Neurospora crassa developmental control mediated by the FLB-3 transcription factor

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ARTICLE INFO

Article history:

Received 25 October 2017

Received in revised form

16 January 2018

Accepted 22 January 2018

Available online 1 March 2018

Corresponding Editor: Drauzio E.N. Rangel

Keywords:

Development control

FLB-3

Neurospora crassa

Protein-binding microarrays (PBM)

ABSTRACT

Here, we report that the *Neurospora crassa* FLB-3 protein, the ortholog of the *Aspergillus nidulans* FlbC transcription factor, is required for developmental control. Deletion of *flb-3* leads to changes in hyphae morphology and affects sexual and asexual development. We identified, as putative FLB-3 targets, the *N. crassa* *aba-1*, *wet-1* and *vos-1* genes, orthologs of the ones involved in *A. nidulans* asexual development and that work downstream of FlbC (*abaA*, *wetA* and *vosA*). In *N. crassa*, these three genes require FLB-3 for proper expression; however, they appear not to be required for normal development, as demonstrated by gene expression analyses during vegetative growth and asexual development. Moreover, mutant strains in the three genes conidiate well and produce viable conidia. We also determined FLB-3 DNA-binding preferences via protein-binding microarrays (PBMs) and demonstrated by chromatin immunoprecipitation (ChIP) that FLB-3 binds the *aba-1*, *wet-1* and *vos-1* promoters. Our data support an important role for FLB-3 in *N. crassa* development and highlight differences between the regulatory pathways controlled by this transcription factor in different fungal species.

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1. Introduction

The FlbC transcription factor was first described in *Aspergillus nidulans*, together with five other developmental activators of asexual spore formation (FluG, FlbA, FlbB, FlbD and FlbE) (Wieser et al., 1994; Adams et al., 1998). Mutation in any of these genes results in undifferentiated masses of vegetative hyphae that lead to the formation of cotton-like colonies described as “fluffy”. FlbC has two C-terminal C₂H₂ zinc finger DNA binding-domains and, in

A. nidulans, is described to regulate development by activating the first gene of the conidiation-specific regulatory pathway, *brlA*, and by inhibiting vegetative growth (Kwon et al., 2010; Park and Yu, 2012). The *brlA* gene encodes a transcription factor that is necessary for development (Adams et al., 1988), activating a cascade of genes that characterize the so-called central regulatory pathway, required for conidiation (Park and Yu, 2012). In *A. nidulans*, the central regulators of conidiation include the *abaA* gene, a transcription factor necessary for normal conidiophore development and conidia formation (Adrianopoulos and Timberlake, 1994) and *wetA*, which is necessary for the synthesis of cell wall layers required for conidia maturation and impermeabilization (Marshall and Timberlake, 1991). The VosA and VelB regulators have more

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recently been shown to be connected to the central regulatory genes, acting as negative regulators of conidiation by exerting negative regulation over *brlA* (Park and Yu, 2012; Ni and Yu, 2007; Park et al., 2012). In addition to its aforementioned role in development, *VosA* has also been described to control trehalose biogenesis, thus coupling trehalose biosynthesis and conidia maturation (Ni and Yu, 2007).

FlbC orthologous have been characterized in only a few fungi. The *fle1* gene encodes the FlbC ortholog in *Podospira anserina* (FLE1), which acts as a repressor of female sexual differentiation (Coppin, 2002). More recently, FlbC orthologs have been characterized in *Fusarium verticillioides* (Ada1) and in *Penicillium oxalicum* (PoFlbC). The Δ *ada-1* mutant strain displays reduced production of conidia and defects in conidia and conidiophore morphology (Malapi-Wight et al., 2014). In *P. oxalicum*, PoFlbC is required for normal growth and asexual development, and gene deletion results in reduced production of cellulases, hemicellulases, and proteins related to lignocellulose degradation (Yao et al., 2016). In *Neurospora crassa*, the *A. nidulans* FlbC orthologous protein [formerly known as *acon-4* (Chung, 2011) and herein termed *flb-3*], encoded by the ORF NCU03043, was identified as a putative transcription factor involved in the control of glycogen metabolism. The Δ *flb-3* strain exhibits higher levels of glycogen and higher expression of the gene encoding glycogen synthase than the wild-type strain under heat stress (Gonçalves et al., 2011).

Although the FLB-3 transcription factor has been described as required for proper regulation of glycogen metabolism (Gonçalves et al., 2011), no reports have evaluated whether it plays any role in development in *N. crassa*. Conidiation in *N. crassa* involves a sequence of events that leads to the production of multinucleated macroconidia, uninucleated microconidia and arthroconidia (Springer and Yanofsky, 1989). Under specific environmental conditions, aerial hyphae grow and branch to form an aerial mass, from which conidiophores develop (Springer and Yanofsky, 1989). Many genes are activated in this process, each one participating at a specific stage. Light promotes this process through the activation of conidiation-specific genes, while the heterotrimeric G protein signaling pathway also plays a role in the regulation of conidiation (Kays and Borkovich, 2004; Park and Yu, 2012). In a screen for genes differently expressed during asexual development, a large number of genes were identified (Greenwald et al., 2010), within which there were many encoding transcription factors, including *flb-3* (NCU03043), suggesting that they may play a role in conidiation.

In this work, we demonstrate that FLB-3 is a sequence-specific DNA-binding protein required for proper vegetative growth and sexual and asexual development in *N. crassa*. We identify the orthologs of *A. nidulans* developmental regulators *abaA* and *wetA* (termed *aba-1* and *wet-1*, respectively), but not the *brlA* ortholog. We further show that both genes, together with *vos-1*, are regulated by FLB-3 during vegetative growth and asexual development in *N. crassa*. In addition, we provide evidences for cross regulation between *aba-1*, *wet-1* and *vos-1* genes, which differs from the one described previously in *A. nidulans*. Interestingly, and contrary to that reported in *A. nidulans*, these genes appear not to be required for development in *N. crassa*. This work supports an important role for FLB-3 in *N. crassa* development and highlights differences between the regulatory pathways of conserved processes among different fungal species.

2. Materials and methods

2.1. *N. crassa* strains and growth conditions

The *N. crassa* wild-type (FGSC#2489), Δ *flb-3* (FGSC#11355, NCU03043), Δ *vos-1* (FGSC#13536, NCU05964), Δ *wet-1* (FGSC#11204,

NCU01033), and Δ *aba-1* (FGSC#15901, NCU02612) strains were purchased from the Fungal Genetics Stock Center (FGSC) (McCluskey, 2003). All strains were maintained on solid Vogel's minimal (VM) medium (Vogel, 1956), pH 5.8 containing 2 % sucrose. To induce protoperithecia formation, the wild-type and Δ *flb-3* strains were grown on 0.1 $\times$ SC medium (Westergaard and Mitchell, 1947) containing 0.5 % sucrose. For microconidiation assay, the same medium was used, but supplemented with 1 mM sodium iodoacetate (Ebbole and Sachs, 1990). Conidia from 10-d cultures were collected, suspended in sterile water and counted in a Neubauer chamber. Microconidia were isolated from 10 to 15-d cultures after filtration using Millex SV (5 μ m) filters.

To induce asexual development, 10^6 conidia ml⁻¹ from the wild-type strain or a conidia suspension (from the Δ *flb-3* strain) were inoculated into liquid VM medium (125 ml) plus 2 % sucrose and grown at 30 °C and 200 rpm for 20 h (control, time 0 h). After this, mycelia were filtered in order to have a thin layer. Mycelial discs were cut and placed onto the surface of a petri dish containing solid VM medium plus 2 % sucrose, over a cellophane membrane, and exposed to constant light at 25 °C for different times (6, 9, 12, and 24 h) in a Percival Scientific incubator. All samples were frozen in liquid nitrogen and stored at -80 °C for RNA extraction.

For microscopy analysis, 10^7 microconidia ml⁻¹ from wild-type and a conidial suspension from the Δ *flb-3* strain were germinated for different times within plates containing coverslips immersed in liquid VM medium plus 2 % sucrose. For morphological hyphae analysis, mycelia were stained with 100 μ L of calcofluor white (3.7 μ g ml⁻¹ final concentration) for 15 min and washed twice with phosphate-buffered saline (PBS). Fluorescence was visualized using a fluorescence microscope with excitation and emission wavelengths of 359 nm and 461 nm, respectively. For nuclei analysis, mycelia were fixed [3.7 % formaldehyde, 50 mM NaH₂PO₄, pH 7.0, 0.2 % (v/v) tween 80], washed twice with PBS, stained with 100 μ L DAPI (0.5 mg ml⁻¹) for 5 min and visualized as described above. In both cases, hyphae were examined in an AXIO Imager.A2 Zeiss microscope. All images were captured with the AxioCam MRm camera and processed using the AxioVision software, version 4.8.2. For apical hyphae analysis, 10^7 conidia ml⁻¹ were inoculated onto plates containing solid VM medium for 20 h. Images of colony morphology were captured with an AxioCam ICc3 camera coupled to the stereoscope trinocular Discovery V8 (Zeiss) at 50 $\times$ magnification.

For vegetative growth experiments, 10^7 conidia ml⁻¹ from wild-type strain or hyphae and conidia homogenates from the Δ *flb-3* strain were inoculated into VM medium (125 ml) plus 2 % sucrose at 30 °C, 200 rpm, and mycelia pads were collected every 12 h. To count microconidia, 25 μ L of suspension with 10^7 conidia from wild-type, the FLB-3 overexpressing strain and the mutant strain were inoculated onto Erlenmeyer flasks containing solid VM medium plus 2 % agar and 2 % sucrose at pH 5.8 for 9 d. Conidia were collected and suspended in 30 ml of sterile water, and 250 μ L from this conidia suspension were filtrated using Millex SV (5 μ m) filters and counted. Experiments were repeated four times.

2.2. RNA isolation and gene expression analysis

Total RNA was prepared from mycelia samples (Sokolovsky et al., 1990). RNA (1 μ g) from each sample was fractionated onto a 2.2 M formaldehyde 1.2 % (w/v) agarose gel, stained with ethidium bromide, and visualized under UV light to assess rRNA integrity. Gene expression analysis was performed by quantitative PCR (RT-qPCR). RNA samples (10 μ g) were treated with RQ1 RNase-free DNase (Promega) and subjected to cDNA synthesis using the SuperScript III First Strand Synthesis kit (Invitrogen) and oligo (dT) according to the manufacturer's instructions. RT-qPCR was performed on a StepOnePlus™ Real-Time PCR System (Applied Biosystems) using

the Power SYBR® Green PCR Master Mix (Applied Biosystems) and specific primers for *flb-3*, *aba-1*, *vos-1*, and *wet-1* mRNA amplicons (see [Supplementary Table S1](#) online, qPCR). Data analysis was performed using the Comparative CT ($\Delta\Delta CT$) method as implemented in StepOne Software (Applied Biosystems) (Livak and Schmittgen, 2001). Data were normalized to the reference gene β -tubulin (*tub-2*, NCU04054). All reaction efficiencies were between 94 and 100 %. For each sample, at least three biological replicates were performed.

2.3. Determination of the sequence preferences of FLB-3 via protein binding microarrays and identification of putative FLB-3 target sites

We used protein-binding microarrays (PBM) (Berger et al., 2006) to determine the sequence preferences of the FLB-3 protein. We designed primers to clone, via conventional PCR methods, a region encompassing the predicted C₂H₂ DNA-binding domain of FLB-3 (as identified using HMMER; Eddy, 2009), from residues 313 to 385, into the T7-GST vector for expression in *Escherichia coli* (Weirauch et al., 2014). The construct was analyzed in duplicate on two different arrays with differing probe sequences (denoted “ME” and “HK”). Calculation of 8-mer Z and E scores, which estimate the strength of binding to each possible 8-mer, was performed as previously described (Berger et al., 2006). The FLB-3 binding motif was derived using the BEEML-PBM method (Zhao and Stormo, 2011; Weirauch et al., 2013).

We analyzed the 3.5 kb upstream region of the genes of interest (3.5 kb upstream of the predicted translational start site, Neurospora genome annotation NC12) for the identification of putative FLB-3 binding sites. Sequences were scored with the PBM-determined PWM using the standard log-likelihood scoring scheme, with a cutoff of 7 bits (Stormo, 1990). Motif comparisons were performed with Tomtom (Gupta et al., 2007), version 4.10.1 (as implemented at <http://meme-suite.org/tools/tomtom>) using the Sandelin-Wasserman similarity function with default settings (Wasserman and Sandelin, 2004).

2.4. Construction of the FLB-3 overexpressing strain

To generate the sfGFP-tagged FLB-3 strain, a DNA fragment of 1269 bp was amplified by PCR with the primers C-sfGFPFLBC-F and C-sfGFPFLBC-R (Table S1) using genomic DNA from the wild-type strain as template. The C-sfGFPFLBC-R oligonucleotide contains the sequence that encodes for 6-Gly that links the FLB-3 coding sequence and sfGFP. PCR was performed using Phusion High-Fidelity PCR kit (Finnzymes) and the DNA fragment was purified with QIAquick Gel Extraction Kit (QIAGEN, CA), according to the manufacturer's instructions. The purified DNA fragment was cloned into the XbaI and PacI sites of the pTSL91-A plasmid (a donation from N. L. Glass, University of California at Berkeley, Berkeley, CA, USA), leading to the pTSL91-A-*flb-3* construct. The pTSL91-A plasmid allows for constitutive expression of the C-terminus tagged FLB-3-sfGFP fusion protein under the control of the *ccg-1* promoter. The pTSL91-A-*flb-3* construct was used to transform competent conidia from a recipient *his-3* mutant strain (FGSC#9716, *mat* a *his-3*[−]). The transformants (*his-3::Pccg-1-flb-3-sfgfp*) were selected on VM media without histidine. The homokaryons were isolated after crossing with the wild-type strain (FGCS#2498, *mat* A), and confirmed by PCR using the primers C-sfGFPFLBC-F and C-sfGFPFLBC-R (Table S1). The *his-3::Pccg-1-flb-3-sfgfp* was named *flb-3*⁺.

2.5. Chromatin immunoprecipitation-PCR assays

Chromatin immunoprecipitation (ChIP) assays were performed as previously described (Tamaru et al., 2003). Briefly, 10⁸ conidia

from the *flb-3*⁺ strain were grown in different flasks containing 125 ml of VM liquid medium with 2 % sucrose, pH 5.8, and incubated at 30 °C and 200 rpm during 12, 24, 48 and 60 h. Samples were fixed by adding formaldehyde (Sigma) to a final concentration of 1 %, and the formaldehyde was quenched with glycine. Samples were suspended in ChIP lysis buffer and the procedure followed the conditions previously described (Freitas et al., 2016). The DNA was quantified using Nanovue Plus (GE Healthcare) and 200 ng of Input DNA (IN), no Ab (N, reaction without antibody) and IP [immunoprecipitated DNAs with anti-GFP antibody (Sigma)] samples were amplified by PCR using primers specific for each promoter (Table S1, ChIP-PCR). Input DNA was used as a positive control and no Ab as negative control of the experiments.

PCR was performed using Phusion High-Fidelity PCR kit (Finnzymes) and specific oligonucleotides (see [Supplementary Table S1](#) online, ChIP-PCR) for *paba-1* (*pabaFLBC-F/pabaAFLBC-R*), *pvos-1* (*pVOS1FLBC-F/pVOS1FLBC-R*) and *pwet-1* (*pWETAFLBC-F/pWETAFLBC-R*) promoters. A region containing a match of the *A. nidulans* FlbC motif in the *vos-1* promoter was analyzed using the primers *pvosA* (*pVosA-F/pVosA-R*). Reactions were performed under the following conditions: 98 °C for 10 s, 35 cycles of 98 °C for 1 s, 60 °C for 5 s and 72 °C for 30 s, and then 72 °C for 5 min. The reaction products were analyzed on a 2 % agarose gel and visualized with ethidium bromide. Densitometry was performed using ImageJ software, and the IP signals were compared to the negative control (N, no Ab).

3. Results

3.1. The ORF NCU03043 encodes the *N. crassa* ortholog of the *A. nidulans* FlbC transcription factor

The NCU03043 gene is annotated as “C₂H₂ finger domain-containing protein FlbC” in the *N. crassa* genome database (<http://fungidb.org/fungidb/app/record/gene/NCU03043>). The predicted protein is composed of 385 amino acid residues and contains two typical C₂H₂ domains separated by a linker, which conforms to the canonical TGE[K/R]P[F/Y]X pattern. This protein exhibits substantial sequence similarity to functionally characterized FlbC orthologs from different fungi (Fig. S1), particularly in the predicted DNA-binding domain, and is syntenic with FlbC (<http://fungidb.org/fungidb/app/record/gene/NCU03043>), suggesting that NCU03043 encodes the *N. crassa* ortholog of the *A. nidulans* FlbC transcription factor.

3.2. Deletion of *flb-3* results in morphological defects and impairment in sexual and asexual development

To characterize the putative *N. crassa* ortholog of the *A. nidulans* FlbC transcription factor, we analyzed the morphological aspects of the *N. crassa* $\Delta flb-3$ (NCU03043) strain. In *A. nidulans*, FlbC deletion results in a *fluffy* phenotype (cotton-like colonies) and defects in vegetative growth and asexual development, with reduced conidiation (Kwon et al., 2010; Park and Yu, 2012). Similarly, deletion of *flb-3* in *N. crassa* results in multiple morphologic defects related to growth and development, including low production of macroconidia and an inability to develop normal aerial hyphae, which lead to the generation of masses of vegetative hyphae with the same *fluffy* phenotype described for the *A. nidulans* mutant strain. In addition, the $\Delta flb-3$ strain appears to have an apparently reduced β -carotene pigmentation (Fig. 1A). Moreover, analysis of apical hyphae in solid VM medium showed a high quantity of short hyphae, most likely resulting from hyperbranching (Fig. 1B, top), and the very few macroconidia produced by the mutant strain displayed abnormal morphology (Fig. 1B, middle). We also evaluated whether FLB-3 impacts sexual development. Induction of female

Fig. 1. The transcription factor FLB-3 is required for normal development in *N. crassa*. (A) The wild-type and $\Delta flb-3$ strains were grown on both solid VM medium plus 2 % sucrose in Erlenmeyer flasks for 10 d and in plates. (B) Top: strains were cultured on plates containing agar VM medium plus 2 % sucrose at 30 °C for 24 h. Hyphae at the colony edge were observed using a stereoscope at 50 $\times$ of magnification. Middle: Conidia from wild-type and $\Delta flb-3$ strains were collected after 10 d of growth and visualized by optical microscopy. Bottom: Protoperithecia of the wild-type and $\Delta flb-3$ strains were observed at a stereoscope at 50 $\times$ of magnification after 15 d after induction. (C and D) Microconidia from wild-type and $\Delta flb-3$ strains were induced and compared. Microconidia from both strains were inoculated onto a coverslip, grown in liquid VM medium plus 2 % sucrose for different times and stained with either calcofluor (C) or DAPI (D). Images were obtained using the microscope AXIO Imager.A2 (Zeiss) with 100 $\times$ magnification. Scale bar, 10 μ m.

sexual development by nitrogen starvation, which normally results in highly pigmented protoperithecia, led to the production of an increased number of translucent protoperithecia in the $\Delta flb-3$ strain compared to the wild-type strain (Fig. 1B, bottom). These were unable to engage in a cross with conidia from the opposite mating type, suggesting female sterility. We further tested the ability of the mutant strain to participate in a cross by using it as the male strain, but no crossing was observed, suggesting that deletion of *flb-3* results in complete sterility.

Conidial germination of the $\Delta flb-3$ strain was also evaluated using microconidia from wild-type and mutant strains. Since the mutant strain produces a few conidia, microconidiation was induced in both strains and the cells were compared by microscopic analysis. Germination of wild-type and $\Delta flb-3$ microconidia was compared by growing the cells in liquid VM medium for different time periods. Calcofluor White staining revealed that the $\Delta flb-3$ microconidia germinated earlier than wild-type microconidia, however, the hyphae were shorter and displayed increased septation (Fig. 1C). After 48 h of germination, the $\Delta flb-3$ strain exhibited severe morphological alterations, such as cytoplasmic constrictions and hyphae swelling (Fig. 1C and D).

3.3. Expression of *flb-3* is regulated during vegetative growth and asexual development

As FLB-3 was demonstrated to be involved in vegetative growth and asexual development, we decided to analyze its expression

under such growth conditions. In *N. crassa*, growth on liquid medium, under agitation, suppresses conidiation, enabling the evaluation of gene expression specifically under vegetative growth. The *flb-3* transcript started to accumulate at an early phase of vegetative growth, being highly expressed at later times and reaching highest levels at the latest time we assessed (60 h, Fig. 2A). To evaluate gene expression during asexual development, we transferred mycelia grown for 20 h on liquid cultures (C, control) to solid media and then exposed the cultures to light, which are conditions that induce conidiation in *N. crassa* (Greenwald et al., 2010). Under this growth condition, *flb-3* expression decreased with time (Fig. 2B). Altogether, these results suggest that the FLB-3 transcription factor may play a function in vegetative growth and asexual and sexual development in *N. crassa*.

3.4. Determination of the DNA-binding specificity of FLB-3

To further characterize this transcription factor, we determined its DNA-binding preferences using Protein Binding Microarrays (PBMs), a robust and powerful system that allows for rapid and unbiased characterization of the sequence specificity of DNA-binding proteins (Andrilenas et al., 2015; Berger et al., 2006; Weirauch et al., 2014). Briefly, PBMs are microarrays containing >40 000 double-stranded DNA probes, each 60 nucleotides in length. The probes are designed such that every possible non-palindromic 8-base sequence appears on the array in 32 different flanking sequence contexts (palindromes occur 16 times). A GST-

Fig. 2. Expression of the *flb-3* gene is developmentally regulated. (A and B) Gene expression analysis of *flb-3* in the wild-type strain during (A) vegetative growth and (B) asexual development. For vegetative growth, conidia were inoculated into liquid VM medium and mycelia samples were harvested at different times. For asexual development, conidia were inoculated into liquid VM medium, collected by filtration and placed onto solid VM medium over a cellophane membrane and exposed to light for different times. Total RNA was extracted and gene expression analyzed by RT-qPCR. The *tub-2* gene (NCU04054) was used as the reference gene in all experiments. Data shown represent the means and standard deviations of the results of three independent experiments. (*) Asterisks indicate the statistically significant differences according to Tukey–Kramer multiple comparison test ($p < 0.001$) between the levels at each time point compared to those after 12 h (A) and to the one in C (B).

tagged protein consisting of the predicted FLB-3 DNA binding domain and flanking sequence is then bound to the array, the amount of binding to each probe is quantified based upon the measured GST fluorescence, and used to construct DNA binding models (see Methods). Through this approach, we determined that FLB-3 is indeed a sequence-specific DNA-binding protein and characterized its binding specificity (Fig. 3A).

The obtained FLB-3 motif is only somewhat similar to the one reported for FlbC (TGACGAT/A) (Kwon et al., 2010), the *A. nidulans* ortholog of FLB-3. This is surprising, considering the high identity exhibited by these two proteins in their DNA-binding domains (93.8 % of their amino acids are identical in this region), which usually results in similarities in their DNA sequence preferences (Weirauch et al., 2014; Bernard et al., 2012). This led us focus on the reported FlbC motif further. The published FlbC motif was derived from the inspection of only three promoter regions, and the motif discovery was limited to searching for sequence matches similar to the characterized DNA-binding preferences of the *Saccharomyces cerevisiae* CMR3/YPR013C (Zhu et al., 2009) (available at <http://cisbp.ccb.utoronto.ca>), a putative C₂H₂ zinc finger protein, which exhibits certain similarity to FlbC within the zinc finger domain (Kwon et al., 2010). The reported FlbC motif however, displays only a minor overall similarity to the one derived for the yeast protein (Tomtom p-value 0.25). The herein determined FLB-3 motif, on the other hand, displays a significantly more similar motif to that determined for YPR013C (Tomtom p-value 3.8e-05), consistent with their similarity in the predicted DNA-binding region, suggesting that the binding specificity we obtained from the PBM may more accurately represent the specificity of FlbC-like proteins.

Several groups have developed methods to predict the DNA-binding specificity of zinc finger proteins based on the specific residues present at canonical positions within the ZF domains. As an additional test to evaluate and compare our motif to that reported for FlbC, we used a recently published random forest-based predictive recognition model for zinc finger proteins, ZFModels (Gupta et al., 2014), which takes the full sequence of a protein of interest, and through an HMM-based algorithm, extracts the determinants in each finger for construction of a recognition motif. The ZFModels-derived motif predicted for either FLB-3 or FlbC is identical (again, consistent with their similarity in the DNA-binding region) and is largely in agreement with our PBM-derived motif for

Fig. 3. FLB-3 is a sequence-specific DNA-binding protein. (A) Affinity logo representing the DNA-binding specificity of the *N. crassa* FLB-3 protein, as determined using protein-binding microarrays. The height of the given base indicates the strength of binding, with bases above the X-axis being preferred, and bases below the X-axis being disfavored. (B) Schematic representation of FLB-3 (*N. crassa*) and FlbC (*A. nidulans*) putative binding sites identified in the 5'-flanking regions of the *aba-1*, *vos-1* and *wet-1* genes. The empty circles indicate the position of predicted *N. crassa* FLB-3 binding sites and the black ones indicate the position of predicted *A. nidulans* FlbC binding sites (5'-TGACGA(T/A)-3'). The binding site sequences analyzed in this work and their genomic locations are shown. Numbers indicate the first nucleotide of the binding sites relative to the ATG start codon in the + strand.

FLB-3 (Tomtom p-value 0.0016). The similarity with the published FlbC motif, however, is low (Tomtom p-value 0.58). As expected, the ZFModels motif for FLB-3 is also significantly similar to the PBM-determined one for YPR013C (Tomtom p-value 0.0053). These results largely agree with those obtained with another predictive method, B1H-RC (Najafabadi et al., 2015) (not shown). Altogether, this suggests that the sequence preference of FlbC and FlbC-like proteins may be somewhat different from what has been reported and may actually resemble more closely the one we determined for FLB-3, which would be consistent with their similarity in the DNA-binding region (Weirauch et al., 2014; Bernard et al., 2012), and general predictive models of the sequence specificity of zinc finger proteins.

3.5. Identification of the *A. nidulans* *AbaA*, *WetA* and *VosA* orthologs in *N. crassa*

In *A. nidulans*, it has been proposed that FlbC activates *brlA*, which encodes for the early regulator of development, *BrlA*, which then activates *abaA* and *wetA*. These three genes coordinately regulate the expression of a variety of conidiation-specific genes (Adams et al., 1988). In addition, the regulators *VosA* and *VelB* are interconnected to these central regulatory genes (Park and Yu, 2012). The *VosA* regulator has additionally been described to control trehalose biogenesis (Ni and Yu, 2007). To further characterize the pathway involving FLB-3, we searched for the *N. crassa* genes that are predicted to be downstream of FLB-3, based on the *A. nidulans* conidiation pathway. A BLASTP search in the *N. crassa* database identified putative orthologs for some of these genes (termed here as *aba-1*, *wet-1* and *vos-1*). We were unable however, to identify a putative *brlA* ortholog or the

putative orthologs of other known *A. nidulans* developmental regulators (*sfgA* and *velB*) (Fig. 4A).

The NCU02612 predicted protein was identified as the *A. nidulans* *AbaA* ortholog (41 % identity, termed here as *ABA-1*). This protein is characterized as a DNA-binding protein belonging to the TEA/ATTS domain family of transcriptional regulators (Adrianopoulos and Timberlake, 1991). This domain has been identified and characterized in a few regulatory proteins (Laloux et al., 1990; Xiao et al., 1991; Campbell et al., 1992). Sequence alignment of *ABA-1* and orthologous proteins from different filamentous fungi is shown in Fig. S2. *ABA-1*, together with the *Sordaria macrospora* *ABAA* protein, are the largest among the analyzed proteins and share identical amino acids residues in the TEA/ATTS domain (Adrianopoulos and Timberlake, 1991). The NCU01033 predicted protein, annotated as a hypothetical protein, was identified as an ortholog of the *A. nidulans* *WetA* protein (65 % identity, termed here as *WET-1*), and Fig. S3 shows the sequence alignment of related proteins from different filamentous fungi. The NCU05964 protein is already annotated in the *N. crassa* database as the developmental regulator *VOS-1*, the putative *VosA* ortholog. This protein contains a *velvet* domain in the N-terminal region, which corresponds to a new class of DNA-binding domain present in transcription factors classified under the *velvet* family (Ni and Yu, 2007). The *velvet* regulators are highly conserved proteins, only found in ascomycetes and basidiomycetes fungi (Bayram and Braus, 2012). They have been described to play a role in various processes, such as developmental control, sporogenesis and secondary metabolism (Park et al., 2012; Bayram and Braus, 2012).

To evaluate the role of these genes in *N. crassa*, the morphology of the $\Delta aba-1$, $\Delta vos-1$ and $\Delta wet-1$ strains was compared to that of the wild-type strain. Despite some slight differences among them

Fig. 4. Identification of the *N. crassa* orthologs of *A. nidulans* genes involved in conidiation. (A) Genetic model for upstream and central regulators of conidiation in *A. nidulans* (Park and Yu, 2012) and *A. fumigatus* (Tao and Yu, 2011). The gray circles represent the orthologs identified in *N. crassa*, each one with its respective ORF number. (B) Phenotypic and morphological analysis of the *N. crassa* $\Delta aba-1$, $\Delta vos-1$ and $\Delta wet-1$ strains. Strains were cultured for 9 d on solid VM medium plus 2 % sucrose. For hyphae analysis, conidia from the strains were germinated in liquid VM medium plus 2 % sucrose for 12 h and stained with calcofluor white.

(e.g. rare hyphae constrictions in the $\Delta wet-1$ strain), they were phenotypically similar to the wild-type strain after growth on minimal solid medium and conidial germination (Fig. 4B). All strains exhibited viable conidia and the conidia germinated well, with the exception of $\Delta vos-1$, which displayed a slight delay in conidia germination, but was able to reach normal hyphae growth after 8 h of incubation in liquid culture (data not shown). We further analyzed conidia viability after storage in all strains and they retained high viability (around 90 %) after up to 21 d at 4 °C (data not shown). The lack of vegetative growth phenotypes in the aforementioned mutants was unexpected, based on what is known about the corresponding orthologs in *Aspergilli* (Adrianopoulos and Timberlake, 1994; Tao and Yu, 2011; Son et al., 2013), and suggests divergent functionality between the *N. crassa* proteins and those from other filamentous fungi regarding the central regulatory pathway of conidiation: *aba-1*, *wet-1* and *vos-1* may not play such important roles in *N. crassa* conidiation as they do in *Aspergilli*. The result related to *aba-1* gene here is consistent with the one reported by Chung (2011). The *N. crassa* gene does not play an analogous role in conidiation as the *A. nidulans* gene; it does not complement defective conidiation in *A. nidulans* *abaA14* mutant, even using different promoters.

3.6. FLB-3 regulates *aba-1*, *wet-1* and *vos-1* expression during vegetative growth and asexual development and binds to their promoters

In *A. nidulans*, FlbC regulates the expression of genes of the conidiation pathway (Kwon et al., 2010). To verify whether FLB-3 is required for the normal expression of *aba-1*, *wet-1* and *vos-1*, we analyzed the expression of all these genes in the wild-type and $\Delta flb-3$ strains by RT-qPCR. The assays were performed during vegetative growth in liquid submerged cultures and under induction of asexual development. During vegetative growth, the *N. crassa* wild-type strain displayed a maximum level of *aba-1* mRNA at 12 h, and a reduction after this time, whereas the expression of *vos-1* and *wet-1* reached their highest levels after 48 h of growth (Fig. 5A). In the $\Delta flb-3$ strain, the levels of the *aba-1* and *wet-1* transcripts were slightly lower than those in the wild-type strain and remained almost constant over the time period analyzed, whereas the expression levels of *vos-1* were generally higher than those in the wild-type strain throughout the time course (Fig. 5A). These results suggest that FLB-3 is required for the proper expression of *aba-1*, *wet-1* and *vos-1* genes during vegetative growth.

The expression of the same genes was also analyzed during induction of asexual development (Fig. 5B). In the wild-type strain, *aba-1* expression decreased with time, while levels of the *vos-1* transcript remained relatively constant during all time points analyzed. The maximum level of the *wet-1* transcript was observed at 9 h after induction of conidiation. In the $\Delta flb-3$ strain however, very low levels of *aba-1* and *wet-1* transcripts were detected throughout the assay, indicating the requirement of FLB-3 for the expression of both genes under this growth condition. On the other hand, levels of the *vos-1* transcript were similar to those observed in the wild-type strain, suggesting that this gene may not be involved in conidiation in *N. crassa* as described in *Aspergillus fumigatus* (Tao and Yu, 2011) and *A. nidulans* (Ni and Yu, 2007). We suggest that FLB-3 directly regulates the expression of *aba-1*, *wet-1* and *vos-1* genes, acting as an activator of *aba-1* and *wet-1* expression under both vegetative growth and asexual development, and as a repressor of *vos-1* expression under vegetative growth. Such regulation would be independent of the *brlA* regulator, whose ortholog was not identified in *N. crassa*. FLB-

3 appears not to be required for normal *vos-1* expression under induction of conidiation.

In *A. nidulans*, using a GST pull-down assay, it was demonstrated that FlbC binds *in vitro* to the promoters of *brlA*, *abaA* and *vosA*, but not to that of *wetA* (Kwon et al., 2010). We identified putative FLB-3 binding sites in the *N. crassa* *aba-1*, *wet-1* and *vos-1* promoters (Fig. 3B), and we investigated whether FLB-3 could bind *in vivo* to DNA fragments containing some of these motifs. Putative *A. nidulans* FlbC binding sites were also identified in the *N. crassa* *wet-1* and *vos-1* promoters (Fig. 3B), and one in the *vos-1* promoter was analyzed. For these experiments, we constructed a GFP-tagged FLB-3 overexpressing strain, by inserting the *flb-3* genomic sequence into the *his-3* locus of a wild-type strain (*his-3::Pccg-1-flb-3-sfgfp*). The homokaryon strain (named as *flb-3⁺*) produces a C-terminal sfGFP-tagged FLB-3 under the control of the *cgg-1* promoter. The FLB-3 overexpressing strain was compared to the wild-type strain under the same conditions, and we observed no differences in colony growth and in the amounts of macroconidia (Fig. S4A). Interestingly, the overexpressing strain grew more in 9-d old cultures and produced a significantly higher number of microconidia than the wild-type strain ($p < 0.01$; approximately 400 times) (Fig. S4B), supporting a role for the FLB-3 transcription factor in *N. crassa* microconidiation.

ChIP-PCR assays were performed to analyze the binding of FLB-3-sfGFP to the *aba-1*, *wet-1* and *vos-1* promoters. Chromatin from mycelia after 12, 24, 48, and 60 h of vegetative growth was used for immunoprecipitation, and binding of FLB-3 was analyzed by PCR using the oligonucleotides described in Table S1. The amplified DNA fragments (Fig. 6A) were quantified by ImageJ, and the results are shown in graphs (Fig. 6B). FLB-3 was able to bind to its predicted sites in the *aba-1* (–2766 bp from the ATG start codon), *vos-1* (–3441 bp from the ATG start codon), and *wet-1* (–2212 bp from the ATG start codon) promoters after 48 and 60 h of growth ($p < 0.01$). However, we did not observe binding when mycelia collected at 12 h (data not shown) and 24 h (Fig. 6A) of vegetative growth were used. It is noteworthy that FLB-3 strongly bound to the *aba-1* and *vos-1* promoters at both time points and binding to the *wet-1* promoter appeared to be stronger at 48 h of growth (Fig. 6A and B). We also analyzed binding to a region of the *vos-1* promoter that contains a match to the reported *A. nidulans* FlbC binding site (located at –759 bp from the ATG start codon). Interestingly, FLB-3 was unable to bind to the *A. nidulans* FlbC site located in *vos-1* promoter, further supporting the FLB-3 DNA binding preferences determined by PBM. The results indicate that FLB-3 directly regulates the expression of these three genes, differently from what has been described in *A. nidulans*, where FlbC directly activates *brlA*, the first gene of the conidiation cascade.

3.7. Cross regulation among the *aba-1*, *wet-1* and *vos-1* genes

The existence of genetic interactions between these genes was previously reported in *A. fumigatus* (Tao and Yu, 2011) and *A. nidulans* (Ni and Yu, 2007). In *A. fumigatus*, the *AfuwetA* transcript is not detected in the *AfuabaA* mutant, and *AfuvosA* expression is reduced in the *AfuwetA* and *AfuabaA* mutants, establishing an order of gene activation shown in Fig. 4A. In addition, in *A. nidulans*, *wetA* levels strongly decrease in the $\Delta abaA$ strain under vegetative growth condition (Ni and Yu, 2007). We thus decided to investigate the existence of genetic interactions among the *N. crassa* genes under vegetative growth and asexual development. We observed that *aba-1* and *wet-1* down regulate each other under vegetative growth but they do not regulate *vos-1* (Fig. 7A), although *VOS-1* negatively regulates *aba-1* and *wet-1* (Fig. 7A). Under the same condition, FLB-3 directly regulates *vos-1*, as demonstrated in

Fig. 5. FLB-3 regulates the expression of *aba-1*, *vos-1* and *wet-1* during vegetative growth and asexual development. Gene expression analysis of the *aba-1*, *vos-1* and *wet-1* genes in the wild-type and $\Delta flb-3$ strains during vegetative growth (A) and under asexual development (B). Conidia were inoculated in liquid VM medium plus 2 % sucrose at 30 °C, 200 rpm for 20 h (control, C). After that, mycelia were collected by filtration and placed onto solid VM medium over a cellophane membrane and exposed to light. Mycelia samples were harvested at different times, total RNA was extracted and gene expression was analyzed by RT-qPCR. The *tub-2* gene (NCU04054) was used as the reference gene in all experiments. Data shown represent the means and standard deviations of the results of at least three independent experiments. (*) and (**) Asterisks represent the statistically significant differences according to Tukey–Kramer multiple comparison test with $p < 0.001$ and 0.01, respectively, between the levels observed in the wild-type and the $\Delta flb-3$ strains, at each time point.

Fig. 5A. Under asexual development, the genetic interactions seem to be less prominent. Under this condition, ABA-1 does not regulate *wet-1* and *vos-1*; however, both genes are required for proper regulation of *aba-1* expression, and a genetic interaction between *wet-1* and *vos-1* was only observed at 24 h of induction of conidiation (Fig. 7B). These results in *N. crassa* show differences in the genetic interaction when compared to *Aspergilli*, and indicate that the signaling pathway under control of FLB-3 is not fully conserved in distinctive filamentous fungi. Based on these results, we propose a model of interaction among *flb-3*, *aba-1*, *wet-1* and *vos-1* genes in *N. crassa*, which is based on a different order of gene activation under both growth conditions (Fig. 8).

4. Discussion

In this study, we investigated the functional role of the *N. crassa* FLB-3 transcription factor in developmental control and demonstrated that it has an important role in sexual and asexual development. In addition, we also characterized downstream genes, which are described as being under FLB-3 control in *Aspergilli*. This transcription factor has been characterized in only a few fungi, and in *N. crassa* it has only been previously identified in a screen for transcription factors regulating glycogen metabolism (Gonçalves et al., 2011), and as a gene regulated by light (Wu et al., 2014) and during development (Greenwald et al., 2010). Deletion of *flb-3*

Fig. 6. -The FLB-3 transcription factor binds to the *aba-1*, *vos-1* and *wet-1* promoters *in vivo*. (A) Genomic DNA samples from *flb-3*⁺ strain under vegetative growth conditions were immunoprecipitated with anti-GFP antibody and subjected to PCR to amplify DNA fragments in the *aba-1*, *vos-1* and *wet-1* promoters. The Input DNA (IN) was used as positive control, and the non-immunoprecipitated reaction (no Ab, N) as the negative control. L, 1 kb DNA ladder. (B) The DNA bands after ChIP-PCR were quantified by ImageJ, and the binding results are shown in graphs as arbitrary units (AU). (*) Asterisks indicate significant differences between no Ab (N) and IP in the same condition (Student's *t*-test, *p* < 0.01). The results represent the average of two independent experiments.

results in severe defects in conidiation and in sterility. While the production of the female sexual structure was increased, similar to the phenotype described for the *A. nidulans* $\Delta flbC$ strain (Kwon et al., 2010), the protoperithecia were unable to cross.

In *A. nidulans*, FlbC is necessary for proper activation of conidiation, acting as an activator of *brlA*, the first gene of the *brlA* → *abaA* → *wetA* cascade that defines the central regulatory pathway, and controls conidiation in this organism (Kwon et al., 2010; Park and Yu, 2012; Mirabito et al., 1989). The *A. nidulans* regulators VosA and VelB have been shown to be interconnected to this regulatory pathway via negative feedback regulation of *brlA* and other developmental genes (Park and Yu, 2012; Ni and Yu,

2007). In addition, they function in spore maturation, by controlling trehalose biogenesis and wall completion (Park and Yu, 2012; Ni and Yu, 2007). In *P. anserina*, a fungus more closely related to *N. crassa*, FLE-1 (the FLB-3 ortholog) coordinates male and female sexual differentiation, by controlling the balance between male and female structure production (Coppin, 2002), and in *F. verticillioides*, this protein, termed Ada1, plays a role in asexual development (Malapi-Wight et al., 2014). Therefore, this transcription factor appears to be involved in the control of the transition from vegetative growth to asexual/sexual development in fungi, but the precise mechanisms through which it performs this function depend on the organism. While in *A. nidulans* *flbC* acts upstream of

Fig. 7. Cross-regulation among *aba-1*, *wet-1* and *vos-1* genes. (A) Gene expression analysis of the genes in the $\Delta aba-1$, $\Delta vos-1$ and $\Delta wet-1$ strains during vegetative growth. Conidia were inoculated in liquid VM medium +2 % sucrose at 30 °C, 200 rpm and mycelia collected after 12, 36 and 60 h of growth. (B) Gene expression analysis of the genes in the same strains under asexual development. Conidia were inoculated in liquid VM medium plus 2 % sucrose at 30 °C, 200 rpm for 20 h (control, C). After that, mycelia were collected by filtration and placed onto solid VM medium over a cellophane membrane and exposed to light. Mycelia samples were harvested at different times, total RNA was extracted and gene expression was analyzed by RT-qPCR. The *tub-2* gene (NCU04054) was used as the reference gene in all experiments. Data shown represent the means and standard deviations of the results of at least three independent experiments. (*, ** and ***) Asterisks represent the statistically significant differences according to Tukey–Kramer multiple comparison test with $p < 0.001$, 0.01 and 0.05, respectively, between the levels observed each time point relative to time 12 h (A) and to C (B).

Fig. 8. Simplified models of the role of FLB-3 in the regulation of *aba-1*, *wet-1* and *vos-1* expression during vegetative growth (A) and under induction of asexual development (B) in *N. crassa*. The genetic interactions among the *aba-1*, *wet-1* and *vos-1* genes are shown. The regulatory mechanisms and the genetic interactions among the genes are presented in the text.

brlA, regulating the expression of conidiation-specific genes, in *P. anserina*, *F. verticillioides* and in *N. crassa* (as shown in this study), its role in conidiation appears to be independent of the *brlA* gene, since this protein was not identified in any of these organisms (Coppin, 2002; Malapi-Wight et al., 2014). Although FlbC has been reported to play a direct role in the expression of the *abaA* and *vosA* genes, independent of *brlA*, activation of *brlA* is required for conidiophore development in *A. nidulans* (Kwon et al., 2010).

The DNA-binding specificity of FLB-3 was determined here by protein binding microarrays and we demonstrated that this transcription factor binds *in vivo* to the predicted *cis* elements present in the *aba-1*, *wet-1* and *vos-1* promoters. However, under the same conditions, it does not bind to a predicted FlbC binding site that exists in the *vos-1* promoter. The FLB-3 motif we determined here was expected to be similar to that determined for FlbC, the *A. nidulans* ortholog of FLB-3 (Kwon et al., 2010), considering the high sequence identity exhibited by these proteins in their DNA-binding regions (Weirauch et al., 2014; Bernard et al., 2012). The motifs, however, are only somewhat similar, and we argue that the motif reported herein is more likely to represent the true DNA-binding specificity of FlbC orthologs. The main reasons are that the reported FlbC motif was based only on a handful of promoters, and that our motif is not only in large agreement with that determined for a related protein (YPR013C) (Zhu et al., 2009), but is also consistent with that derived from predictive models for DNA-binding specificity of zinc finger proteins (Gupta et al., 2014; Najafabadi et al., 2015). The previously reported motif for FlbC, on the other hand, performs poorly in both analyses. Interestingly, an alternative motif for FlbC, GTAGATC, has previously been presented that better resembles the one of YPR013C, and consequently, also of FLB-3 (Kwon et al., 2010). Based on our results, we consider it to be a more accurate representation of its DNA-binding preference. Altogether, our analyses suggest that the FLB-3 motif we report here better represents the DNA-binding preference of FlbC orthologs, including that of *A. nidulans* FlbC.

The *N. crassa* *aba-1*, *wet-1* and *vos-1* genes were identified in this work, and we showed that these genes may not play a role in the induction of conidiation in *N. crassa*, differing from what has been described for their orthologs in *A. nidulans* (Adrianopoulos and Timberlake, 1994), *A. fumigatus* (Tao and Yu, 2011) and *Fusarium graminearum* (Son et al., 2013). Loss-of-function mutation in *abaA* of *A. nidulans* and *A. fumigatus* has been shown to result in aberrant conidiophores exhibiting cylinder-like terminal cells without conidia (Adrianopoulos and Timberlake, 1994; Tao and Yu, 2011). Deletion of *vosA* in *A. nidulans* leads to a drastic reduction in conidia viability and apparent lack of cytoplasm and organelle (Ni and Yu, 2007). In addition, deletion of the *A. fumigatus* *wetA* gene (*Afu-wetA*) affects conidia wall formation, resulting in incomplete conidial separation and maturation, and reduced viability when compared to the wild-type strain (Tao and Yu, 2011). In *F. graminearum*, deletion of *wetA* results in longer conidia, containing fewer septa than those of the wild-type strain. Additionally, the conidia are sensitive to oxidative and heat stresses (Son et al., 2014). The corresponding *N. crassa* knockout strains, on the other hand, conidiate well and their conidia show high viability over time. These genes may play additional roles in *N. crassa*, similar to what has been described for *abaA* in *A. nidulans*, where deletion of *abaA* affects autolysis by reducing chitinase production (Emri et al., 2008). Although seemingly not involved in conidiation in *N. crassa*, these genes do appear to rely on FLB-3 for proper expression, which we report here as a regulator of conidiation, during both vegetative growth and in the induction of asexual development. Alternatively, these proteins may indeed play a role in conidiation, but additional proteins may compensate for their function in their absence. It is important to emphasize that although FLB-3 regulates the expression of *aba-1*, *wet-1* and *vos-1* genes in the absence of *brlA*, its role in the regulation of conidiation is, most likely, independent of these genes considering the phenotypes of the three mutants.

We demonstrated here that FLB-3 is involved in sexual and asexual development in *N. crassa*, and the gene overexpression data suggests that it specifically impacts the microconidiation process. Moreover, FLB-3 binds to the promoter of the *aba-1*, *wet-1* and *vos-1* genes and their expression is misregulated in the $\Delta flb-3$ strain, suggesting that they are direct targets of this transcription factor and their function might be connected to FLB-3. However, mutant strains in these three genes conidiate well. Our data also show that regulatory interactions among the *aba-1*, *wet-1* and *vos-1* genes are less prominent under asexual development than those observed during vegetative growth, which reinforces that these genes may not play an important role in conidiation. Therefore, the role of FLB-3 in conidiation is independent of these three genes. Their promoters can be bound *in vivo* by FLB-3, which raises questions about the functions of these genes in *N. crassa*. The *velvet* regulators, which include the VOS-1 protein, are involved in important biological process including tolerance to stress (Park et al., 2012; Lan et al., 2014). However, in *N. crassa*, a systematic analysis of the $\Delta vos-1$ strain under different stress conditions revealed that VOS-1 may not play a role in stress response (unpublished results). Considering that the role of these genes may differ from what has been described in other filamentous fungi, investigating their targets may be useful in fully understanding these proteins in *N. crassa*. Although much work remains to be done, this report represents a first step in understanding the functional role of the FLB-3 transcription factor in *N. crassa*, which can be further used as an entry point for the study of how regulatory circuits involved in development change among different fungal species.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

We thank the Fungal Genetics Stock Center (Manhattan, KS, USA) for the *N. crassa* strains. We would like to thank the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, Proc. 2013/24705-3) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (Grant no. 444068/2014-7) for providing research grants to MCB and fellowships to DLA, ACB, FBC, SV, FZF, FAC, RDG, and MCB. LFL and AMM are supported by grants from Millennium Institute for Integrative Systems and Synthetic Biology (MIISB) and Fondo Nacional de Desarrollo Científico y Tecnológico (FONDECYT 1171151). TRH acknowledges support from the Canadian Institutes of Health Research (FDN-148403). We also acknowledge the support for the International Symposium on Fungal Stress (ISFUS)-2017 meeting from the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) of Brazil, PAEP 88887.126652/2017-00 and the Fundação de Amparo à Pesquisa do Estado de Goiás of Brazil, 201710267000110.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.funbio.2018.01.004>.

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