Characterization of the *Aspergillus flavus rdiA* gene with a *rdi1Δ* mutant of *Cryptococcus neoformans*.

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Introduction

Several members in the *Aspergillus* genus produce aflatoxin, the most potent naturally occurring carcinogen known. While all the biosynthetic steps have been characterized, much remains to be elucidated regarding the regulatory network controlling its production. Deletion of *rdiA* resulted in increased septation interval and branching as well as severely decreased AF production. The *ΔrdiA* mutant phenotype is similar to the *rdi1Δ* mutant of *C. neoformans* and the *bem4Δ* mutant in *S. cerevisiae*. *A. flavus rdiA* expression at least partially rescues the cold-sensitive phenotype of the *S. cerevisiae bem4Δ* mutant, however, the mutant of *C. neoformans RDI1* exhibits discernable morphological phenotypes similar to the *rdiA* mutant of *A. flavus*. Therefore, we will complement the *rdiA* mutant of *A. flavus* to confirm the observed phenotypes in *A flavus*. Furthermore, we will complement the *C. neoformans rdi1Δ* mutant with *A. flavus rdiA* to establish the identity of the *A flavus rdiA* gene.

Background

Regulation of aflatoxin (AF) biosynthesis has been studied for several decades as this mycotoxin is currently regulated by the USDA. Regulation of AF production is complex, involving transcriptional and post-transcriptional regulation focused mainly through the pathway specific transcriptional regulator *aflR*. An investigation into the nature of the transcriptional regulation of AF production in *Aspergillus flavus* by comparing conducive and non-conducive culture conditions revealed a clade of genes with a similar transcription profile to that of *aflR*. One of these genes, a putative Rho-GDP dissociation inhibitor, was characterized by gene deletion and shown to regulate AF production in *Aspergillus flavus*. The protein encoded by this gene, *rdiA*, showed 45% identity to Rdi1p in *S. cerevisiae*. Rho-GDP dissociation inhibitors like *RDI1* in *S. cerevisiae* interact with monomeric GTPases such as Rho1 and Cdc42 and inactivate them by holding them in the cytosol bound to GDP.

Cryptococcus neoformans is an opportunistic pathogen due to its preference in targeting immunocompromised patients. Highly conserved signal transduction pathways have been observed to be key in many of the fundamental cellular processes that allow for a microbial pathogen's survival inside of their host, and these pathways have also been shown to regulate growth signals in a multitude of microorganisms. One of these conserved signaling molecules is Cdc42, a member of the G-protein superfamily that has been shown to be involved in the foundation and preservation of cell polarity in yeast.

Methods

- *A. flavus rdiA* including 1kb upstream and downstream sequence was cloned into plasmid pAB520 to create plasmid pRT1 that contains the phleomycin resistance gene *ble* and will be used to reconstitute the *ΔrdiA A. flavus* mutant. Transformations will be performed using an established PEG transformation protocol (He et al., 2007). Growth phenotypes will be assessed using standard growth media at 30°C and 37°C, and aflatoxin will be measured via enzyme-linked immunosorbent assay.
- The *A. flavus rdiA* gene was cloned into the *C. neoformans* vector pSDMA57 to create the plasmid pSCB1 that contains the G418 resistance gene *NEO* and will be used for transformation into the rdi1Δ mutant of *C. neoformans*. Transformation will be performed using electroporation as described elsewhere (Huang et al., 2022). The ability of the *A. flavus rdiA* gene to complement the *C. neoformans rdi1*Δ mutant phenotypes will be assessed including virulence (Price et al., 2008).

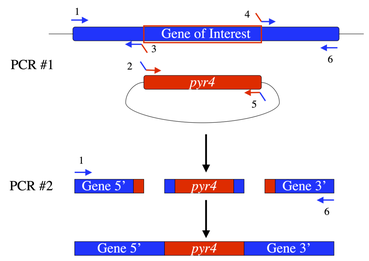


Figure 1. Construction of linear gene-deletion construct for CA747847. PCR primers were synthesized to amplify the 5' and 3' flanking regions surrounding the putative CA747847 coding region. Internal primers were synthesized as hybrid sequences between the 5' or 3' region and the *pyr4* marker gene from *N. crassa*. The final construct is produced during a PCR reaction utilizing the products of PCR #1 as template, and the forward and reverse primers 1 & 6.

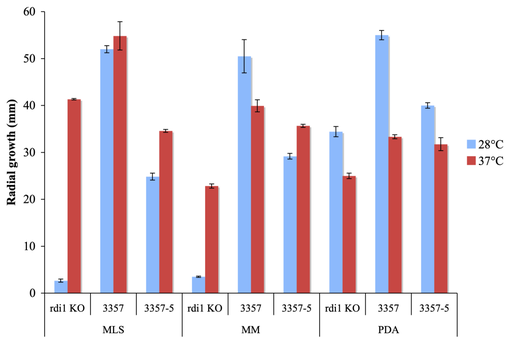


Figure 3. Growth of Δ*rdiA* on MM, MLS, and PDA at either 28°C or 37°C. Spores of each strain were inoculated onto plates of MM, MLS, or PDA by toothpick. The plates were incubated at either 28°C or 37°C for 5 days.

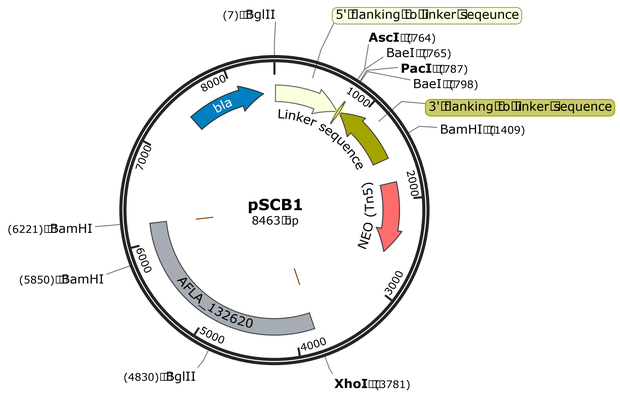
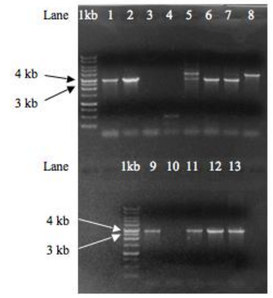


Figure 5. Plasmid map of pSCB1 for expression of *A. flavus rdiA* in *C. neoformans*. *A. flavus rdiA* (GeneID AFLA_132620) was cloned into plasmid pSDMA57 for introduction into *C. neoformans* strain MPC2 (*rdi1Δ*::NAT). The resulting transformant will be assessed for complementation of *rdi1Δ* mutant phenotypes including virulence defect in a mouse model of disease.

Figure 2. PCR screen of Δ*rdiA* gene-deletion mutants. Transformants exhibiting prototrophic growth on medium lacking uracil were analyzed by PCR to identify those containing the deletion construct. The predicted size of the deletion amplicon is 3.7 kb and the wild-type amplicon is 2.9 kb. 13 out of 45 transformants are shown. One transformant out of forty-five was observed to possess the gene deletion.



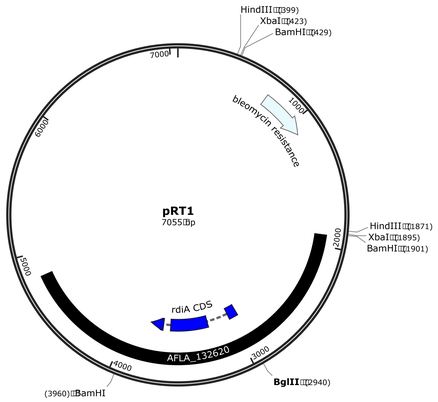


Figure 4. Plasmid map of pRT1 for complementation of *A. flavus* Δ*rdiA*. The *A. flavus rdiA* gene was cloned into plasmid pAB520 for transformation into *A. flavus* strain MPA1 (Δ*rdi1*::pyr4) using phleomycin.

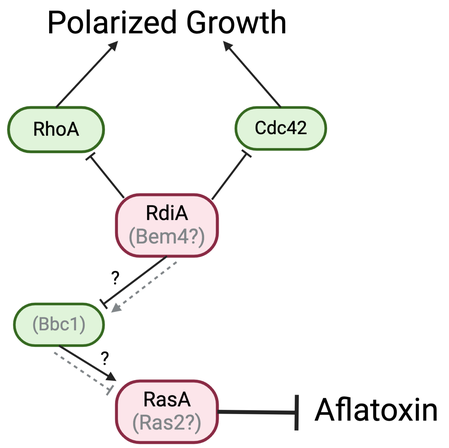


Figure 6. Proposed gene interaction network in *A. flavus* for control of AF biosynthesis via RdiA. The *RDI1* ortholog in *A. flavus*, *rdiA*, may control AF production in *A. flavus* by modulating the activity of a putative Bbc1 ortholog in *A. flavus*, which in turn would regulate the activity of RasA in the fungus. RasA inhibits transcription and activity of *aflR* and AflR, respectively, in *A. nidulans,* inhibiting sterigmatocystin biosynthesis (an intermediate in aflatoxin biosynthesis) (Shimizu et al., 2003). *S. cerevisiae* orthologs indicated in grey font. Created with BioRender.com.

Results

- The *rdiA* gene was successfully deleted from *A. flavus* (Fig. 1 & Fig. 2), and exhibited the predicted size of PCR products. However, the transformation did not produce an abundance of transformants.
- The *rdiA* mutant displayed a temperature sensitive phenotype (Fig. 3). On both MLS and minimal media (MM) at 28°C, the growth of the *rdiA* KO grew significantly less than at 37°C.
- For complementation of *rdiA* in *A. flavus* strain MPA1, a plasmid was developed that housed the *rdiA* gene and contained a phleomycin resistance (Fig. 4).
- For complementation and assessment of virulence in *C. neoformans*, a plasmid was developed containing the *A. flavus rdiA* gene and a neomycin resistance marker (Fig. 5).
- These data implicate a putative GDP-dissociation inhibitor, *rdiA*, in the control of AF biosynthesis in *A. flavus*. Figure 6 proposes a regulatory network in which the deletion of RdiA would allow Bbc1 to promote RasA, which inhibits AF production.

Future Work

- Reconstitute the *ΔrdiA A. flavus* mutant with *rdiA* to assess growth phenotypes
- Use a transformed *rdi1Δ* mutant of *C. neoformans* to help characterize *A. flavus rdiA* gene using a growth phenotype
- Assess the effect of *rdiA* deletion on virulence in an invertebrate model of disease

References

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