δ*mus-52* mutant strain, is strain-specific, highlighting that the phenotypic impact may be attributed to *pac-3* absence despite the combined *mus-52* deletion.

Keywords: RNA-seq, conidiation, hyphal development, transcription factor, inorganic phosphate.

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Introduction

To survive and proliferate, fungi must interact with and sense changes in their environment (Trevisan et al., 2011). The adaptive success in different niches is resultant of the ability to scavenge for nutrients and respond to several challenging factors such as extreme temperature, carbon source, and pH changes (Han et al., 1987; Bahn et al., 2007; Martinez-Rossi et al., 2017). Fungal sensing of the environment leads to the activation of intracellular signaling pathways, which are mediated by transcription factors. This network results in stress-associated transcriptional patterns which support the adaptation to specific stimuli (Braunsdorf et al., 2016; Simaan et al., 2019). The pH fitness of fungi is directly mediated by a C2H2 zinc finger transcription factor encoded by the *pac-3/rim101/pacC* gene, which is activated through the highly conserved Pal/Rim signaling pathway (Trevisan et al., 2011; Rossi et al., 2013). The pH signaling cascade activates gene regulation in response to acidic to alkaline pH shifts, which can extensively alter metabolic events (Rossi et al., 2013).

Using mutant strains carrying the Δ*pac-3* (or Δ*rim101/pacC*) revealed that the PAC-3 transcription factor correlates with fungal traits beyond pH signaling, impacting fundamental biological processes, including cell morphology, hyphal growth, conidiation, and adaptation to the host or nutritional variances (Ferreira-Nozawa et al., 2006; Mendes et al., 2012; O’Meara et al., 2014; Martins et al., 2018, 2019; Rascle et al., 2018). In *Neurospora crassa*, the deletion of *pac-3* and the *pal* cascade-associated genes, except for Δ*pal-9/pall*, diminished aerial growth, reduced conidiation, and resulted in high production of melanin, in comparison with that in the wild-type strain (Virgilio et al., 2016). A decrease in conidiation was also observed in the *Trichophyton interdigitale* H6 *pacC* mutant (Ferreira-Nozawa et al., 2006). Additionally, the absence of *pacC* led to low conidiation in *Botrytis cinerea* (Rascle et al., 2018), *Magnaporthe oryzae* (Landraud et al., 2013), and *Aspergillus nidulans* (Tilburn et al., 1995). Thus, there is a strong connection between the PAC-3 transcription factor and fungal development. However, the regulatory effect resultant of PAC-3 deletion on genes responsible for fungal development remains unexplored.

The expression of *pac-3/rim101/pacC* is modulated in response to environmental conditions, including variations in inorganic phosphate (Pi), carbon sources, and pH fluctua-
tions, which may impair critical physiological functions (Ferreira-Nozawa et al., 2006; Trevisan et al., 2011; Mendes et al., 2012; Martins et al., 2019). In this study, we used RNA-seq analysis to assess the genes related to morphogenesis and development of the N. crassa mutant Δpac-3 cultivated in media containing sufficient or limited Pi, an essential constituent of biomolecules (Gras et al., 2013). The purpose was to obtain evidence for transcriptional contribution to the observed phenotypical patterns. Pi is involved in diverse metabolic pathways and thus functions as a growth-limiting factor in microorganisms (Dick et al., 2011; Vicent et al., 2015). To determine whether pac-3-regulated genes are involved in the N. crassa phenotype, we studied the transcriptional impact that resulted from both the deletion and nutrient adaptive response simultaneously, as well as the changes that occurred exclusively due to the gene deletion independently of the nutritional condition. The results obtained provide evidence of the role of pac-3-mediated regulation in N. crassa growth and development, and indicate the genes possibly associated with the phenotypical effects observed in the Δpac-3 mutant.

Material and Methods

Culture conditions of N. crassa knockout strains

N. crassa mus-52Δkoi (FGSC#9568) parental and Δpac-3 (NCU00090) knockout strains (Cupertino et al., 2012) were maintained on solid Vogel’s Minimal (VM) medium, pH 5.8 (Vogel, 1956) containing 2% sucrose at 30 °C. Approximately 10^7 cells/mL−1 conidia were germinated in an orbital shaker for 5 h at 30 °C (200 rpm), as previously described (Martins et al., 2019), in low- and high-Pi media (final concentrations, 10 μM or 10 mM Pi, respectively). The media was supplemented with 44 mM sucrose as the carbon source and adjusted to pH 5.4 with 50 mM sodium citrate (Nyc et al., 1966; Gras et al., 2007). The resulting mycelia was collected by filtering through 0.22-μm size filters (Milipore Corp., USA), frozen in liquid nitrogen, and stored at -80 °C until RNA extraction. Experiments were performed in three biological replicates.

RNA extraction, sequencing, data analysis, and functional enrichment

Total RNA was isolated using TRIZol Reagent (Invitrogen, USA) according to the manufacturer’s instructions and treated with DNase I, RNase-free (Thermo Fisher, USA). The RNA concentration was quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher). The RNA integrity was determined by agarose formaldehyde gel electrophoresis and using the Agilent Bioanalyzer platform 2100 (Agilent, USA). Total RNA was reverse transcribed into cDNA using oligo(dT) primers and Superscript II (Invitrogen). The cDNA was used as the template for amplification using the pair of primers GCCARG-3' (Tilburn et al., 1995), in the 5' upstream regions of the PAC-3 motifs, and NCU08351 and NCU01064, and two Ca^2+-ATPases (E1-E2 ATPase-1, NCU05046; and calcium-transporting ATPase3, NCU07966) were repressed in both Pi conditions. Among the genes that were upregulated in response to pac-3 deletion.

Selection of morphology- and development-related genes

After functional annotation analysis with the Blast2GO tool, the genes identified in the RNA-seq data were filtered with a customized R script, mapping modulated Genes and Gene Ontology (GO) terms. The script detected the descendant terms of the Gene Ontology nodes “cell wall,” “developmental process,” “cellular developmental process,” “regulation of biological process,” and “anatomical structure development” with the Bioconductor GO.db package (Carlson, 2018). We further identified morphology and development-related genes using literature data. The identified genes are listed in Table 1.

In silico evaluation of the putative PAC-3-binding sites

The N. crassa OR74A genome (Ensembl Fungi) was used to search for the occurrence of the PAC-3 motif, 5'-GCCARG-3' (Tilburn et al., 1995), in the 5' upstream regions (1 kb) of each gene (Table S1). The pursuit was determined using an ad hoc Perl script (Martins et al., 2019).

Results

Global DEG identification and selection of genes associated with morphogenesis and development in the RNA-seq libraries

To perform a comprehensive analysis of the impact of Δpac-3 knockout on gene modulation, and to determine the effects of Pi variation on that response, we evaluated the results obtained by high-throughput sequencing (RNA-seq) using the N. crassa mus-52Δkoi background strain as the control. By applying a cut-off threshold of at least 2.8-fold difference and a statistical significance threshold of P<0.05, 427 genes were identified as differentially modulated in response to both of the analyzed Pi conditions (Martins et al., 2019). The results identified 55 genes that are associated with morphogenesis and development (Table 1). A heatmap of gene expression of the identified genes is shown in Figure 1. MultiExperiment Viewer (MeV) was used for hierarchical clustering by average linkage clustering based on Pearson correlation.

Gene modulation in response to pac-3 deletion

Among the identified genes that associated with the N. crassa developmental progress, catalase-3 (NCU00355), ornithine-N5-oxygenase (NCU07117), 1,3-beta-glucanosyltransferase gell (NCU07253), two hypothetical proteins (NCU08351 and NCU01064), and two Ca^2+-ATPases (E1-E2 ATPase-1, NCU05046; and calcium-transporting ATPase3, NCU07966) were repressed in both Pi conditions. Among the genes that were upregulated in response to pac-3 deletion...
deletion, regardless of the Pi condition, we identified CipC protein (NCU04197), GAL10 (NCU04442), GTPase Ras2p (NCU06111), and the anchored cell wall protein-12 (NCU08171).

Gene modulation in response to Pi variation

The cell wall protein PhiA (NCU00399), fluffy (NCU08726), three hypothetical proteins identified as NCU09629, NCU04493, and NCU04605, a non-anchored cell wall protein-6 (NCU00586), and the galactose oxidase (NCU09209) were identified among the Pi-dependent genes.

Discussion

Deciphering the regulatory mechanisms underlying the developmental programs in fungi can contribute to the elucidation of events that are unique and essential to these
microorganisms. In this study, we aimed to identify the pac-3-regulated genes involved in N. crassa development in a transcriptional scenario, and thus contribute to the molecular understanding of the phenotype observed in the Δpac-3 mutant. Reduced growth and low conidiation were not observed in the parental background strain pacman150 4 (pac-3). Although pac-3 deletion drastically affected the N. crassa phenotype, only a small number of genes associated with cell morphology, or hyphal and conidial development, were modulated in the evaluated conditions of Pi variance (Table 1). The phenotypic effect in the Δpac-3 strain may be the result of a more complex combined regulatory mechanism. Among the 55 genes that were identified, 31 possessed a binding motif for PAC-3 in their promoter regions (Table S1). The deletion of pac-3 resulted in the differential modulation of 12 additional transcription factors, seven of which contained the PAC-3 binding motif.

N. crassa gene downregulation is reflected in its phenotype

The transcriptional response that resulted explicitly from the absence of PAC-3, with Pi conditions being not a determinant of gene modulation, involved the downregulation of two catalase genes. Catalase-3 gene deletion provoked enhanced conidial production, hyphal adhesion, and more aerial hyphae in N. crassa (Michan et al., 2003; Sun et al., 2012), whereas catalase-1 deletion increased germination rate in Metarhizium anisopliae (Morales Hernandez et al., 2010). Since catalase activity is protective against cellular component damage, the gene repression observed in this study may have led to oxidative stress toxic effects, which would impair N. crassa development.

Our results also identified that the ornithine-N5-oxgenase gene is downregulated. The encoded enzyme catalyzes the first step in the microbial-exclusive hydroxamate siderophore biosynthesis system (Eisendle et al., 2003; Hisen et al., 2005). Disruption of orthologue genes in A. nidulans and Nomuraea rileyi correlated the activity of the siderophore biosynthesis to diminished conidiation and defective hyphal elongation (Eisendle et al., 2003; Li et al., 2016).

Figure 1 - Heatmap of morphology and development regulation-associated genes from N. crassa mutants. The hierarchical clustering of expression patterns for the 55 differentially expressed genes identified in the Δpac-3 Δmus-52 mutant strain versus N. crassa wild-type strain. Expression levels were loaded into the MultiExperiment Viewer (MeV) and analyzed using average-linkage hierarchical clustering with a Pearson correlation coefficient distance metric. The intensity of color represents the value of upregulation (red) or downregulation (green) in log2FC.
Δpac-3-mutant attempts to restore developmental deregulation

We identified that CipC (from concanamycin-induced protein) protein was highly upregulated in both Pi conditions. This protein, exclusively found in fungi (Asif et al., 2006), was associated with filamentous growth in Ustilago maydis (Rodriguez-Kessler et al., 2012), and with hyphal development and conidial surface interactions in the pathogenic mold Aspergillus fumiatus (Asif et al., 2006; Bauer et al., 2010). In A. nidulans, the upregulation of CipC protein in a mutant strain deficient in glucosidase I could be one of the factors that contributed to the hyperbranching, resultant of its activity in polarizing growth (Zhang et al., 2009).

The UDP-galactose-4-epimerase (GAL10), which was also upregulated, codes for an enzyme of the galactose metabolism. Its activity is associated with cell-wall integrity, morphology, and induces excessive filamentation in C. albicans (Singh et al., 2007), and results in highly branched hyphae and reduced conidiation in A. nidulans (El-Gammy et al., 2010). We also identified the induction of the GTPase Ras2p, which codes for GTPase signal transducer proteins and acts as a regulator of growth and development (Fortwendel, 2015). In N. crassa, this protein is reported to be involved in the regulation of cell morphology, which affects the apical growth of hyphae and conidium formation (Kanauchi et al., 1997).

Proteomic analysis identified proteins that are secreted by the vegetative hyphae of N. crassa and expressed in a cell-type-specific manner (Maddi et al., 2009). One such protein, anchored cell wall protein-12, is implicated in cell wall remodeling and in the growth of the hyphae (Potapova, 2014). Our results revealed that the gene coding for this protein is induced in N. crassa in both Pi conditions. Our findings identified the overexpression of some developmental genes in the mutant strain, which indicates a possible attempt of the fungi to compensate for pac-3 deletion.

Δpac-3-mutation is responsive to Pi variation

Among the genes presenting the pac-3 binding motif, upon induction, the up-modulation occurred in high-Pi as a pattern. When repressed, with four exceptions (NCU02235, NCU01931, NCU00399, and NCU02167), the downregulation occurred in low Pi. Thus, our results suggest a pac-3 dependency in gene regulation in a Pi-dependent manner.

The cell wall protein PhiA, an essential gene for conidia development and healthy growth in A. nidulans (Melin et al., 2003), is repressed exclusively in high Pi conditions, suggesting that the Pi restriction directly impacts gene modulation. The Fluffy gene is induced in response to sufficient Pi. This gene codes for a C6 zinc finger transcription factor active in the regulation of two out of the five genes that act as specific regulators of conidiation in N. crassa (Mendes et al., 2016). Fluffy is expressed at a basal level in vegetative hyphae, and is transcriptionally activated in the aerial hyphae formation (Bailey and Ebbole, 1998; Rerngsamran et al., 2005; Mendes et al., 2016). The expression pattern of PhiA and Fluffy genes in high Pi suggests that Δpac-3 strain shows an attempt to restore conidiation by activating genes other than PhiA in the herein evaluated conditions.

Nineteen conidia-specific cell wall proteins were identified using a proteomic approach with N. crassa (Ao et al., 2016). The promoter regions of each coding gene were analyzed to determine whether they were expressed in a conidia-specific manner (Ao et al., 2016). Among these genes, four were modulated in our results. Two of them, identified as non-anchored cell wall protein-6 (NCU00586) and the hypothetical protein NCU04605 were downregulated in response to low-Pi. The other two, hypothetical protein NCU04493 and galactose oxidase (NCU09209), were induced in high-Pi. The two upregulated genes identified had conidiation-specific expression using the promoter approach, and the two downregulated genes, did not give a conidia-specific developmental expression pattern. These complementary results support the feasible role of the transcription factor pac-3 in conidia-related regulation in high Pi.

The hypothetical protein NCU09629 presented with inverted modulation in Pi conditions. In limited Pi availability, this gene is repressed, and under sufficient Pi, it is induced. This gene shows the HET (for heterokaryon incompatibility [HI]) domain, a regulator of HI, and is associated with severe growth inhibition and negatively affects conidiation and hyphal compartmentation that leads to programmed cell death (Demethon et al., 2006).

Genetic interactions render unexpected phenotype

The strain under analysis is a double mutant (Δpac-3Δmus-52). As described for negative genetic interactions (Mani et al., 2008), the resultant phenotype was more substantial than expected. These genetic interactions frequently involve genes presenting leastwise partially overlapping functions, which may compensate for the deletion mutually (Hartman et al., 2001). Growth-based gene interaction profiling is reasonable to subdivide the observed negative to positive interactions, although expression-based genetic interaction profiling provides a more specific understanding of the genetic interaction patterns (Amini et al., 2019).

A heatmap that depicts the relative expression levels of the growth and development-associated genes identified in the Δpac-3Δmus-52 mutant strain compared to the expression in the corresponding single mutant Δmus-52 is shown in Figure 1. As a pattern, the modulatory response observed in the single mutant was not sustained in the double mutant strain. Two profiles could be highlighted: in one of them, the modulatory pattern was strain-specific, as observed in the induced cluster including the gene identified as CipC protein (NCU04197) and the hypothetical protein NCU00282, or in the repressed cluster including the calcium-transporting ATPase 3 (NCU07966) and Krev-1-like (NCU02167) genes. In the second explicit expression profiling, the pattern skipped between high to low Pi in the different strains, as observed in a cluster, including the hypothetical protein NCU09629 and catalase-1 (NCU08791). In only a few genes, the expression pattern was maintained in both conditions and strains as for the pyridoxine kinase (NCU10351).
Our results provide evidence of the role of the double mutant-mediated regulation in *N. crassa*. In the same proportion that *mus-52* deletion incurs underestimated consequences to the organism (Martins *et al.*, 2018), the associated *pac-3* deletion reflects a pervasive and profound effect in *N. crassa* development, bringing relevant insights regarding biological networks.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

MPM conducted the experiments, analyzed the data, and drafted the manuscript. PRS performed computational and statistical analyses. AR and NMM-R designed the project, supervised the research, and prepared the manuscript. All authors read and approved the final version.

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Internet Resources
Carlson M (2018) GO.db: A set of annotation maps describing the entire Gene Ontology. R Package Version 3.7.0. Ensembl Fungi, https://fungi.ensembl.org/index.html.
Supplementary Material

The following online material is available for this article:

Table S1 - Genes of *N. crassa* modulated in response to mutant strain ΔpacC (test) compared with the control strain (∆mus-52) in medium containing low-Pi or high-Pi.

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