Fungal Genetics and Biology 94 (2016) 47–53

Contents lists available at ScienceDirect

Fungal Genetics and Biology

journal homepage: www.elsevier.com/locate/yfgbi

A proteomic and genetic analysis of the *Neurospora crassa* conidia cell wall proteins identifies two glycosyl hydrolases involved in cell wall remodeling

Jie Ao [a,1], Mash'el Aldabbous [b,1], Marysa J. Notaro [a], Mark Lojacono [a], Stephen J. Free [a,*]

[a] Department of Biological Sciences, SUNY, University at Buffalo, Buffalo, NY 14260, United States
[b] Department of Biological Sciences, Faculty of Science, Khaldiya, Kuwait University, Safat 13060, Kuwait

ARTICLE INFO

Article history:
Received 6 June 2016
Revised 30 June 2016
Accepted 1 July 2016
Available online 2 July 2016

Keywords:
Fungal cell wall
Asexual development
Conidia
Glycosyl hydrolase
Chitinase
Glucanase

ABSTRACT

A proteomic analysis of the conidial cell wall identified 35 cell wall proteins. A comparison with the proteome of the vegetative hyphae showed that 16 cell wall proteins were shared, and that these shared cell wall proteins were cell wall biosynthetic proteins or cell wall structural proteins. Deletion mutants for 34 of the genes were analyzed for phenotypes indicative of conidial cell wall defects. Mutants for two cell wall glycosyl hydrolases, the CGL-1 β-1,3-glucanase (NCU07523) and the NAG-1 exochitinase (NCU10852), were found to have a conidial separation phenotype. These two enzymes function in remodeling the cell wall between adjacent conidia to facilitate conidia formation and dissemination. Using promoter::RFP and promoter::GFP constructs, we demonstrated that the promoters for 15 of the conidia-specific cell wall genes, including *cgl-1* and *nag-1*, provided for conidia-specific gene expression or for a significant increase in their expression during conidiation.

1. Introduction

The formation of asexual spores is an important part of the life cycles of many fungi. The asexual spore is a differentiated cell type adapted for dissemination and for the establishment of a new vegetative hyphal network. Asexual spores are generated in very large numbers, and can be rapidly and widely disseminated in the environment, to allow the fungi to colonize new sites and substrates. The formation of asexual spores has been examined in many fungi, and the details of how asexual spores are generated varies widely. In *Neurospora crassa* the process of conidiation has been extensively studied (Springer, 1993; Springer and Yanofsky, 1989). A number of proteins have been shown to be expressed during conidiation, and transcription factors needed for conidial development have been identified (Greenwald et al., 2010; Lee and Ebbole, 1998; Sachs and Yanofsky, 1991; Springer and Yanofsky, 1992). Our analysis of conidiation focused on the question of how the cell wall proteome changes as cells go through conidial development. We have previously demonstrated that the conidial cell wall contains α-1,3-glucan, a glucan not found in the vegetative cell wall (Fu et al., 2014).

The cell wall plays a critical role in fungal cell biology. Not only does the wall protein protect the cell from environmental factors, such as osmotic stress, desiccation, heat, freezing, and attacks from other microbes, the cell wall also allows the fungus to assess its environment and activate signaling pathways in response to a changing environment (Free, 2013). The wall consists of a cross-linked matrix of glucans, chitins, and cell wall proteins. The proteins are particularly important in allowing the fungus to assess and respond to its environment. Cell wall proteins function in cross-linking cell wall components together, and are therefore essential for cell wall biogenesis.

In order to do a proteomic analysis of fungal cell wall proteins, the proteins, (or peptides derived from them) need to be obtained free of the cell wall glucans and chitin to which they are covalently attached. Several different approaches for obtaining cell wall proteins for proteomic analyses have been used over the years (Chaffin, 2008; Free, 2013; Klis et al., 2006; Latge, 2007; Lesage and Bussey, 2006; Ruiz-Herrera et al., 2006). Cell wall proteins have been isolated from among the proteins being secreted by sphaeroplast that are regenerating their cell walls. Some cell wall proteins have been isolated from among the proteins being released into the medium, suggesting that either the incorporation of the proteins into the wall isn't 100% efficient or that some

* Corresponding author at: SUNY, University at Buffalo, 109 Cooke Hall, Buffalo, NY 14260, United States.
E-mail address: free@buffalo.edu (S.J. Free).
[1] Co-first authors.

http://dx.doi.org/10.1016/j.fgb.2016.07.003
1087-1845/

proteins that have been incorporated into the wall are subsequently released into the medium. Some cell wall proteins can be released from the wall by chemical treatment of the wall or by digesting the wall with glucanase and chitinase preparations, and these can then be isolated and subjected to proteomic analysis. Yet another approach has been to treat the cell wall with trypsin, and to use the released tryptic peptides for proteomic analyses. Recently we introduced the use of trifluoromethanesulfonic acid (TFMS), which digests the glycosidic bonds found in the glucan and chitin cell wall components and releases deglycosylated cell wall proteins for proteomic analysis (Birkaya et al., 2009; Bowman et al., 2006; Liu and Free, 2015; Maddi et al., 2009, 2012; Maddi and Free, 2010).

In this study, we have used TFMS to release cell wall proteins from the cell wall of *N. crassa* asexual spores (macroconidia or more simply referred to as conidia) and compared the cell wall proteome with the cell wall proteins found in vegetative hyphae. After identifying the proteins in the conidia cell wall, we then used a genetic analysis to determine whether these proteins were important for conidia formation, and to determine if the genes encoding these proteins were transcribed in a conidia-specific manner. We report that the conidia cell wall contains 15 conidia-specific proteins, and that the genes encoding these proteins are transcribed in a conidia-specific manner. We demonstrate that two of these conidia-specific cell wall proteins, the NGA-1 exo-chitinase and the CGL-1 β-1,3-glucanase, play critical roles in remodeling the cell walls between adjacent conidia to allow the conidia to separate.

2. Materials and methods

2.1. Proteomic analysis of the conidia cell wall

Conidial cell walls were obtained by growing a wild type isolate (FGSC #2400) on six 3 ml slants of Vogel's sucrose medium and collecting the conidia in water. The conidia were passed through 4 layers of cheese cloth to remove hyphae. The conidial cells were broken by subjecting them to two 45 s treatments in a Fast Prep machine with glass beads (1.0 mm in diameter) at a setting of 6.6. The cell walls were collected by a 10 min centrifugation at 5000*g*, and washed two times with sterile distilled water. The cell walls were then resuspended in PBS containing 1% SDS and subjected to a 15 min incubation in a boiling water bath. The cell walls were then washed once with PBS and three additional times with sterile distilled water. The cell walls were then lyophilized before being subjected to deglycosylation using the trifluromethanesulfonic acid (TFMS) digestion method, as previously described (Maddi et al., 2009). At the conclusion of the TFMS digestion, the cell wall proteins were collected by trichloroacetic acid precipitation, resuspended in PBS with 1% SDS, subjected to a 15 min incubation in a boiling water bath, loaded onto an SDS PAGE, and subjected to a 5 min electrophoresis step. After staining the gel with Coomassie brilliant blue, the top 5 mm of the gel, which contained the cell wall proteins, was excised from the gel and used for proteomic analysis. The proteomic analysis was carried out at the Fred Hutchinson Cancer Center Proteomic Facility (Seattle, WA). Trypsin digestion and Orbitrap Elite Mass Spectrometry analysis were used for peptide sequence determination. To identify conidia cell wall proteins, peptide sequences were matched to the *N. crassa* proteome by a search of the annotated *N. crassa* genome at the National Center for Biotechnology Information database. The Big P and Signal P predictor programs were used to predict the presence of GPI anchor signals and N-terminal signal peptides (Eisenhaber et al., 2004; Petersen et al., 2011).

2.2. Strains and culture conditions

The deletion mutants for the genes encoding conidia cell wall proteins were taken from the single gene deletion library, which was obtained from the Fungal Genetics Stock Center. The individual mutants were routinely maintained on Vogel's sucrose medium slants. Matings were carried out as described by Davis and DeSerres (1970).

2.3. Testing for mutant phenotypes

The individual deletion mutants were tested for a number of conidial mutant phenotypes. The ability to generate conidia and the ability of the conidia to separate from one another were assessed microscopically. The ability of the conidia to survive an overnight freezing at −20 °C, the ability of the conidia to survive heating at 45 °C for 60 min, and the ability of the conidia to survive a 15 s sonication were all assessed by preparing conidial suspension at a concentration of 10^5 conidia/ml and then subjecting the conidia to the stress treatment. The percentage of the conidia that survived the treatment was determined by comparing the number of viable conidia (determined by plating the conidia on a Vogel's sorbose plate) from an aliquot of the conidia that had been taken prior to the treatment with the number of viable conidia from an aliquot of the conidia taken after the treatment. Germinating conidia were examined under the microscope to look for germling lysis phenotypes and for altered growth morphologies. Lastly, the linear growth rates for all the mutants were determined on Vogel's sucrose agar medium.

For each of the mutants that had a mutant morphology, co-segregation experiments were conducted to determine whether the mutant phenotypes co-segregated with the deletion mutations, which were marked by the presence of the hygromycin-resistance cassette. Twenty-four progeny from a mating between the deletion mutant and a wild type isolate of the opposite mating type were analyzed for the mutant phenotype and for the ability to grow on a hygromycin-containing Vogel's sucrose slant. The co-segregation of the mutant phenotype with the deletion mutation was taken as evidence that the deletion mutation was responsible for the mutant phenotype.

To further verify that the conidial separation phenotypes associated with deletions of *nag-1* (NCU10852) and *cgl-1* (NCU07523) were caused by the deletion mutations, complementation experiments were carried out. For *nag-1* the NAG-1CF and NAG-1CR primers (Table S1) were used to PCR amplify the gene from the wild type genome. The PCR amplified gene was inserted into the pMF272 plasmid (Bowman et al., 2009) that had been digested with NotI and EcoRI using the Clonetech In-Fusion cloning kit (Clontech, Mountain View, CA). The pMF272 plasmid allows for the selected integration of the plasmid into the *his-3* locus (Bowman et al., 2009; Freitag et al., 2004). The cloned gene was then used to transform a *Δnag-1*, *his-3* isolate and the ability of the cloned gene to complement the conidial separation mutant phenotype was assessed microscopically. Similarly the *cgl-1* gene was amplified with the CGL-1CF and CGL-1RF primers (Table S1) and inserted into the NotI and EcoRI digested pMF272. The cloned genes were then used to transform a *Δcgl-1*, *his-3* isolate and the ability of the cloned gene to complement the mutant phenotype was determined by microscopic examination.

2.4. Promoter cloning and expression patterns

To examine the expression patterns for the conidia cell wall proteins, the promoter regions from approximately 1800 base pairs upstream of the start of translation site to the start of translation site were inserted upstream of the GFP and RFP coding regions in