Published in final edited form as:

Fungal Genet Biol. 2016 September ; 94: 47–53. doi:10.1016/j.fgb.2016.07.003.

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

Jie Ao^{*,1}, Mash'el Aldabbous^{*,2}, Marysa J. Notaro¹, Mark Lojcono¹, and Stephen J. Free^{1,3}

¹Department of Biological Sciences, SUNY, University at Buffalo, Buffalo, NY 14260

²Department of Biological Sciences, Faculty of Science, Khaldiya, Kuwait University, Safat 13060, Kuwait

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.

Keywords

fungal cell wall; asexual development; conidia; glycosyl hydrolase; chitinase; glucanase

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.

³Corresponding author. Address: SUNY University at Buffalo, 109 Cooke Hall, Buffalo, NY 14260, United States., free@buffalo.edu (S.J. Free).

*Co-first authors.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

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 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; Maddi and Free, 2010; Maddi et al., 2012).

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 exochitinase 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 second 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 minute centrifugation at $5,000 \times G$, and washed two times with sterile distilled water. The cell walls were then resuspended in PBS containing 1% SDS and subjected to a 15 minute 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 trifluoromethanesulfonic 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 minute incubation in a boiling water bath, loaded onto an SDS PAGE, and subjected to a 5 minute 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 (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 minutes, and the ability of the conidia to survive a 15 second 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 Clontech 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 *nag-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 *cgl-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 pMF272 and pMF334 (Bowman et al., 2009; Freitag et al., 2004; Freitag and Selker, 2005). The primers used for PCR amplifying and cloning the promoter regions are given in Table S1. Using the InFusion cloning kit (Clontech, Mountain View, CA) we inserted the PCR amplified promoter regions into pMF272 that had been cut with NotI and SmaI or into pMF334 that had been cut with ApaI and EcoRI. The primers were designed such that the start of translation site from the promoter was fused in frame with the GFP or RFP coding region. The pMF272 and pMF334 plasmids are designed for insertional mutagenesis into the intergenic region downstream of the *his-3* gene during *N. crassa* transformation experiments. The plasmids contain the 3' portion of the *his-3* gene, and when used to transform a *his-3* mutant (FGSC# 6103) a wild type *his-3+* gene is generated via homologous recombination. This allows for the isolation of transformants containing the promoter::GFP or

promoter::RFP sequences as histidine prototrophs (Margolin et al., 1997). Promoter constructs were used in transformation experiments with a *his-3* isolate. Individual transformants containing the promoter::GFP and promoter::RFP constructs were rendered homokaryotic by 5 rounds of streaking conidia on sorbose plates (Davis and DeSerres, 1970). Expression of the promoter::RFP or promoter::GFP was assessed by examination of living cells under the fluorescence microscope.

3. Results

3.1. Proteomic analysis to identify cell wall proteins

Integral cell wall proteins are covalently attached to the glucan/chitin cell wall matrix. To release these cell wall proteins from the wall and to deglycosylate the proteins, we used the trifluoromethanesulfonic acid (TFMS) technique described in the Materials and Methods. TFMS cleaves glycosidic bonds, but leaves peptide bonds intact. The proteins released from the cell wall can then be directly used for proteomic analysis. Using this approach, we have been able to identify 35 cell wall proteins from the conidial cell wall (Table 1 and Table 2). Seventeen of these proteins have a C-terminal signal sequence for the addition of a GPI anchor (glycosylphosphatidylinositol anchor), a feature frequently found in cell wall proteins. Sixteen of the cell wall proteins have been previously identified in hyphae cell wall proteomic analyses (Maddi et al., 2009) and represent proteins that are in common between the two cell wall types (Table 1). The proteins found in common between the two types of cell walls included some cross-linking enzymes (glycosyl hydrolases/glycosyl transferases) that function to cross-link the glucan, chitin and cell wall proteins together to create the cell wall matrix (see Table 1). These include members of the GH-17, GH-18, and GH-72 families of cross-linking enzymes. The cross-linking proteins not only function to cross-link the matrix together, they are incorporated into the matrix, and thus serve as cell wall components as well as functioning as cross-linking enzymes.

In addition to these cross-linking enzymes we found eight “structural” cell wall proteins that were in common between the two types of cell walls (Table 1). These proteins do not have identified enzymatic functions and they have therefore been considered as being important “structural” elements of the wall. The proteins that hyphae and conidia share were among the most abundant conidia cell wall proteins as judged by the number of peptides we observed for these proteins in our analysis. Thirteen of these shared proteins have GPI-anchors.

What was particularly interesting in the analysis was that the conidia cell wall contained 19 proteins that were not found in the vegetative hyphae cell wall proteomic analysis (Table 2). These are likely to represent conidia-specific cell wall proteins and we found some additional glycosyl hydrolases from GH families not found in the hyphal cell wall, some additional “structural” cell wall proteins, and other miscellaneous proteins (Table 2). We hypothesize that some of these conidia-specific cell wall proteins may play roles in conidia differentiation and in mediating interactions between the conidia and their environment.

3.2. Phenotypic characterization of deletion mutants

In order to determine whether any of the proteins in the conidial cell wall were important for the differentiation and viability of the conidia, we carried out an extensive phenotypic analysis on mutants lacking these proteins. Deletion mutants for the genes encoding 34 of the 35 conidial cell wall proteins were taken from the *N. crassa* single gene deletion library (Colot et al., 2006), and examined for conidia-based phenotypes. Conidia from these deletion mutants were tested for viability, for the ability to survive a 45°C heating treatment, for the ability to withstand freezing at –20°C, for the ability to survive a sonication treatment, for vegetative hyphae linear growth rate, and for any morphological differences. We found that two of the mutants, NCU10852 and NCU07523, showed a very obvious conidial separation mutant phenotype (Fig. 1). All of the mutants were indistinguishable from the wild type in all of the other tests conducted except for NCU00673, which had a sensitivity to freezing. In the CAZy glycosyl hydrolase database, NCU10852 encodes a glycosyl hydrolase family 20 (GH-20) exochitinase, and the NCU07823 encodes a glycosyl hydrolase family 55 glucanase (GH-55 includes both endo and exo β -1,3-glucanases). These results suggest that the exochitinase and the glucanase are both required for the cell wall remodeling between adjacent conidia that is needed for the conidia to separate from one another to facilitate dispersal. Tzeleis et al (2012) previously demonstrated that NCU10852 was induced in fungal/fungal interactions and named the gene *nag-1* (N-acetylglucaminidase-1). We have opted to name the NCU07523 gene *cgl-1* (conidia β -glucanase gene 1).

To demonstrate that the deletions of *nag-1* and *cgl-1* were responsible for the conidia separation phenotypes, co-segregation experiments, as described in the Materials and Methods, were carried out. The deletion mutations are marked by the incorporation of the hygromycin-resistance cassette in place of the gene deletion. We found that in 24 of 24 progeny the mutant phenotype co-segregated with deletion mutations, as assessed by hygromycin resistance, which strongly suggests that the deletions were responsible for the conidia separation phenotypes.

Complementation experiments were carried out to verify the roles of NAG-1 and CGL-1 in modifying the conidia cell wall to facilitate cell separation. The DNA sequences from 1763 base pairs upstream of the *nag-1* coding region to 561 base pairs downstream of the stop codon were cloned, sequenced, and used to transform a *nag-1, his-3* isolate. The wild type gene was able to complement the conidia separation phenotype, demonstrating that the loss of NAG-1 caused the mutant phenotype (Fig. 1). Similarly, the DNA sequences from 3068 base pairs upstream of *cgl-1* coding region to 473 base pairs downstream of the gene were cloned, sequenced and used to transform a *cgl-1, his-3* isolate. We found that the conidia separation mutant phenotype was complemented by the wild type *cgl-1* gene. We conclude that both the exochitinase and the β -1,3-glucanase are required to digest the cell wall septum between adjacent conidia cells to allow the two conidia cells to separate from one another and facilitate their dispersal.

3.3. Analysis of conidia-specific cell wall protein promoters

In our proteomic analysis 19 cell wall proteins were identified that were present in the conidia cell wall, but not identified in the hyphal cell wall. To determine whether these represent proteins that are being expressed in a conidia-specific manner, we prepared a promoter::GFP or promoter::RFP construct for each of the genes encoding these proteins. Promoter regions containing approximately 1,800 base pairs of genomic DNA were used for these experiments. The promoter regions (which included the start of translation sites) were PCR amplified and inserted upstream of the GFP or RFP coding regions in pMF272 or pMF334. Table S1 give the primers used for the PCR amplifications of the promoter regions. The promoter::GFP and promoter::RFP plasmid constructs were used to transform *N. crassa* and the GFP and RFP expression patterns were assessed by fluorescence microscopy. We carefully examined the expression of RFP or GFP from representative promoter::RFP and promoter::GFP constructs in liquid-grown hyphae, hyphae grown on an agar medium, and during the process of conidiation (aerial hyphae and conidia) (Fig. 2 and 3). Fig. 2 contains images of a *rds-1* promoter::GFP-containing isolate grown on an agar medium. The merged DIC/GFP image shows that the *rds-1* promoter is not active in the large vegetative hyphae, the thinner meandering aerial hyphae, and the early stage conidia cells found in short conidia chains, but is highly expressed in the mature conidia. Fig. 3 contains images for a *cgl-1* promoter::RFP isolate. Brilliant RFP expression is evident in the mature conidia, while the large vegetative hyphae, thinner meandering aerial hyphae, and early stage short conidia chains show a minimal amount of RFP expression. The expression for each of the 19 promoters under each of the tested conditions is given in Table 2. We found that 15 of the 19 promoters gave a conidiation-specific expression. The remaining four promoters (from the *acw-4/ham-11*, *acw-12*, *ncw-7*, and NCU09209 genes) did not give a conidia-specific developmental expression pattern. We concluded that the genes encoding most of the conidia-specific cell wall proteins we found in our proteomic analysis were being transcribed in a conidia-specific manner and represent cell wall proteins that are unique to the conidia. These conidiation-specific proteins included the CGL-1 β -1,3-glucanase and the NAG exochitinase needed to remodel the cell wall to provide for conidia cell separation.

4. Discussion

Using a combination of proteomic and genetic approaches, we have characterized the *N. crassa* conidia cell wall. We found that like other fungal cell walls, the conidia cell wall contains a number of commonly-found structural proteins and cross-linking enzymes. These structural and cross-linking proteins are encoded by multi-gene families in the *N. crassa* genome and in the genomes of other fungi (Free, 2013). These cross-linking and structural proteins are found in virtually all cell wall proteomes and have been shown to play vital roles in the cell wall (Chaffin, 2008; Free, 2013; Klis et al., 2006; Latge, 2007). In most of the cell wall proteomes analyzed, multiple members from these cross-linking enzyme and structural gene families are found in a cell wall. For example, in an analysis of the GH-76 family of cross-linking enzymes used in incorporating proteins into the cell wall, we have previously shown that two enzymes (DFG-5 and DCW-1) with partially overlapping substrate specificity are expressed, which provide the cell wall with a redundancy of cross-linking activity (Maddi et al., 2012). We hypothesize that this strategy of using multiple

cross-linking enzymes with overlapping activities provides a robust system for generating the cell wall.

Our data shows that although the conidia and vegetative hyphae contain some cross-linking and structural proteins in common, each of the cell wall types has some cross-linking enzymes lacking in the other cell type. The data demonstrates that different combinations of cross-linking enzymes from these multigene families are being used in different cell types and that some of these cross-linking genes are being expressed in a cell type-specific manner. What our analysis of the conidia cell wall proteome adds to the conversation about these cross-linking enzymes is experimental evidence demonstrating that different members from these multi-gene families are being used at different points in the organism's life cycle and provides an additional rationale for the existence of these multigene families.

In this study we identified 35 cell wall proteins in conidia, and demonstrate that the conidia cell wall contains at least 15 conidia-specific cell wall proteins. We looked for conidial mutant phenotypes for 34 of the identified cell wall proteins and found that 32 of the cell wall protein mutants generated conidia that were indistinguishable from wild type conidia. There are at least three possible reasons why we were unable to identify a mutant phenotype for most of the conidia cell wall proteins. One possible reason is that there may be redundancy in the functions played by some of the proteins. The second possible reason is that our phenotypic analysis may not have included a set of conditions that would allow us to see a mutant phenotype. Finally, some of the proteins may play more minor roles in the conidial cell wall and loss of these proteins might not generate an easily observed mutant phenotype.

We found that there were substantial differences in the proteomes between the conidia and hyphal cell walls. We have previously demonstrated that there was a major difference in the glucans present in hyphae and conidia (Fu et al., 2014). The *N. crassa* conidial cell wall contains α -1,3-glucan, a glucan which is not found in the hyphal cell wall. The α -1,3-glucan was shown to play an important role in conidia development. Mutants that lack the glucan produce less than 5% of the conidia produced by wild type cells, and most of the mutant conidia are inviable. What was interesting is that two of the conidia-specific cell wall glycosyl hydrolases identified in our study were α -glucanases (NCU06010 and NCU09281) (Table 2). These enzymes may play a role in cross-linking the conidia-specific α -1–3-glucan into the cell wall matrix.

Although most of conidial cell wall protein mutants did not give a clear mutant phenotype, mutants for two enzymes, the NAG-1 exochitinase and the CGL-1 β -1,3-glucanase, had a dramatic conidia cell separation phenotype (Fig. 1). These proteins were among the more highly expressed conidia-specific cell wall proteins (Table 2). Co-segregation and complementation experiments demonstrated that the loss of the exochitinase or loss of the glucanase was responsible for the mutant phenotype. We concluded that the two enzymes play important roles in the differentiation of the conidia. These enzymes are needed to remodel the cell wall between adjacent conidia to allow for conidia separation and dissemination. The details of how the cell wall is remodeled during the terminal stages of differentiation to facilitate the separation between adjacent conidia has not been previously

characterized from an enzymatic perspective, although the importance of cell wall remodeling enzymes was clearly demonstrated in the analysis of the *csp-1* and *csp-2* mutants (Selitrennikoff et al., 1974). Our report provides the first identification of specific enzymes that function in the process of cell wall modification to facilitate cell separation during conidial development. Consistent with their role in remodeling the cell wall between adjacent conidia, the exochitinase and β -glucanase are expressed in a conidia-specific manner (Table 2, Fig. 3) and have the types of enzymatic activity that would be needed to digest the cell wall glucan/chitin matrix in the septa connecting adjacent conidia during their maturation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding for this study was provided by grant RO3-A1103897 from the National Institutes of Health, by the UB Foundation, and by Kuwait University. We thank Alan Segal for help with the confocal microscope imaging and Jim Stamos for help with manuscript preparation.

References

- Birkaya B, Maddi A, Joshi J, Free SJ, Cullen PJ. Role of the cell wall integrity and filamentous growth mitogen-activated protein kinase pathways in cell wall remodeling during filamentous growth. *Eukaryot Cell*. 2009; 8:1118–33. [PubMed: 19502582]
- Bowman BJ, Draskovic M, Freitag M, Bowman EJ. Structure and distribution of organelles and cellular location of calcium transporters in *Neurospora crassa*. *Eukaryot Cell*. 2009; 8:1845–55. [PubMed: 19801418]
- Bowman SM, Piwowar A, Al Dabbous M, Vierula J, Free SJ. Mutational analysis of the glycosylphosphatidylinositol (GPI) anchor pathway demonstrates that GPI-anchored proteins are required for cell wall biogenesis and normal hyphal growth in *Neurospora crassa*. *Eukaryot Cell*. 2006; 5:587–600. [PubMed: 16524913]
- Chaffin WL. *Candida albicans* cell wall proteins. *Microbiol Mol Biol Rev*. 2008; 72:495–544. [PubMed: 18772287]
- Colot HV, Park G, Turner GE, Ringelberg C, Crew CM, Litvinkova L, Weiss RL, Borkovich KA, Dunlap JC. A high-throughput gene knockout procedure for *Neurospora* reveals functions for multiple transcription factors. *Proc Natl Acad Sci USA*. 2006; 103:10352–7. [PubMed: 16801547]
- Davis RH, DeSerres FJ. Genetic and microbiological research techniques for *Neurospora crassa*. *Meth Enzymol*. 1970; 27:79–143.
- Eisenhaber B, Schneider G, Wildpaner M, Eisenhaber F. A sensitive predictor for potential GPI lipid modification sites in fungal protein sequences and its application to genome-wide studies for *Aspergillus nidulans*, *Candida albicans*, *Neurospora crassa*, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. *J Mol Biol*. 2004; 337:243–53. [PubMed: 15003443]
- Free SJ. Fungal cell wall organization and biosynthesis. *Adv Genet*. 2013; 81:33–82. [PubMed: 23419716]
- Freitag M, Hickey PC, Raju NB, Selker EU, Read ND. GFP as a tool to analyze the organization, dynamics and function of nuclei and microtubules in *Neurospora crassa*. *Fungal Genet Biol*. 2004; 41:897–910. [PubMed: 15341912]
- Freitag M, Selker EU. Expression and visualization of red fluorescent protein (RFP) in *Neurospora crassa*. *Fungal Genet Newsl*. 2005; 52:14–17.

- Fu C, Tanaka A, Free SJ. *Neurospora crassa* 1,3- α -glucan synthase, AGS-1, is required for cell wall biosynthesis during macroconidia development. *Microbiology*. 2014; 160:1618–27. [PubMed: 24847001]
- Greenwald CJ, Kasuga T, Glass NL, Shaw BD, Ebbole DJ, Wilkinson HH. Temporal and spatial regulation of gene expression during asexual development of *Neurospora crassa*. *Genetics*. 2010; 186:1217–30. [PubMed: 20876563]
- Klis FM, Boorsma A, De Groot PW. Cell wall construction in *Saccharomyces cerevisiae*. *Yeast*. 2006; 23:185–202. [PubMed: 16498706]
- Latge JP. The cell wall: a carbohydrate armour for the fungal cell. *Mol Microbiol*. 2007; 66:279–90. [PubMed: 17854405]
- Lee K, Ebbole DJ. Analysis of two transcription activation elements in the promoter of the developmentally regulated con-10 gene of *Neurospora crassa*. *Fungal Genet Biol*. 1998; 23:259–68. [PubMed: 9680956]
- Lesage G, Bussey H. Cell wall assembly in *Saccharomyces cerevisiae*. *Microbiol Mol Biol Rev*. 2006; 70:317–43. [PubMed: 16760306]
- Liu L, Free SJ. Characterization of the *Sclerotinia sclerotiorum* cell wall proteome. *Mol Plant Pathol*. 2015
- Maddi A, Bowman SM, Free SJ. Trifluoromethanesulfonic acid-based proteomic analysis of cell wall and secreted proteins of the ascomycetous fungi *Neurospora crassa* and *Candida albicans*. *Fungal Genet Biol*. 2009; 46:768–81. [PubMed: 19555771]
- Maddi A, Free SJ. α -1,6-Mannosylation of N-linked oligosaccharide present on cell wall proteins is required for their incorporation into the cell wall in the filamentous fungus *Neurospora crassa*. *Eukaryot Cell*. 2010; 9:1766–75. [PubMed: 20870880]
- Maddi A, Fu C, Free SJ. The *Neurospora crassa* *dfg5* and *dcw1* genes encode α -1,6-mannanases that function in the incorporation of glycoproteins into the cell wall. *PLoS One*. 2012; 7:e38872. [PubMed: 22701726]
- Margolin BS, Frietag M, Selker EU. Improved plasmids for gene targeting at the *his-3* locus of *Neurospora crassa*. *Fungal Genet Newsl*. 1997; 44:34–36.
- Petersen TN, Brunak S, von Heijne G, Nielsen H. SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nat Methods*. 2011; 8:785–6. [PubMed: 21959131]
- Ruiz-Herrera J, Elorza MV, Valentin E, Sentandreu R. Molecular organization of the cell wall of *Candida albicans* and its relation to pathogenicity. *FEMS Yeast Res*. 2006; 6:14–29. [PubMed: 16423067]
- Sachs MS, Yanofsky C. Developmental expression of genes involved in conidiation and amino acid biosynthesis in *Neurospora crassa*. *Dev Biol*. 1991; 148:117–28. [PubMed: 1834495]
- Selitrennikoff CP, Nelson RE, Siegel RW. Phase-specific genes for macroconidiation in *Neurospora crassa*. *Genetics*. 1974; 78:679–90. [PubMed: 4280981]
- Springer ML. Genetic control of fungal differentiation: the three sporulation pathways of *Neurospora crassa*. *Bioessays*. 1993; 15:365–74. [PubMed: 8357339]
- Springer ML, Yanofsky C. A morphological and genetic analysis of conidiophore development in *Neurospora crassa*. *Genes Dev*. 1989; 3:559–71. [PubMed: 2524423]
- Springer ML, Yanofsky C. Expression of con genes along the three sporulation pathways of *Neurospora crassa*. *Genes Dev*. 1992; 6:1052–7. [PubMed: 1534304]

Highlights

- Proteomic analysis of the *N. crassa* conidia cell wall identified 35 cell wall proteins.
- GFP and RFP promoter tagging experiments demonstrated that the conidia cell wall contains 15 conidia-specific cell wall proteins that are likely to carry out cell type-specific functions.
- The conidia-specific proteins including the NAG-1 exochitinase and the CGL-1 β -glucanase.
- The NAG-1 exochitinase and the CGL-1 β -glucanase are needed to remodel the conidial cell wall so that adjacent conidia cells can separation from each other and be disseminated

Fig. 1.

The NAG-1 exochitinase and CGL-1 β -glucanase are required for conidia cell separation.

DIC images of 7 day old conidia from wild type cells, the *-cgl-1* mutant, the *nag-1* mutant, and a *nag-1* mutant that has been transformed with a wild type copy of the *nag-1* gene showing that the wild type gene complements the mutant allele. The black bars represent a distance of 40 μ m.

Fig. 2.

Cell type-specific expression of the *rds-1* gene (NCU05143). The promoter region preceding the *rds-1* gene was cloned in front of the GFP coding region and the expression of the gene assessed in developing conidia. The left panel shows the GFP image, the middle panel a DIC image, and the right panel shows a merged image. The promoter directs expression in the maturing conidia, but not in vegetative hyphae or in early stage conidial development. The black bars represent a distance of 40 μm.

Fig. 3.

Cell type-specific expression of the *cgl-1* gene (NCU07523). The promoter region preceding the *cgl-1* gene was cloned in front of the RFP coding region and the expression of the gene assessed in developing conidia. The left panel shows the RFP image, the middle panel a DIC image, and the right panel shows a merged image. The promoter directs high level expression in the maturing conidia, but not in vegetative hyphae or in early stage conidial development. The black bars represent a distance of 40 μm.

Table 1

Proteomic analysis of cell wall proteins found in vegetative and conidial cell walls.

NCU# & Name ¹	Protein type ²	Peptides found ³
NCU08936* ACW-1 (CCG-15)	GPI-anchored cell wall protein	7 peptides
NCU07253* GEL-2	GH-72 β -1,3-glucanotransferase	9 peptides
NCU08755 GH3-3	GH-3 β -glucosidase	13 peptides
NCU05667* ACW-3	GPI-anchored cell wall protein 3	7 peptides
NCU06781* GEL-5	GH-72 β -1,3-glucanotransferase	10 peptides
NCU08909* GEL-1	GH-72 β -1,3-glucanotransferase	8 peptides
NCU09175* EGL-C	GH-17 β -1,3-glucanase	4 peptides
NCU05974* CRH-1	GH-16 β -1,3-glucanase	6 peptides
NCU09326 BGL-2	GH-17 β -1,3-glucanase	1 peptide
NCU09133* ACW-7	GPI-anchored cell wall protein	1 peptide
NCU07776* ACW-5	GPI-anchored CFEM protein	1 peptide
NCU02184* CHIT-1	GH-18 chitinase	1 peptide
NCU03013* ACW-10	anchored cell wall protein-10	2 peptides
NCU00957* ACW-2	GPI-anchored small protein	1 peptide
NCU03530* ACW-6	CFEM-domain-containing protein	1 peptide
NCU00716 NCW-5	small cell wall protein	1 peptide

Cell wall proteins identified in the proteomic analyses of conidial and vegetative cell walls.

¹ The name and NCU# for each of the proteins is given in the left-most column. An asterisk by the NCU # in the left-most column denotes that the protein is a GPI-anchored cell wall protein.

² Functional information about the role of the protein is found in the second column.

³ The third column indicates how many peptides were identified from the protein in the proteomic analysis.

Table 2

Proteins identified only in the conidial cell wall analysis.

NCU# & Name ¹	Protein type ²	Peptides found ³	GFP or RFP Expression ⁴
NCU10852 NAG-1	GH-20 exochitinase (mutant has a conidia separation phenotype)	10 peptides	C+, A-, V-, L-
NCU07523 CGL-1	GH-55 β -glucanase (mutant has a conidia separation phenotype)	8 peptides	C+++ , A-, L-, V-
NCU09791	GH-55 β -1,3-glucanase	5 peptides	C+, A-, V-, L-
NCU00673	protease k-like (mutant is sensitive to freezing)	2 peptides	C++, A-, V-, L-
NCU01162* GEL-3	GH-72 β -1,3-glucanosyltransferase	5 peptides	C+, A+, V-, L-
NCU00586 NCW-6	SUR7/PalI family	3 peptides	C+, A-, V-, L-
NCU04930	lipase	3 peptides	C++, A+, V-, L-
NCU03125 HET-C	HET-C protein	4 peptides	C+++ , A+, V+, L+
NCU05143 RDS-1	ferritin-like cell wall protein	1 peptide	C++, A-, V-, L-
NCU04605 NCW-8	Small cell wall protein	2 peptides	C+++ , A+, V-, L-
NCU01080*	GH-64 β -1,3-glucanase	1 peptide	C+, A+, V-, L-
NCU06010	GH-71 α -1,3-glucanase	1 peptide	C++, A-, V-, L-
NCU03083 NCW-9	Cell wall protein	1 peptide	C+, A-, V-, L-
NCU09281	GH-31 α -glucosidase	1 peptide	C+, A+, V-, L-
NCU02948 NCW-4	Cell wall protein	1 peptide	C++, A-, V-, L-
NCU09263* ACW-4 (HAM-11)	GPI-anchored small cell wall protein	1 peptide	C+, A+, V+, L+
NCU04493* ACW-12	GPI-anchored small cell wall protein	1 peptide	C+, A+, V+, L+
NCU09209	Galactose oxidase	1 peptide	C+, A+, V+, L-
NCU08907 NCW-7 (CCG-13)	Cell wall protein	1 peptide	C+++ , A+++ , V+++ , L-

Cell wall proteins identified in the proteomic analysis that were found in the conidial cell wall but not in previous analyses of the vegetative cell wall.

¹The name and NCU# for each of the proteins is given in the left-most column. An asterisk by the NCU # denotes that the protein is a GPI-anchored cell wall protein.

²Functional information about the role of protein is found in the second column.

³The third column indicates how many peptides were identified from the protein in the proteomic analysis.

⁴The fourth column indicates the cell types in which a promoter::RFP or promoter::GFP construct from the gene was expressed. C denotes expression in conidia. A denotes expression in aerial hyphae (the aerial hyphae give rise to conidia). V denotes expression in vegetative hyphae grown on an agar surface. L denotes express in liquid-grown vegetative hyphae. A- (minus sign) after the cell type designation indicates that the promoter is not expressed. The + (plus sign) after the cell type designation indicates that the promoter is expressed. A subjective measure of the level of expression is provide by the number of +'s (+ for weak expression, ++ for stronger expression, +++ for very strong expression).