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Trifluoromethanesulfonic acid-based proteomic analysis of cell wall and secreted proteins of the ascomycetous fungi *Neurospora crassa* and *Candida albicans*

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Abstract

Cell wall proteins from purified *Candida albicans* and *Neurospora crassa* cell walls were released using trifluoromethanesulfonic acid (TFMS) which cleaves the cell wall glucan/chitin matrix and deglycosylates the proteins. The cell wall proteins were then characterized by SDS PAGE and identified by proteomic analysis. The analyses for *C. albicans* identified 15 cell wall proteins and 6 secreted proteins. For *N. crassa*, the analyses identified 26 cell wall proteins and 9 secreted proteins. Most of the *C. albicans* cell wall proteins are found in the cell walls of both yeast and hyphae cells, but some cell type-specific cell wall proteins were observed. The analyses showed that the pattern of cell wall proteins present in *N. crassa* vegetative hyphae and conidia (asexual spores) are quite different. Almost all of the cell wall proteins identified in *N. crassa* have close homologs in the sequenced fungal genomes, suggesting that these proteins have important conserved functions within the cell wall.

Index descriptors

trifluoromethanesulfonic acid; fungal cell wall; cell wall protein; proteome; secretome; *Neurospora*; *Candida*; glycoprotein; cell wall organization

1. Introduction

The fungal cell wall comprises 30% of the dry weight of the cell and is a vital organelle (Klis, 1994). It provides mechanical strength and plays a major role in protecting the cell from various environmental stresses (de Nobel et al., 2000). It is a dynamic structure that is modified continuously to accommodate the growth of the cell and the transformation among the various fungal cell types (Bowman and Free, 2006). The components of the cell wall include polysaccharides like glucans, mannans and chitin, and cell wall proteins. The cell wall glycoproteins are extensively modified by the addition of N-linked and/or O-linked carbohydrates. Some of the glycoproteins receive a glycosylphosphatidylinositol (GPI) anchor at their C-termini while others do not. In *Saccharomyces cerevisiae* and *Candida albicans*, the

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GPI-anchored cell wall proteins (GPI-CWPs) and proteins with internal repeats (PIR-CWPs) are covalently attached to the cell wall glucan/chitin matrix, while other glycoproteins may be bound to cell wall proteins by disulfide bonds (de Groot et al., 2005; Yin et al., 2008). In *S. cerevisiae*, the GPI-anchored proteins are attached to the polysaccharides by a linkage to β -1,6-glucan while PIR-CWPs are attached by a linkage to β -1,3-glucan (Kapteyn et al., 1999).

Cell wall proteins play important roles during the life of fungi. They function in cell wall biosynthesis, adhesion, biofilm formation, interactions with the external environment, and activation of signal transduction pathways inside the cell (Bowman et al., 2006; Chaffin, 2008; Yin et al., 2008). In pathogenic fungi, cell wall proteins take part in various virulence mechanisms including invasion of the host tissue as well as evasion of the host immune surveillance. In the past, fungal cell wall proteins have been released from the cell wall by treatments with chemical reagents and enzymes that digest the cell wall glucan/chitin matrix (Klis, 1994). Enzymes like β -glucanases have been used for releasing covalently linked mannoproteins, alkali has been used for releasing PIR-CWPs, and treatment with hydrofluoric acid and pyridine have been used for extracting GPI-anchored CWPs (Castillo et al., 2008; de Groot et al., 2004). Reducing agents like β -mercaptoethanol (β ME) or dithiothreitol (DTT) have been used to isolate cell wall proteins from live fungal cells, where cells are incubated with β ME or DTT at 37°C for at least 30 min (Cappellaro et al., 1994; Casanova et al., 1992). However, β ME and DTT are moderately lipophilic (Klis et al., 2007), and extraction of cell wall proteins from live yeast cells may result in contamination of the cell wall protein extract with cytoplasmic proteins by leakage through the plasma membrane. Moreover incubation of live cells at high temperatures with lipophilic chemicals might arouse a stress response in the cells that could alter the cell wall composition.

Identification of the isolated cell wall proteins has been done traditionally by 2D gel electrophoresis where proteins are separated by their mass and charge. However, there are inherent problems in the separation of glycoproteins due to the variability in their charge as a result of the type of modification and the variability in their mass due to the extent of the modification (Gemmill and Trimble, 1999; Yin et al., 2008; Zeng and Biemann, 1999). To overcome these shortcomings “cell wall shaving” and “cell shaving” techniques have been proposed where fungal cell walls or cells respectively are incubated with proteolytic enzymes to release peptides that are identified by mass spectrometry (Yin et al., 2008). These techniques might be useful for releasing peptides from the outer or the inner surface proteins but may not be effective at penetrating the cell wall of living cells to release those proteins that are embedded within the cell wall. Moreover the heavy glycosylation may render the glycoproteins inaccessible to the proteases. To overcome such shortcomings we have turned to the technique of complete deglycosylation of the cell wall by using trifluoromethanesulfonic acid (TFMS), which cleaves all glycosidic linkages and releases whole proteins (Bowman et al., 2006; Edge 2003). The released proteins can be readily separated by SDS PAGE and subjected to trypsin digestion followed by nano-LC/MS/MS for their identification. The cell walls of *S. cerevisiae* and *C. albicans* have been extensively analyzed, but a very limited amount of information is available on the cell wall proteins of filamentous fungi (Bowman et al., 2006). We report a comprehensive analysis of the *Neurospora crassa* cell wall from both vegetative cells and conidia. In our analysis of the *C. albicans* cell walls, we identified a number of cell wall proteins previously identified by others (Castillo et al., 2008; Chaffin, 2008; de Groot et al., 2004; Ebanks et al. 2006), corroborating their results and verifying the utility of the TFMS procedure. A comparison of the covalently linked cell wall proteins from the *C. albicans* yeast and hyphae forms and from the *N. crassa* vegetative hyphae and conidia cells shows that some cell wall proteins are expressed in a cell-type specific manner.

Many cell wall proteins are released or shed into the culture medium. The set of proteins released or secreted into the external environment or culture media is referred to as the

secretome. The secretome may contain important cell wall proteins that function in cell separation, adhesion, cell fusion, biofilm formation, and virulence (Chaffin, 2008; Firon et al., 2007; Norice et al., 2007). Previous studies have identified the presence of classical proteins (proteins with an N-terminal signal peptide directing them into the secretory pathway) and non-classical proteins (proteins lacking an N-terminal signal peptide) as part of the *C. albicans* secretome (Chaffin, 2008; Hiller et al., 2007; Pitarch et al., 2005). In our analyses, most of the proteins identified in the *C. albicans* and *N. crassa* secretomes, were also found in the cell wall proteomes.

Our results show that TFMS, which cleaves the glucan-chitin cell wall matrix, is effective at releasing cell wall proteins in a single extraction step. TFMS was effective in deglycosylating the cell wall and the secretome proteins. The deglycosylated cell wall and secretome proteins can be readily separated by SDS PAGE and be identified by mass spectrometry.

2. Materials and Methods

2.1 Strains and Culturing Conditions

N. crassa wild-type strain 74-OR23-IVA was cultured in Vogel's-2% sucrose medium at room temperature (Davis and DeSerres, 1970). The *C. albicans* wild type strain SC5314 was cultured at 30°C in yeast nitrogen base (YNB) supplemented with complete amino acid supplement mixture (Q-Biogene) as described previously (Vylkova et al., 2007) to obtain yeast cells. For obtaining hyphae cells, *C. albicans* yeast cells were inoculated and grown in YNB medium supplemented with 20% fetal bovine serum (GIBCO-BRL) at 37°C. The cultures were examined visually to check for clumping of cells as well as under the microscope to confirm the transition to hyphae.

2.2 Cell Wall Preparations for *N. crassa*

N. crassa vegetative hyphae cells were grown in 1 L of Vogel's-2% sucrose liquid medium for 18 h at 25°C in a shaking incubator (150 rpm) and harvested by filtration on Buchner funnels. The harvested vegetative hyphae cultures were frozen with liquid nitrogen in a mortar, and ground to a fine powder with a pestle while intermittently adding liquid nitrogen. The ground hyphae were then resuspended in an extraction buffer consisting of PBS (Maniatis et al, 1982) containing 1% SDS.

Conidia were obtained by inoculating five 1 L Erlenmeyer flasks containing 200 mL of Vogel's-sucrose agar medium, and allowing the cultures to grow and produce conidia during a seven day incubation at room temperature. The conidia were harvested by adding sterile water to the Erlenmeyer flasks and vigorously mixing. The released conidia were freed of contaminating hyphae by pouring the released conidia through four layers of cheesecloth. The conidia were then collected by a centrifugation step, washed with PBS, and resuspended at a titer of 1.0×10^8 conidia per mL. One mL aliquots of the conidia were transferred to ice-cold Fast Prep tubes (MP Biomedicals, Solon, OH) and prepped for 6-12 cycles at 6.0 speed for 20 s, in a FastPrep machine (MP Biomedicals, Solon, OH) maintained at 4°C, with 1 min cooling on ice after each cycle.

To purify cell walls, disrupted hyphae and conidia preparations were first subjected to a centrifugation step (4,000g for 5 min) to separate the cell walls from the cytosolic proteins. The crude cell walls were washed once with the extraction buffer, and resuspended in extraction buffer. The resuspended cell wall pellets were boiled in the SDS-containing extraction buffer for 15 min, allowed to cool, and collected by centrifugation (10,000g for 5 min). The supernatants were collected as "SDS-solubilized cell wall protein" and the cell wall pellets were washed twice with ice-cold PBS and once with distilled water at 10,000g for 5 min. The

washed pellets were considered as “purified cell wall” and were lyophilized. We typically obtained 40 mg of vegetative hyphae purified cell wall and 30 mg of conidia purified cell wall. The purified cell walls were subjected to deglycosylation using TFMS, which completely solubilized the cell wall. Purified cell wall was also directly treated with trypsin (cell wall shaving) to compare the abilities of TFMS and trypsin to release proteins from purified cell walls.

2.3 Cell wall Preparations for *C. albicans*

C. albicans yeast and hyphae cells were cultured in 500 mL of YNB culture medium and collected in the late exponential growth phase by centrifugation at $4000 \times G$ for 5 min. The collected cells were then washed once in ice-cold PBS and resuspended in 10 mL of ice-cold PBS (approximate titer of 1.0×10^8 cells per mL). One mL samples were transferred to ice-cold Fast Prep tubes and prepped for 36-40 cycles at 6.0 speed for 20 s, in a FastPrep machine maintained at 4°C, with 1 min cooling on ice after each cycle. The resulting lysate was collected and transferred to 1.5 mL Eppendorf tubes on ice and centrifuged at 4,000g for 5 min at 4°C. The resulting supernatant was collected as cytosolic fraction and the pellets were washed two times with ice cold PBS and once with ice cold distilled water at 4,000g for 5 min. These pellets were considered as “cell walls”. The pellets were then resuspended in 1% SDS in PBS and boiled for 15 min. The samples were cooled to room temperature and centrifuged at 10,000g for 5 min. The supernatant was collected as “SDS-solubilized cell wall protein” in a separate tube and the pellets were considered as “SDS-resistant cell walls”. The “SDS-resistant cell walls” were washed twice with ice cold PBS and once with ice cold distilled water at 10,000g for 5 min, resuspended in ammonium carbonate buffer (1.89 g/liter; pH 8.29) with 1% (v/v) β -mercaptoethanol (β ME) and incubated at 37°C with shaking at 150 rpm for 30 min (Li et al., 2006). The extract was centrifuged at 10,000g for 5 min at 4°C and the supernatant collected as β ME-solubilized cell wall protein. The pellet was collected as “purified cell wall”. The pellet was washed twice with ice cold PBS and once with ice cold distilled water at 10,000g for 5 min. The purified cell wall preparations were lyophilized and subjected to TFMS. A typical 500 mL culture of yeast or hyphae gave 30-40 mg of purified cell wall.

2.4 Isolation of proteins released into the medium

Secreted proteins from *C. albicans* yeast cells were obtained from YNB medium in which wild-type cells had grown for 18 h in a 30°C shaking incubator. The cells were removed by centrifugation and proteins were collected for analysis by TCA precipitation. The proteins released into the medium from *N. crassa* vegetative cells growing for 18 h at 25°C in 500 mL of shaking Vogel's liquid medium were obtained by filtering the medium through three layers of Whatman #1 paper on a Buchner funnel, to remove the vegetative hyphae and subjecting the filtrate to TCA precipitation. To determine if we could see differences in the *N. crassa* secretome under different medium conditions, we also purified proteins from a starvation medium. *N. crassa* cells were grown in 500 mL of Vogel's liquid sucrose medium for 18 h in a shaking incubator, collected on a Buchner funnel, and resuspended in 500 mL of Vogel's liquid medium that was devoid of a carbon/energy source. After 2 h of incubation in the shaking starvation medium, the cells were collected again on a Buchner funnel and the proteins released into the medium were collected by TCA precipitation.

Trichloroacetic acid (TCA) precipitation was used to collect the secreted proteins from the *C. albicans* and *N. crassa* media. Acetone and TCA were added to the media to a final concentration of 50% acetone, 12.5% TCA, and the proteins were allowed to precipitate for 24 h at -20°C. The precipitated proteins were collected by centrifugation, washed twice with -20°C acetone, and lyophilized. The lyophilized protein samples were then subjected to TFMS. We obtained between 10 and 15 mg of secreted protein from 500 mL cultures of *C. albicans* and *N. crassa*.

2.5 Trifluoromethanesulfonic acid (TFMS) treatment of cell walls and secreted protein

To analyze the cell wall and secreted proteins, the samples were treated with TFMS using a procedure described previously (Bowman et al., 2006; Edge, 2003). TFMS, anisole, and pyridine were obtained from Sigma Aldrich Chemical Company (St. Louis, MO). Secreted protein samples and cell wall pellets were lyophilized overnight to ensure complete dryness of the samples. To maintain anhydrous conditions during the TFMS treatments, all glass tubes and syringes used were dried under a vacuum and the procedures were performed in a chamber being continually purged with liquid nitrogen. Initially, a solution of 16% anisole in TFMS acid was prepared and 1.25 mL of this mixture was added to 20 mg of “purified cell wall” sample or to a sample of secreted protein collected from 500 mL of culture medium. The samples were then purged with liquid nitrogen, quickly covered with parafilm, and placed in the N₂-filled chamber at 4°C. During the course of the reaction, the samples were periodically mixed with a Pasteur pipette, purged with N₂ gas, and covered again with parafilm. The *N. crassa* cell wall samples and the samples of secreted protein were completely solubilized in 5 h, as assessed by visual inspection. The *C. albicans* cell walls, however, required an 18 h treatment before they were completely solubilized. After the cell walls had been solubilized, 3.75 mL of a solution of pyridine/methanol/H₂O (3:1:1) were added in a drop-wise fashion to each of the digests, which were continually swirled in a dry ice-ethanol bath. The samples were then left in the dry ice-ethanol bath for 20 min, followed by incubation for another 20 min at -20°C. The samples were removed from -20°C, allowed to thaw, and 1 mL of 5% ammonium bicarbonate solution was added to each. The released proteins were then precipitated by adding 6 mL of 25% TCA in acetone and incubating at -20°C for 24 h. The precipitated proteins were collected by centrifugation at 10,000g, washed three times in ice-cold 100% acetone, briefly dried, and resuspended by boiling in 1% SDS. Protein concentrations of all samples were determined using the Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hercules, CA). The amount of protein released from 5 mg of starting cell wall material was separated by SDS-PAGE on a 4 to 12% Bis-Tris NuPAGE gel and visualized using the SilverQuest silver staining kit (Invitrogen Life Technologies, Carlsbad, CA) or with Coomassie blue (Maniatis et al., 1982).

2.6 Nano-LC/MS/MS analysis of peptides

Samples of β ME-solubilized cell wall proteins and integral proteins released by TFMS treatment were subjected to SDS PAGE. For nano-liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS)-based identification the protein samples were loaded onto an SDS-polyacrylamide gel and subjected to a very brief electrophoresis step. The electrophoresis was stopped when the dye front had traveled 5 mm into the gel. The gels were stained with Coomassie brilliant blue and 5 mm gel slices containing the proteins were sent to Midwest Bio Services (Overland Park, KS). The samples were treated with trypsin to generate peptide fragments. The mixture of peptides resulting from trypsin digestion was then concentrated on a peptide trap column followed by a wash to remove any salts and impurities. The resulting peptides were then separated on a microcapillary C18 reverse-phase chromatography column. PicoFrit columns (New Objective, Inc, Woburn MA) were used to directly spray the peptides into the mass spectrometer without any post-column losses. Precursor peptide ions were isolated and fragmented inside the ion trap of the mass spectrometer (LCQ Deca XP Plus ion trap mass spectrometer, Thermofannigan). The resulting daughter ions were detected in a tandem mass spectrometry experiment to obtain the full MS/MS spectra. Typically the samples were analyzed for 90 min to acquire at least 1200 MS/MS spectra, which is sufficient for the identification of hundreds of peptides. The dynamic exclusion function of the instrument helps in the detection of low abundance peptides.

2.7 Data analysis (MS/MS database search)

The peptide sequences were inferred by matching the MS/MS spectra to the protein sequences at the *C. albicans* genome database (www.candidagenome.org) or to the protein sequences for the *N. crassa* genome available at the Broad Institute (www.broad.mit.edu) using the TURBOSEQUENT software. Only those proteins with multiple peptides and/or single peptides with a score XC (Correlation coefficient) of >2.5 for 2 ions or >3.0 for 3 ions were accepted as accurate identifications in this analysis.

3. Results

3.1 Proteins identified in the *C. albicans* yeast and hyphae cell walls

TFMS can be used to specifically cleave glycosidic linkages while leaving peptide bonds intact. TFMS treatment of glycoproteins leaves a single N-acetyl-glucosamine residue from N-linked oligosaccharides attached to the asparagine residue, and removes all of the sugars associated with O-linked oligosaccharides (Bowman et al., 2006; Edge 2003). As a way to evaluate and validate our TFMS-based procedure for identifying cell wall proteins, we carried out an analysis of the *C. albicans* cell wall. The proteins of *C. albicans* cell wall have been previously identified in a number of studies, including studies in which the glucan/chitin matrix was removed by enzymatic treatment (de Groot et al., 2004). *C. albicans* cell walls from yeast and from hyphae were prepared as described in the Material and Methods section. The purification included a step in which proteins were extracted from the cell wall with a boiling SDS treatment to release any proteins that were not covalently attached to the cell wall. We also included a second extraction step using β -mercaptoethanol (β ME) in an alkaline solution to extract proteins that were attached to the cell wall by disulfide bonds and alkali-labile linkages. Each of these extraction steps released a number of proteins from the cell wall preparation. As shown in Fig. 1, there are clear differences in the pattern of proteins released as “SDS-solubilized cell wall proteins”, “ β ME-solubilized cell wall proteins” and the “integral cell wall proteins” that are only released after TFMS treatment of the glucan/chitin cell wall matrix. This result demonstrates that the purification procedure was effective at fractionating the proteins associated with the cell wall. Note that the proteins observed in the TFMS-solubilized protein lane appear as sharp bands suggesting that the oligosaccharides, which usually give size heterogeneity to individual glycoproteins, have been removed.

The hyphae cell walls were subjected to the same purification procedure as the yeast cell walls. The purified cell walls were digested with TFMS and the purified proteins were subjected to electrophoresis in SDS-polyacrylamide gels. As shown in Fig. 1 there are a number of covalently linked cell wall protein bands that are in common between the yeast and hyphae cell walls, but there are also some differences. We considered the proteins released by β ME and the proteins that remained in the cell wall as being cell wall proteins. To identify these *C. albicans* cell wall proteins, we carried out LC/MS/MS analyses on the proteins released by β ME and TFMS treatments from the yeast and hyphae cell walls. We considered the proteins released by the boiling SDS to be a mixture of cell wall proteins and cytosolic proteins that associate with the wall upon cell lysis, and these proteins were not further characterized in our analysis. As detailed in Table 1, we identified 15 cell wall proteins that were released by the TFMS treatment. Four of these proteins (Mp65/Scw1, Pga29/Rhd3, Phr2, and Crh11), were found in both yeast and hyphae cell walls. Five of the proteins (Pga24/Ywp1, Pir1, Cht2, Als4, and Pga2/Sod4) were identified in the yeast cell wall but not hyphae cell wall. Six of the proteins (Pga62/Flo1, Csa1/Wap1, Als3, Hwp1, Rbt1, and Tos1) were identified in the hyphae cell wall but not in the yeast cell wall. Some of the proteins that were identified in only one of the two cell types might have a cell-type specific expression pattern, as has been shown immunologically for Hwp1 and Ywp1 (Granger et al., 2005; Staab and Sundstrom, 1998). However, because the analysis isn't able to routinely identify all of the cell wall proteins present

in a sample, additional experiments will be needed before we can definitively conclude that the proteins we identified in only one of the two cell types have a cell-type specific expression pattern. Hwp1 is a cell wall protein that is required for normal cell wall development and its null mutants exhibit a virulence defect in mice (Plaine et al., 2008; Sundstrom et al., 2002). Hwp1 was previously shown to localize to hyphae surfaces by using immunofluorescent antibodies raised against its recombinant protein produced in *Pichia pastoris* (Staab and Sundstrom, 1998). Rbt1, a cell wall protein with homology to Hwp1, has been found to be required for virulence in a mouse systemic candidiasis model (Braun et al., 2000). Rbt1 null mutants have an abnormal cell wall (Plaine et al., 2008). Pga62 is a putative GPI-anchored protein and its null mutants have an abnormal cell wall (Plaine et al., 2008). Tos1 is a secretory protein with an N-terminal signal peptide whose function is not known. All of the proteins we identified in our TFMS-based analysis have been previously identified as being components of *C. albicans* cell walls, and most of the proteins others have identified by proteomic and immunological analyses were found in our analysis (Castillo et al., 2008; Chaffin, 2008). We conclude that the TFMS-based approach is effective at releasing and identifying cell wall proteins. Many of these proteins are thought to be involved in cell wall biogenesis and repair, and the remaining proteins are thought to be cell wall structural elements.

We identified 3 classical cell wall proteins, Scw1, Cht2 and Tos1, in the yeast β ME-solubilized cell wall fraction. Two of these proteins, Scw1 and Cht2, had been previously identified as being released by β ME treatment by Castillo et al. (2008). We found that the proteins from the fetal bovine serum became major contaminants in the β ME-solubilized fraction of the hyphae cell wall, but found no serum proteins in the cell wall proteins released by the TFMS treatment (Fig. 1) indicating that β ME was effective at removing proteins not integrated into the cell wall. We ascribe the presence of serum proteins in the cell wall to trapping and perhaps cross-linking of these proteins, which are found at high concentrations in the medium, into the cell wall. We also identified 50 “non-classical” cell wall proteins in our preparation (Supplemental data). Most of these had been identified previously in other studies (Castillo et al, 2008; Ebanks et al, 2006). A majority of these “non-classical” cell wall proteins (45 of the 50) were identified in the β ME- solubilized fractions, but 13 of these were identified in the cell wall protein fraction and released after the TFMS treatment, suggesting that some of these “non-classical” proteins may be tightly associated with the cell wall and might be cross-linked into the glucan/chitin matrix.

To compare the identification of proteins released from *C. albicans* cell walls by the TFMS-based approach and by a trypsin digestion of the cell wall (cell wall shaving) without having removed the glucan/chitin matrix, we treated 20 mg samples of purified yeast and hyphae cell walls with trypsin, and subjected the released peptide fragments to nano-LC/MS/MS. We were able to identify 4 proteins by this method, as compared to the 15 proteins identified after TFMS treatment. All 4 of the proteins we identified by treating the purified cell walls with trypsin (Mp65/Scw1, Pga29/Rhd3, Phr2, and Pga2/Sod4), were ones we had identified in the TFMS-released protein fraction. We identified 28 peptides from the TFMS-released protein fractions as compared to 5 peptides from the trypsin digestion of the purified cell walls (Table 1). In our analysis, the TFMS-based treatment of the glucan/chitin matrix was superior to “cell wall shaving” in identifying cell wall proteins.

We conclude that the TFMS-based treatment is an effective way to digest the *C. albicans* glucan/chitin cell wall matrix and release deglycosylated proteins for proteomic identification. Inspection of the amino acid sequences from many of the *C. albicans* peptides we identified from the TFMS-treated cell wall shows many of these peptide fragments have serine and threonine residues, residues that might well have had O-linked glycosylation. It is interesting to note that 12 of the 15 proteins we identified in our analyses are predicted to be GPI-anchored proteins (de Groot et al., 2003, Eisenhaber et al., 2004). Others have previously noted that most

of the “classical” *C. albicans* cell wall proteins identified by proteomic analysis are predicted to be GPI-anchored proteins (Castillo et al., 2008; de Groot et al., 2004).

3.2 TFMS-based analysis of protein released into the medium by *C. albicans* yeast cells

Many cell wall proteins are shed into the medium along with secreted proteins. To examine these proteins, we carried out an analysis of the proteins released from *C. albicans* yeast cells grown in YNB medium. In a previous analysis, Hiller et al. (2007) used trypsin digestion of the proteins released from yeast cells into YNB medium and identified 5 classical cell wall proteins (Mp65/Scw1, Tos1, Ywp1/Flo1, Sim1/Sun42, Sun41). Using the TFMS-based treatment approach, we were able to identify 11 proteins from the growth medium (Table 2). Of these 11 proteins, 5 were in common with the proteins we had identified in the yeast cell wall fraction (Mp65/Scw1, Tos1, Cht2, Pir1, and Pga24/Ywp1) and two of these five (Cht2 and Pga24/Ywp1) are predicted to have GPI-anchors (de Groot et al., 2003; Eisenhaber et al., 2004). These GPI-anchored proteins would have to be cleaved from their GPI-anchor before being released into the medium. Five of the remaining 6 proteins identified in the medium were proteins that have previously been identified in *C. albicans* analyses (Exg1/Xog1, Scw11, Sim1/Sun42, Cht3, and Bgl2). Two of these proteins, Sim1/Sun42 and Bgl2, have been identified as cell wall proteins (Castillo et al., 2008; Pitarch et al., 2006; Sosinska et al., 2008). The final protein we identified in the medium was Msb2. In *S. cerevisiae*, Msb2 is involved in signal transduction events, and a cleavage product of Msb2 is released into the medium (Vadaie et al., 2008).

We did not find any non-classical cell wall proteins in the yeast growth medium, although others have reported finding such proteins in the medium (Chaffin, 2008). We were unable to get proteomic data on the proteins being released into the hyphae growth medium. The hyphae growth medium contains 20% fetal calf serum, and all of the proteins identified in the analysis came from the serum.

Among the peptide fragments we obtained in the analysis of hyphae cell walls, yeast cell walls, and yeast secreted protein (see Tables 1 and 2), were many with likely candidate sites for serine and threonine O-glycosylation. We ascribe the ability to identify so many such fragments to the fact that TFMS is effective in removing glycosylation. This was helpful in the identification of cell wall proteins released into the growth medium as well as for the identification of cell wall glycoproteins.

3.3 Proteins identified in the cell wall preparations from *N. crassa* vegetative hyphae and conidia

The *N. crassa* cell wall is known to be a dynamic structure that changes during the life cycle. For example, the hydrophobin, EAS, is expressed at high levels on the surface of the conidia (asexual spores), but not at other times in the life cycle (Beever and Dempsey, 1978). Similarly, melanization of the cell wall occurs during the maturation of the perithecia (female mating structure) and in the cell walls of the ascospores (sexually produced spores), but not in cell walls of other cell types. To identify and characterize cell wall proteins that are integrated into the glucan/chitin polymer matrix we purified cell walls from vegetative hyphae grown in a shaking liquid medium and from conidia, and carried out a proteomic analysis on these proteins. These two cell types were chosen for the analysis because they can be isolated without contamination from other cell types. To remove any proteins that were not covalently linked to the polymer matrix and restrict our analysis to covalently linked cell wall proteins, the cell walls were boiled in an SDS solution, as part of the purification process. Fig. 2 shows that the boiling SDS step removes a large number of proteins from the cell wall preparation. Some of these proteins may be cytosolic proteins that adhere to the cell wall after the cell is lysed, while others are cell wall proteins that are not cross-linked into the glucan/chitin cell wall matrix.

Treatment of *N. crassa* cell walls with β ME in an alkaline solution does not release a significant amount of proteins from the *N. crassa* cell wall (personal observation), and so this step was not included in the *N. crassa* cell wall purification. A 5 h treatment with TFMS completely solubilizes the *N. crassa* cell wall. Following TFMS treatment, the released cell wall proteins were precipitated and subjected to SDS-PAGE. Fig. 2 shows the pattern of cell wall proteins released from the vegetative hyphae and conidia cell walls. A comparison of the pattern of proteins in the SDS-gels shows that there are clear differences in the covalently linked cell wall proteins found in these two cell types. Note that the released proteins are found as sharp bands on the gel, indicating that TFMS treatment successfully removed the oligosaccharides that would otherwise have given a larger range of molecular weights for each of the released proteins.

To identify some of these proteins, samples of the TFMS-released proteins were sent for nano-LC/MS/MS analysis. The analyses identified 26 proteins from the *N. crassa* genome database which contained typical N-terminal signal peptide sequences. These proteins are listed in Table 3 and include both cell wall structural proteins and proteins that have homology with cell wall biogenesis and remodeling enzymes found in other fungi. We have used the designation of ACW (GPI-anchored cell wall protein) and NCW (non-anchored cell wall protein) to name *Neurospora* cell wall proteins that do not have an associated enzymatic activity as defined by a homology search. Ten of the identified proteins, ACW-1/CCG-15 (NCU08936), ACW-2 (NCU00957), ACW-3 (NCU05667), ACW-6 (NCU03530), GH17-3/glucan- β -glucanase (NCU09175), GH55-3/GEL-2 (NCU07523), GH72-2/GEL-5 (NCU06781), CAT-3/catalase (NCU00355), NCW-3 (NCU07817), and NCW-5 (NCU000716) were found in vegetative cell walls and conidia cell walls. Twelve of the identified proteins were found only in the cell walls from vegetative cells. These were ACW-5 (NCU07776), ACW-7 (NCU09133), ACW-8 (NCU07277), ACW-9 (NCU06185), ACW-10 (NCU03013), ACW-11 (NCU02041), GH16-1/mixed linkage glucanase (NCU01353), GH16-7/glycoside hydrolase (NCU05974), CHIT-1/endochitinase (NCU02184), NCW-1 (NCU05137), β -glucosidase (NCU09326), and NCW-2 (NCU01752). Four of the proteins were found in conidia cell walls but not in the vegetative hyphae cell walls. These proteins were GH3-3/ β -glucosidase 1 precursor (NCU08755), NCW-4 (NCU02948), NCW-6 (NCU00586) and NCW-7/CCG-13 (NCU08907). All 26 of these identified cell wall proteins are either likely cell wall structural proteins or cell wall biosynthetic/remodeling enzymes. Sixteen of these proteins are predicted to have GPI anchors (de Groot et al., 2003, Eisenhaber et al. et al., 2004). These GPI anchored proteins include ACW-1/CCG-15 (NCU08936), ACW-2 (NCU00957), ACW-3 (NCU05667), ACW-5 (NCU07776), ACW-6 (NCU03530), ACW-7 (NCU09133), ACW-8 (NCU07277), ACW-9 (NCU06185), ACW-10 (NCU03013), ACW-11 (NCU02041), GH17-3/glucan- β -glucanase (NCU09175), GH16-1/mixed linkage glucanase (NCU01353), GH16-7/glycoside hydrolase (NCU05974), CHIT-1/endochitinase (NCU02184), GH55-3/GEL-2 (NCU07523), and GH72-2/GEL-5 (NCU06781). The data, along with an inspection of the SDS gels, clearly indicates that the cell walls from the two types of cells differ in their protein content. Some of the proteins identified in only one of the two cell types might be cell type-specific cell wall proteins. The SDS gels contain more protein bands than we were able to identify with the LC/MS/MS analysis. This is a common finding with LC/MS/MS analysis because only a subset of the fragments released by the trypsin digestion used to generate material for the analysis are able to be analyzed. The analysis would also miss minor constituents of the cell wall, whose concentration is below the level detected in the analysis. Because the analysis isn't able to routinely identify all of the cell wall proteins present in a sample, additional experiments will be needed before we can conclude that any of the proteins have a cell-type specific expression pattern. In addition to the proteins mentioned above, we also identified many "non-classical" cell wall proteins (proteins without a signal peptide) in our analyses (Supplemental data). Such "non-classical cell wall proteins" are routinely found in fungal cell wall preparations. Our analysis of the *N. crassa* cell wall was confined to looking at integral cell wall proteins, and

would miss many fungal cell wall proteins that can be removed from the cell wall by boiling in an SDS solution. This explains why our analysis failed to identify the conidia-specific hydrophobin EAS.

Since the TFMS treatment leaves a single N-acetyl-glucosamine residue attached to the asparagine of N-linked oligosaccharide sites, we reasoned we might be able to identify such sites by looking for peptides with a modified asparagine residue. We analyzed our *N. crassa* proteomic data for the presence of peptide fragments containing such asparagine modifications and found three such peptide fragments. These were two fragments from the GPI-anchored protein ACW-1 (FDNNKFDSFSFPN*LTETK and NIDAIN*VTSIK) and a fragment from the CAT-3 protein (GYPAQAN*QTVGR). The N* in these sequences denotes an asparagine residue coupled to N-acetyl-glucosamine. These glycosylated fragments come from proteins we had already identified in our analysis. In each of these cases, the context of the modified asparagine was a predicted site for N-linked glycosylation (N-X-S/T). We conclude that the TFMS treatment is able to help identify N-linked glycosylation sites.

Some cell wall proteins have been identified by treating purified cell walls with trypsin (cell wall shaving), without having first removed or digested the glucan/chitin cell wall matrix. Regions of cell wall proteins that are accessible to the trypsin can be released and identified by LC/MS/MS analysis. To compare the cell wall shaving approach with the TFMS approach, we treated 20 mg of purified conidia and vegetative hyphae cell walls with trypsin and analyzed the released peptide fragments with LC/MS/MS. As shown in Table 3, we identified a much larger number of peptide fragments in the TFMS-based analysis than from the cell shaving approach. In comparison with the 26 proteins (from 76 peptides) we identified from TFMS-treated cell walls, we were able to identify 5 proteins (from 8 peptides) using this approach. Four of these 5 had been identified in the TFMS treated samples. The only new protein identified by the cell wall shaving approach, GH3-3/ β -glucosidase 1 precursor (NCU08755), was released from the conidia cell wall and identified by a single peptide fragment (Table 3). We conclude that the TFMS treatment to remove the glucan/chitin matrix and deglycosylate cell wall proteins greatly enhances the identification of *N. crassa* cell wall proteins. Inspection of the amino acid sequences from many of the peptides we identified from the TFMS-treated cell wall shows many of these peptide fragments have serine and threonine residues that might well have had O-linked glycosylation, and identification based on such peptides requires that all of the O-linked glycosylation was removed.

3.4 TFMS analysis of proteins released into the medium by vegetative *N. crassa* cells

To identify some of the major proteins released from *N. crassa* vegetative cells into the medium, we grew vegetative hyphae in a shaking liquid culture and purified the proteins with a TCA/acetone precipitation step as described in Materials and Methods. To determine if the release of protein into the medium was dependent upon the culture conditions, we grew hyphae in a liquid culture and transferred the cells to a carbon source-free medium (starvation medium) for 2 h and then purified the proteins secreted into the starvation medium with TCA/acetone precipitation. The purified protein samples were treated with TFMS and analyzed with LC/MS/MS to identify the proteins released into the medium under these two conditions. SDS PAGE analysis of the proteins from the two media (Fig.2) shows that there are clear differences in the pattern of proteins released into the medium from actively growing vegetative cells and from cells that have been placed in a starvation medium. From the analysis of these two growth media, we identified 17 proteins (Table 4). Five of these were found in the sucrose-containing growth medium, 2 in the starvation medium, and 10 were found in both media. The identified proteins included three enzymes (GLA-1/glucamylase (NCU01517), INV/invertase (NCU04265) and ASD-1/SDV-10/rhamnogalacturonidase (NCU05598), which might function in releasing sugars from oligosaccharides present in the environment (Sigmund et al.,

1985). Eight of the proteins had been identified as cell wall proteins in the TFMS treatment of the cell wall. These were ACW-1/CCG-15 (NCU08936), ACW-2 (NCU00957), ACW-3 (NCU05667), ACW-7 (NCU09133), GH17-3/glucan-B-glucanase (NCU09175), GH16-7/glycoside hydrolase (NCU005974), NCW-1 (NCU05137), and CAT-3/Catalase (NCU00355). Eight of the proteins we identified in the medium (Table 4) are predicted to be GPI-anchored (de Groot et al., 2003; Eisenhaber et al., 2004), and would need to be cleaved from their GPI-anchors in order to be released into the medium. We also identified, in the starvation medium, 12 “non-classical” cell wall proteins (Supplemental data). These could have been “non-classical cell wall proteins” that were shed from the cell wall into the medium or proteins that were released into the medium by cell lysis. Based on the SDS PAGE analysis, and the identification of proteins from the medium, we conclude that the release of proteins into the medium is dependent upon the culture conditions and that a number of cell wall proteins are being shed into the medium.

4. Discussion

The fungal cell wall proteins carry out a number of important functions in pathogenic and non-pathogenic fungi. They provide for structural integrity, protect the cell from oxidative stress (Fradin et al., 2005), take part in flocculation and mating (de Groot et al., 2005), signal transduction (Cullen et al., 2004), adhesion to epithelial cells and biofilm formation (Li et al., 2007; Nobile et al., 2006), and help in the invasion of host tissues (Phan et al., 2000). Additional functions may well be discovered as research progresses. Cell wall components are thought to be ideal for vaccine development. The isolation and identification of cell wall proteins have been experimentally challenging because the integral proteins are cross-linked to the glucan/chitin matrix of the cell wall and have to be released before they can be analyzed and identified. Cell wall proteins are heavily glycosylated, which protects them from enzymatic digestion. In the present study we have performed an extensive proteomic analysis to identify the cell wall proteomes of the ascomycetous fungi *N. crassa* and *C. albicans* by a method which involves the cleavage of the cell wall glucan/chitin matrix and deglycosylation of cell wall proteins with the chemical agent, TFMS.

C. albicans is a clinically important dimorphic pathogenic fungus. It causes mucosal infections like oropharyngeal candidiasis (OPC) in immunocompromised patients and serious blood stream infections in hospitalized patients. Approximately 70% of American women suffer from vaginal infections caused by *C. albicans* during their life time (Fidel, 2004). In this study we have developed a sequential extraction protocol that includes a boiling SDS step to remove proteins not attached covalently to the cell wall, an alkali/βME extraction step to remove proteins attached to the cell wall through bonds that are susceptible to alkali or reducing conditions, and a TFMS treatment to digest the glucan/chitin matrix of the cell wall and release integral cell wall proteins. We found that treatment of the cell wall using TFMS was effective at releasing cell wall proteins. In the present study we confirm many of the previously identified *C. albicans* cell wall proteins (Castillo et al., 2008; de Groot et al., 2004). The yeast to hyphae transition in *C. albicans* is associated with virulence in the human host (Kumamoto and Vines, 2005). Previous studies that have been done to elucidate the differences in the cell wall proteomes of the yeast and the hyphae forms have mainly identified non-classical cell wall proteins (Ebanks et al., 2006; Pitarch et al., 2002; Urban et al 2003). Our analysis of the covalently linked cell wall proteins by deglycosylation of cell walls from *C. albicans* yeast and hyphae forms demonstrated that the two cell wall types share a number of cell wall proteins, but that there could also be some cell-type specific differences (Fig. 1). Proteomic analyses of the two types of cell walls identified covalently linked cell wall proteins that might be expressed in a cell-type specific manner (Table 1). We also identified a large number of non-classical cell wall proteins in *C. albicans* yeast and hyphae cell walls (Supplemental data).

As part of our study, we documented the secretome of the *C. albicans* yeast cell. Previous studies have identified the presence of non-classical proteins in the secretome, along with a few classical cell wall proteins (Chaffin, 2008; Hiller et al., 2007; Pitarch et al., 2006). In our analysis, we identified 11 proteins with predicted signal peptides that had been shed or released into the medium. These included 2 GPI-anchored proteins (Table 2). As opposed to the cell wall fraction, where most of the proteins we identified were putative GPI-anchored proteins, most of the *C. albicans* secretome proteins lack a putative GPI-anchor signal peptide. In this study, Msb2, a signaling protein that belongs to the mucin family of proteins was identified in the *C. albicans* secretome. Msb2 is known to be part of the filamentous growth signal transduction pathway in *S. cerevisiae* (Cullen et al., 2004). The peptide fragment we identified from Msb2 is located in the carboxyl terminal domain, which is released into the medium by the cleavage of Msb2 in *S. cerevisiae* (Vadaie et al., 2008). It would be interesting to see if a similar function for Msb2 exists in *C. albicans*. In the combined cell wall proteome and secretome, we identified a total of 21 proteins with predicted signal peptides for secretion. The detailed functions of many of the identified cell wall proteins are yet to be elucidated (Table 1).

N. crassa is a haploid filamentous fungus with a well characterized life cycle including asexual and sexual stages (Davis and De Serres, 1970). During the different phases of the life cycle, twenty eight different cell types have been identified (Bistis et al., 2003). The availability of a library of gene knockout mutants for most of the identified *N. crassa* genes makes it an ideal organism in which to study the functions of cell wall proteins. Previously we have identified a few cell wall proteins from a proteomic analysis of the cell wall from vegetative hyphae (Bowman et al., 2006). In the present study we report a more comprehensive proteomic analysis of the cell walls in vegetative hyphae, and for the first time we report an analysis of the conidia cell wall and the *N. crassa* secretome. We were able to identify a total of 26 cell wall proteins of which 16 are putative GPI-anchored proteins (Table 3). The analysis of proteins released into the medium showed that 8 of the proteins we identified in the cell wall analysis were also identified as having been released into the medium. The analysis of proteins released into the medium identified 9 additional secreted proteins. The SDS PAGE (Fig. 2) and proteomic analyses (Table 3) indicate that there are differences in the cell wall proteomes of the vegetative hyphae and conidia cells. Almost all of the identified *N. crassa* cell wall “structural proteins” and “enzymes thought to be involved in cross-linking of glucans, chitins, and glycoproteins” have close homologs in the sequenced genomes of other fungi (Table 5), suggesting that these cell wall proteins are critical to the survival of the fungal cell and have been conserved through evolutionary time. Some of these *N. crassa* cell wall proteins also have homologs in the well characterized cell walls of *S. cerevisiae* and *C. albicans* (Table 5). For example, in our analysis of the *N. crassa* cell wall, we identified GEL1, GEL2 and GEL5, which are putative 1,3- β -glucanase transglucosidases. These proteins are homologs of Gas2 and Gas4 in *S. cerevisiae* and the GEL proteins of *Aspergillus fumigatus* that have been shown to be involved in cell wall biogenesis (Mouyna et al., 2000; Mouyna et al., 2000; Ragni et al., 2007). It may be noteworthy that some of the *N. crassa* cell wall proteins do not have close homologs in *S. cerevisiae* or *C. albicans*, but do have close homologs in other filamentous fungi (Table 5). Our data suggests that the *N. crassa* cell wall would be an excellent model for the cell walls of filamentous fungi. Studies using knockout mutants provided by the Neurospora genome project are underway to define the roles of many of these cell wall proteins.

We found a large number of non-classical or atypical proteins in *N. crassa* and *C. albicans* cell wall fractions (Supplemental data). These included glycolytic enzymes, heat shock proteins, mitochondrial proteins, and ribosomal proteins. A similar array of non-classical cell wall proteins have been reported previously in *C. albicans* and *S. cerevisiae* cell walls (Castillo et al., 2008; de Groot et al., 2004; Ebanks et al., 2006; Pardo et al., 2000). Our study corroborates the many other reports in which such proteins have been found “moonlighting” in the cell wall.

In our *C. albicans* data, most of these proteins were identified in the β ME-solubilized cell wall fraction. However, some of these atypical proteins were released after cleavage of the glucan/chitin matrix with TFMS (Supplemental data). There have been reports that these atypical proteins may function in binding to host proteins and also in the transport of peptides (Crowe et al., 2003; Sun et al., 2008). How such atypical proteins are incorporated into the cell wall is not understood.

In our analysis, the TFMS-based method of releasing cell wall proteins was more effective than the “cell wall shaving” approach. We were able to identify a much larger number of peptides in TFMS-released protein than we did from the direct trypsin digestion of the purified cell wall (Table 1 and Table 3). Among the peptides we identified in the TFMS-released protein fraction were many peptides with serine and threonine residues, whose identification might have been dependent upon the complete removal of O-linked oligosaccharides by the TFMS treatment.

A close inspection of SDS PAGE analysis of the TFMS-released proteins from the *C. albicans* cell wall (Fig. 1) and the TFMS-released protein and *N. crassa* cell wall (Fig. 2) show a background smearing in the SDS gels. This background smearing could be due to some limited clipping of the cell wall proteins during the TFMS treatment, or it might reflect the normal clipping of cell wall proteins by secreted proteases during the growth of the cells. The SDS PAGE analysis of TFMS treatment of secreted proteins has a much cleaner background (Fig. 1 and 2), suggesting that the TFMS does not cleave the secreted proteins and that the smearing seen in the cell wall protein fractions may be due to the *in vivo* clipping of cell wall proteins by secreted proteases.

The SDS PAGE analyses (Fig. 1 and 2) show the presence of more protein bands than the number of proteins we identified in the nano-LC/MS/MS analysis, which suggests that there may be some cell wall proteins which we were not able to identify in our analysis. This is not surprising because the tryptic digestion of some proteins may not give peptide fragments that can be easily identified in the MS/MS analysis. If a battery of proteases with defined substrate specificities were used to generate a much larger number of peptide fragments, additional peptides that could be identified would be generated. These additional peptide fragments could then be analyzed by nano-LC/MS/MS analysis and would allow for the identification of proteins we missed in our analyses.

The TFMS method of releasing cell wall proteins from the cell wall glucan/chitin matrix and identification of cell wall proteins has many advantages. We see eight such advantages: 1) The deglycosylation technique using TFMS provides for relatively fast and easy isolation and identification of cell wall proteins from a wide variety of pathogenic and non-pathogenic fungi, 2) There is no requirement to understand the carbohydrate composition of the cell wall or to add glucanases capable of digesting the particular type of glucan present in the wall, 3) A relatively small amount of starting material is needed (we used 20 mg of SDS-boiled and lyophilized cell wall for our analysis), 4) All cell wall proteins, irrespective of how they are cross-linked into the cell wall, can be released and deglycosylated in a single step, 5) The technique releases the cell wall proteins which can be easily separated and analyzed in SDS PAGE, 6) The effective deglycosylation of cell wall proteins by TFMS releases all O-linked glycosylation, which increases the number of fragments that are available for detection by mass spectrometry, 7) The released proteins are in a form that can be easily used in proteomic analyses, and 8) Asparagine residues which have been modified by N-linked glycosylation can be identified. We find that these advantages make TFMS-based method for releasing cell wall proteins ideal for proteomic analysis of fungal cell walls.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1A

Fig. 1B

Fig. 1C **Fig. 1.**

C. albicans cell wall and secretome preparations: (A) Silver-stained gel of yeast cell wall fractions. Marker size is indicated on the left. Lane 1, cytosolic fraction; lane 2, SDS-solubilized fraction; lane 3, β ME-solubilized fraction. (B) Silver-stained gel of TFMS-solubilized cell wall proteins. Lane Y, cell wall proteins from yeast cells; lane H, cell wall proteins from hyphae cells. Marker size is indicated on the left. (C) Silver-stained gel of the yeast cell secretome after deglycosylation by TFMS.

Fig. 2A

Fig. 2B

Fig.2C **Fig. 2.**

N. crassa cell wall and secretome preparations. (A) Silver-stained gel of cell wall fractions from the conidia cell wall. Lane 1, cytosolic protein; lane 2, SDS-solubilized protein; lane 3, TFMS-solubilized cell wall protein. (B) Silver-stained gel of TFMS-solubilized cell wall proteins from vegetative hyphae. (C) Silver-stained gel of deglycosylated proteins released into the medium by vegetative hyphae. Lane, 1- 2% sucrose-containing medium in which cells had grown for 18 h; lane 2 - starvation medium in which 18 h old vegetative hyphae were incubated for 2 h.

Table 1
Proteomic analysis of proteins from cell wall fractions of yeast and hyphal forms of *Candida albicans*.

Protein Name	Description/(Mol.wt)	Peptide Sequence	Residues	Y	H	CWF	p-Value
Mp65/Scw1	Glycoside hydrolase/Cell wall mannoprotein (39 kDa)	SNQAAISSIK	61-71	■		1	7.45E-06
		YWGYSNK	99-106	■		1	5.36E-05
		SESQIASEIAQLSGFDVIR	142-160	■	■	1, 3	2.31E-10
		IFAGIFDVSSITSGIESLAEEAVK	182-204	■		1	3.35E-11
		SNQAAISSIK	334-344	■	■	1, 2	7.45E-06
Pga29/Rhd3*	Putative GPI anchored (21 kDa)	YWGYSN [#]	372-378	■		1	1.78E-03
		HEGAAIDYLFGLGK	40-52	■	■	2, 3	1.26E-02
		HEGAAIDYLFGLKNGADLK	40-58		■	2	1.53E-03
		QSFTLGGDVVELGATDNFIPVTINK	79-103		■	2	6.95E-04
		TDDSAPIITIVAK	139-150		■	2	2.44E-02
Pga62/Flo1*	Putative GPI anchored (21 kDa)	PAESSPVPTTAAESSPAK	140-157		■	2	6.27E-05
		TTAAESSPAQETTPK	158-172		■	2	1.44E-04
Pga24/Ywp1*	Yeast form wall protein (54 kDa)	TTAVTTHGSTFETVAYITVTK	393-415	■		2	5.72E-06
		GGEQHQPGSPAGAAATAPGAPAGASGAHASTANK	419-455	■		2	4.19E-05
		VTVEAQATPGTLTPENTVAGGVNGEQVAVS AK	456-487	■		2	3.01E-03
		TTISQTTVAK	488-497	■		2	3.34E-03
		DIPYLEAVDTNVIR	75-88	■	■	2, 3	4.87E-06
Phr2*	pH regulated wall protein (39 kDa)	SIPVGYSANDDSAIR	191-205	■		2, 3	8.40E-05
Pir1	Protein with internal repeats (41 kDa)	ACSSANNLEMTLHDSVLK	249-266	■		2	1.03E-02
		LSVIEFVNC [#]	338-346	■		2	1.24E-02
Chit2*	Chitinase (61 kDa)	TVLLSLGGGVGDYGFSDVASATK	99-121	■		1, 2	1.19E-02
		LFVGVPATSNIAGYVDTSK	240-258	■		2	7.60E-03
		LAISTVTDVQK	404-414	■		2	3.03E-02
Csa1/Wap1*	Candida surface antigen (104 kDa)	SSEA VEVAQTA VAEASK	938-954		■	2	4.74E-05
		AGDEISTEIVNITK	955-968		■	2	3.23E-03
Als3*	Agglutinin like protein (123 kDa)	FTTSQTSVDLTAHGVK	77-92		■	2	4.74E-05
		NSDAGSNGIVIVATTTR	317-332		■	2	3.32E-03
Als4*	Agglutinin like protein (221 kDa)	FTTDQTSIDL VADGR	77-91	■		2	1.71E-07
Hwp1*	Hyphal wall protein (65 kDa)	SSAPATEPSPVAPGTESAPAGPGASSSPK	516-544		■	2	1.68E-05

Protein Name	Description/(Mol.wt)	Peptide Sequence	Residues	Y	H	CWF	p-Value
Rbt1 [*]	Hyphal wall protein (77 kDa)	SSAPATEPSPV/APGTESAPAGPGASSSPK	597-624		■	2	1.68E-05
Crh11 [*]	Glycoside hydrolase (47 kDa)	SVLVADYSSGK	234-246	■	■	2	3.80E-02
Pga2/Sod4 [*]	Superoxide dismutase (24 kDa)	TPAALELGDLSGR	105-117	■		2, 3	1.03E-04
Tos1	Protein similar to alpha agglutinin anchor subunit (49 kDa)	SGEYYIIFSGSK	288-299		■	1, 2	1.58E-02

Y- yeast; H-hyphae; CWF-cell wall fraction; 1-βME-solubilized cell wall proteins from yeast form only; 2-TFMS-solubilized protein; 3- protein released by trypsin treatment of purified cell wall (cell wall shaving);

^{*} Putative GPI-anchored proteins;

[#] C-terminal sequences. Only peptides with *p-Values* <0.05 were considered to be significant and included.

Table 2Proteomic analysis of *Candida albicans* proteins released into the medium by yeast cells.

Protein Name	Description/(Mol. Wt)	Peptide Sequence	Residues	p-Value
Mp65/Scw1	Glycoside hydrolase (39 kDa)	SESQIASEIAQLSGFDVIR	142-160	2.14E-12
		NVLITETGWPSR	182-204	7.31E-06
		NVLITETGWPSRGDSNGVAVPSK	311-322	5.19E-06
		IFAGIFDVSSITSGIESLAEAVK	311-333	3.55E-08
		SNQQAASSIK	334-344	1.17E-05
		YWGIIYSNK	372-379	3.11E-03
Exg1/Xog1	Putative β -1,3-glucanase (50 kDa)	VWIDLHGAPGSQNGFDNSGLR	168-188	1.57E-03
		YGGNEYSADVIGIELLNEPLGPVLNMDK	213-240	4.91E-09
		TENAPEWSFQTLTYNGLFPQPVTDNR	405-429	1.01E-04
Tos1	Protein similar to alpha-agglutinin anchor subunit (49 kDa)	GIGFSGSYMDVTNMDENTGK	42-61	1.33E-03
		FGNSLSYANADNSGGSSTPVPLEETIK	260-287	1.02E-03
		SGEEYIIFSGSK	288-299	2.35E-05
Scw11	Cell wall protein (35 kDa)	SDIQLINSK	85-93	1.64E-03
		SVLITETGYPSK	255-266	7.59E-05
		GSTLGVNVPSPENQEIAISSIK	267-289	3.25E-04
Sim1/Sun42	Hypothetical protein (39 kDa)	TDYPGSENMNIPTLLSAGGK	233-252	4.43E-07
		TYLSLIPNPNK	312-325	1.24E-05
Cht3	Chitinase (60 kDa)	TILLSLGGAGSYGFSDDATAK	100-121	1.43E-07
		AAGSGYNDPSAVSQYLTSIDLNSK	256-279	2.72E-08
Bgl2	Putative glycoside hydrolase (34 kDa)	EALQNYLPK	97-105	7.16E-03
		IFLVGSEALYR	113-123	1.04E-05
Cht2*	Chitinase (61 kDa)	TVLLSLGGGVGDYGFSDVASATK	99-121	7.09E-08
Pga24/Ywp1*	Yeast form wall protein (54 kDa)	NLYGAGAVPFFQVHLEK	94-110	1.34E-05
Pir1	Structural wall protein (41 kDa)	ASATPVQQIGDGGIQQHTTAAATASAK	169-198	2.28E-04
Msb2	Signaling Mucin (141 kDa)	LIPYTASNIDYTITVAEVYFPK	1197-1218	4.23E-03

* Putative GPI-anchored proteins. Peptides with *p-Values* <0.05 were considered to be significant.

Table 3
Proteomic analysis of cell wall proteins from *Neurospora crassa* vegetative hyphae and conidia.

Protein Name/Description/(Mol. Wt)	Gene Locus No.	Peptide Sequence	Residues	V	C	CWF	p-Value
ACW-1* (49 kDa)	NCU08936	EVAGDFQLSGPQITGDLK	57-74	■	■	1	1.36E-03
		IENPNINSVGSDTLEQIGGEFR	76-98	■		1, 2	2.33E-06
		LVETTQLNAVNMMLK	99-112	■	■	1, 2	8.66E-07
		VDEITSDSHLR	138-149	■		1	2.00E-03
ACW-2* (28 kDa)	NCU00957	DATAPITITLMTGPDADHMTPEK	40-62	■		1	2.73E-05
		TIASGVTDGSDYTWTPEDVPSGTAFK	63-88	■	■	1	8.85E-05
		FPYVGSAAAATTGASSTLSTVTK	104-125	■		1	2.36E-08
		KNDVVDPSGSGVEVK	27-39	■	■	1, 2	1.64E-04
ACW-3* (28 kDa)	NCU05667	NDVVDPSGSGVEVK	28-39	■		1	2.47E-03
		WSTVSTDPEK	40-48	■	■	1	4.28E-07
		SAHLVLVNMASGHTPYTK	49-66	■	■	1, 2	2.58E-03
		DLGAVDLTK	67-75	■	■	1, 2	1.83E-04
ACW-5* (18 kDa)	NCU07776	FESAAATALEIAGVVEGK	51-68	■		1	3.10E-04
ACW-6* (62 kDa)	NCU03530	DCSIAVCSESADTTIDWAKK	64-84	■	■	1	3.87E-02
ACW-7* (25 kDa)	NCU09133	IDVLGGTTPQTLDK	58-71	■		1	4.91E-05
		VGTVASGVDSSK	72-83	■		1	3.13E-03
ACW-8* (73 kDa)	NCU07277	APVESSESSEWTTSTIIATTTR	365-388	■		1	8.78E-08
ACW-9* (24 kDa)	NCU06185	STVVTSVVYPVTSTIYSTSVR	46-67	■		1	4.29E-08
ACW-10* (25 kDa)	NCU03013	YASLVEGSDAYFLDR	156-170	■		1	3.54E-08
ACW-11* (20 kDa)	NCU02041	VSSSSAAAITSAAKPTQTK	133-150	■		1	2.28E-09
GH17-3/Glucan-β-glucanase* (49 kDa)	NCU09175	DWVQEFFTAQNLIK	42-54	■		1	1.49E-07
		ILLGWASGNTNIEPEIK	92-109	■	■	1	1.66E-04
		LTDLHGASIGSEDL YR	122-138	■		1	
		VSVTGQNK	139-147	■		1	1.44E-02
GH16-1/Mixed linked glucanase/MLG* (80 kDa)	NCU01353	GNNIENSGYLFDR	215-227	■		1	7.53E-06
		TYQLSESYNSANFADK	30-45	■		1	
		TYQLSESYNSANFADKFNFFEGHDEK	30-56	■		1	1.61E-08
		TKDPNSGFVK	57-66	■		1	
		DAAVSSGLFK	71-80	■		1	4.66E-04

Protein Name/Description/(Mol. Wt)	Gene Locus No.	Peptide Sequence	Residues	V	C	CWF	p-Value
GH16-7/Glycoside hydrolase * (38 kDa)	NCU05974	IGVDSTTSGVAGRK	88-101	■		1	7.02E-05
		LESTATYNNGLFIK	105-119	■		1	5.62E-03
		ETDAPTVASSR	76-86	■		1	7.66E-03
		GGFSTVANPQNEFHITYTIK	147-165	■		1	7.07E-03
		GCAGFPQTPMQIK	190-202	■		1	7.70E-04
		IQDFMGGDGTGAK	241-254	■		1	1.76E-03
		VLGTSTTTEDVGTK	273-286	■		1	7.06E-06
		VNVYWGQR	25-32	■		1	1.07E-04
		VLLSLDGEVHMGSR	90-103	■		1	3.48E-07
		AEEFAAFLWGAFGPYDAK	122-129	■		1	3.10E-10
CHIT-1/Endochitinase * (135 kDa)	NCU02184	VSVDFGNLDGELK	143-155	■		1	7.79E-09
		VSVDFGNLDGELKLNAGEGAYAAAMAK	143-169	■		1	7.76E-09
		LNAGAGEGAYAAAMAK	156-169	■		1	2.08E-05
		LFHGLAGASDAAGSGYIEPLEAAALINTYK	245-274	■		1	7.78E-04
		SSFGGAMVLDADR	277-289	■		1	4.80E-07
		VPSVTDGAHEVNPTIVDTSITSPV ALPSGDR	383-417	■		1	9.90E-04
		SGVVPSGVMPTGALPSGLVPSGDR	443-466	■		1	4.33E-02
		SDIIGAIPSGIPLGSPVSGVVPSGDR	539-565	■		1	9.48E-10
		SGLVPSGAVPTGDR	587-600	■		1	1.00E-03
		SDIIGAIPSGIPLGSAFSLVPSGAVPSGDR	601-632	■		1	5.55E-07
GH55-3/GEL-2 * (85 kDa)	NCU07523	SEIIGAIPSGIPLGSPVSGA VPSGDR	633-659	■		1	1.24E-06
		ITGPINGHNVAVPSAGVPSGVVPSGILSNVGSK	917-949	■		1	1.73E-04
		GIAYQPGGSSANLDPADPK	52-71	■		1	2.44E-04
		YPAPTGDGGYSQTTK	338-352		■	1	1.70E-04
		DAAILQILGVNTIR	69-82		■	2	5.86E-03
		NADGSVISDLAVKPLSNDESNSPGTNDK	408-436	■		1	6.29E-04
		SAADVMITDIK	376-385	■		1	2.09E-04
		AIANAGFTTLR	386-396	■		1	2.01E-06
		TALAAIK	579-585	■		1	3.14E-04
		TALAAIKQEMGK	579-590	■		1	8.30E-04
GH72-2/GEL-5 * (53 kDa)	NCU06781	VVFFSFDDHWK	592-603	■		1	4.84E-07
β-glucosidase (64 kDa)	NCU09326						

Protein Name/Description/(Mol. Wt)	Gene Locus No.	Peptide Sequence	Residues	V	C	CWF	p-Value
GH3-3(β -glucosidase 1 precursor (96 kDa)	NCU08755	SSGLHHPGPNPALYDIMYTVTADITNTGK	764-792		■	2	3.53E-07
CAT-3 (80 kDa)	NCU00355	LKEVEVDNGQFMITTDFGNIEEQFSLK	43-70	■	■	1	1.05E-08
		GSTLLEDFIR	75-85	■		1	3.80E-04
		FYTDEGNFDIVGNNIPVFFIQDAIR	163-187	■		1	8.67E-04
		FLPEEFAPLQVLGEMTLNR	333-351	■		1	4.26E-06
		FEASHVTNEQVK	502-515	■		1	4.20E-05
NCW-1 (74 kDa)	NCU05137	TGAGVSTQGLWHYPK	166-281	■		1	1.70E-06
		ISDLETHATWQSNINTR	283-299	■		1	1.70E-06
		SGDVDQITVGSVSGK	551-573	■		1	
NCW-2 (63 kDa)	NCU01752	TEELAALPTEALATTVVTR	128-146	■		1	2.43E-13
		TEDLATLPTEALATTVVTR	241-259	■		1	1.20E-06
		VTSTAVETLTTPK	291-303	■		1	4.54E-07
NCW-3 (25 kDa)	NCU07817	INPLPSGTVGAWQYT#	235-249	■	■	1	5.09E-08
NCW-4 (22 kDa)	NCU02948	AGGTADLYQVPETLSDEVLAK	30-50		■	1, 2	1.90E-02
NCW-5 (17 kDa)	NCU00716	EQFAVGPPQPIDSVR	97-111	■	■	1	4.45E-05
NCW-6 (30 kDa)	NCU00586	PALPLGDAWDSNPAGAPAGLTGK	77-99		■	1	9.17E-07
		ATYATEGHHDHVVADGPTGPSERR	224-247		■	1	1.60E-06
		GTGSCGAIEWAEGTQPAGSQTK	126-147		■	1	2.06E-04

V-Vegetative hyphae; C-conidia; CWF-cell wall fraction: 1-TFMS-solubilized protein; 2- protein released by trypsin digestion of cell wall (cell wall shaving);
* Putative GPI-anchored proteins; ACW – Anchored cell wall protein; NCW – Non-anchored cell wall protein. Peptides with *p-Values* <0.05 were considered to be significant.

Proteomic analysis of *Neurospora crassa* proteins released into the medium by vegetative hyphae.

Protein Name/Description/(Mol.Wt)	Gene Locus No.	Peptide Sequence	Residues	C+	C-	p-Value
ACW-1/CCG-15* (49 kDa)	NCU08936	EVAGDFQLSGPQITGDLK	57-74	■	■	3.54E-05
		IENNPINISVGSDTLEQIGGEFR	76-98	■	■	7.04E-06
		LVEITQLNAVNMKL	99-112	■	■	2.35E-03
ACW-2* (28 kDa)	NCU00957	DATAPITITLMTGPDADHMTPEFK	40-62	■	■	5.89E-05
		TIASGVTDGSYTWTPEDVPSGTYAFK	63-88	■		4.79E-06
		FPYVGSAAATTGASSTLSTVTK	104-125	■		2.02E-07
ACW-3* (28 kDa)	NCU05667	KNDVDPSSGVEVK	27-39	■	■	1.78E-02
		DLGAVDLTK	67-75	■		4.02E-03
ACW-7* (25 kDa)	NCU09133	IDVLGGTTPQTLDK	58-71	■		1.98E-04
		PLTVAAPATTK	147-157	■		7.20E-03
ACW-12* (53 kDa)	NCU08171	LTEQADNVNVTSLSDIFSAAAGK	43-64	■		2.65E-04
		VVSVADVFNDSGNHQMTTDSYGR	66-87	■		6.23E-04
		KLWQHTSDFNDENTTK	88-103	■		2.56E-05
		HALLVYPHASDDFR	153-166	■		2.39E-07
		VFDLTNWQVSGDDVVGK	193-210	■	■	3.73E-06
GH17-3/Glucan-β-glucanase* (49 kDa)	NCU09175	VSSGVYSAAGYK	211-222	■		2.12E-03
		TTTPDSLIVGEYQTSTSDPIR	249-270	■	■	1.15E-07
		YELDYTTR	273-280	■	■	2.05E-03
		GDLWAWVPGGAAK	324-336	■		9.15E-04
		SPEDLSYDKR	345-354	■		3.91E-06
		DWVQEFTTAQNLIK	42-54	■		8.21E-07
		LYTNIQAYSQTSEPIEAFEAIEITNTK	65-91	■		1.72E-06
		ILLGVWASGTNTIEPEIK	92-109	■		1.03E-06
		LTDLIGASIGSEDLYR	122-138	■		2.28E-06
		VSVTGIQNK	139-147	■		1.33E-04
		FIADWK	160-165	■		1.64E-02
		AYDAIEGAVGGK	228-239	■		1.28E-04
GH16-7/Glycoside hydrolase* (38 kDa)	NCU05974	PWVTETGWPPYVGGTWDQAAATIK	240-264	■		7.27E-06
		EIINIDFTK	39-47	■	■	3.20E-02
		ETDAPTVASSR	76-86		■	3.37E-02

Protein Name/Description/(Mol.Wt)	Gene Locus No.	Peptide Sequence	Residues	C+	C-	p-Value
GH72-5/GEL1* (58 kDa)	NCU08909	GGFSTVANPQNEFHTYTIK	147-165	■	■	1.72E-03
		WTPTQLDWIDGAVVRT	166-181	■	■	1.52E-04
		IQDFMGGDGTTGAK	241-254	■		6.05E-04
		DATEYQYGDK	255-264	■		6.07E-04
		VLGTSTTTEDVGTK	273-286	■		1.07E-04
		DVPIMAAAGTNAIR	76-89	■		1.55E-02
		LLDEAGIYVISDLSEPSTINR	105-126	■	■	5.28E-06
		WDITLYKR	131-137	■	■	1.16E-02
		GSGYQDQIDFFK	240-250	■	■	1.47E-04
		SLTNTILILAR	27-37	■	■	1.74E-02
NCW-1 (74 kDa)	NCU05137	SAITAAQWEITYTYQTNFGVR	99-119		■	2.04E-04
		TGAGVSTQGLWHYPAK	166-181	■	■	6.54E-04
		PIATFAPSSDGTFTTSVAAIINTFGTR	190-217	■	■	3.86E-05
		ISDLETHATWQSNINTR	283-299	■		5.50E-06
		LPAGSHYFVEMGHNGNGDFIEGLPKA	300-324	■		2.22E-03
		KPLGTGLTLWK	351-361		■	3.94E-05
		NPENSFWPATINVAATNGYDGLVIIPR	473-498	■	■	1.06E-04
		SGDVDQITVGSVSGK	559-573	■	■	2.77E-08
		LSLQIWWVEITTEMVR	574-590		■	2.34E-07
		LTNWPITSLK	591-600	■		3.40E-04
GLA-1/Glucamylase (66 kDa)	NCU01517	SVDSYIQTETPIAQK	36-50	■	■	2.44E-05
		ASGAASGVVVASPSK	63-77	■	■	5.54E-04
		LQGVSNPSGSLNGAGLGEPK	123-143		■	1.25E-02
		WLVSNGYADTAK	176-187		■	1.93E-02
		SIIWPIVK	188-195		■	4.97E-03
		ALVEGSFAK	230-239		■	2.19E-05
		VTTSYGQTVK	534-543	■	■	1.79E-02
		VVGSIAALGNWAPASGVTLsAK	544-565		■	2.72E-07
		QYSSSNPL WSTTIALPQGTsFK	566-587		■	9.44E-06
		YVVVNSDGSVK	590-600		■	1.70E-05
ASD-1/SDV-10/Rhamnogalactorunase (61 kDa)	NCU05598	LPYEYPYGEVSTTK	144-157	■		4.53E-05
		TVDISWFGELGVTGYVPDSQR	268-288	■		3.86E-06

Protein Name/Description/(Mol.Wt)	Gene Locus No.	Peptide Sequence	Residues	C+	C-	p-Value
Predicted protein (28 kDa)	NCU04603	FNLGSAQSQAATTLR	437-450	■		4.56E-07
		SLVTEWVTETVYETVTK	37-53	■	■	2.21E-05
		AFLDVFGALTDGR	244-256	■		1.92E-02
		KVEWAFSD	258-265	■		4.10E-02
Predicted protein (21 kDa)	NCU00399	GIFINLSK	58-65		■	4.59E-02
		VDGTLSLYTEGKPWQQLFVDR	86-106	■		3.32E-03
		VVVAENPVK	184-192	■	■	1.24E-03
		GSTLLEDFIR	75-85	■		9.79E-03
CAT-3 (80 kDa)	NCU00355	ADIAHILELLEVK	155-167	■		4.57E-05
Predicted protein (31 kDa)	NCU00265	SLGQTDVFGTYEVGFTDTAR	551-570		■	8.99E-03
INV/Invertase/β-fructofuranosidase (75 kDa)	NCU04265	SITATGSTIVK	128-138		■	3.32E-03
Predicted protein (31 kDa)	NCU08720					

C+: 18 hr culture in 2% sucrose-containing media, C-: 2 hr culture in carbon/energy source-free medium following 18 hr culture in 2% sucrose-containing media;

* Putative GPI-anchored proteins; Peptides with *p-Values* <0.05 were considered to be significant.

Table 5Homologs of *Neurospora crassa* cell wall proteins in other fungi

Protein name/Description	Gene Locus No.	<i>S. cerevisiae</i>	<i>C. albicans</i>	Other fungi with homologs
ACW-1/CCG-15*	NCU08936	Ecm33	Orf19.4955	1, 2, 3, 4, 5, 6, 7, 8, 9, 10
ACW-2*	NCU00957			1, 2, 3, 4, 7, 8, 9
ACW-3*	NCU05667			1, 2, 5, 7, 10
ACW-5*	NCU07776			5
ACW-6*	NCU03530			1, 5
ACW-7*	NCU09133			1, 2, 3, 4, 5, 7
ACW-8*	NCU07277			1, 4, 5, 6, 7
ACW-9*	NCU06185			1, 2, 3, 4, 5, 6, 7
ACW-10*	NCU03013	Sod1	Sod6/Pga9	1, 2, 3, 4, 5, 6, 7, 8, 9
ACW-11*	NCU02041			1, 2, 3, 4, 5, 6, 7, 9
GH17-3/Glucan- β -glucanase*	NCU09175	Bgl2	Bgl2	1, 2, 3, 4, 5, 6, 7, 8
GH16-1/Mixed linked glucanase/Mlg*	NCU01353			1, 2, 3, 4, 5, 6, 9, 10
GH16-7/Glycoside hydrolase*	NCU05974	Crh1	Crh12	1, 2, 3, 4, 5, 6, 8, 9
CHIT-1/Endochitinase*	NCU02184	Cts1	Chit3	1, 2, 3, 5, 6, 8, 9
GH3-3/ β -glucosidase 1 precursor	NCU08755		Glycoside hydrolase/Orf19.1664	1, 2, 3, 4, 5, 6, 7, 8, 9, 10
β -glucosidase	NCU09326	Scw11	Scw4	1, 2, 3, 4, 8, 9
GH55-3/GEL-2	NCU07253	Gas2	Phr2	1, 2, 3, 4, 5, 6, 7, 8, 9
GH72-2/GEL-5	NCU06781	Gas4	Phr3	1, 2, 3, 4, 5, 6, 7, 8, 9
NCW-1	NCU05137			1, 5, 6, 7
NCW-2	NCU01752	Dan4	Predicted protein/Orf19.3621	2, 3, 4, 6, 9
NCW-3	NCU07817			1, 2, 3, 9
NCW-4	NCU02948	Pst2	Pst1	1, 2, 3, 4, 5, 6, 7, 8, 9, 10
NCW-5	NCU00716			1, 2, 6
NCW-6	NCU00586			1, 2, 3, 4, 5, 6

Protein name/Description	Gene Locus No.	<i>S. cerevisiae</i>	<i>C. albicans</i>	Other fungi with homologs
NCW-7/CCG-13	NCU08907			1, 2, 3, 4, 5, 7
CAT-3	NCU00355	Cta1	Cat1	1, 2, 3, 4, 5, 6, 7, 8, 10

Fungal species used in the analysis are: 1- *Podospora anserina*; 2- *Chaetomium globosum*; 3- *Magnaporthe grisea*; 4- *Gibberella zeae*; 5- *Aspergillus fumigatus*; 6- *Botryotinia fuckeliana*; 7- *Sclerotinia sclerotiorum*; 8- *Yarrowia lypolytica*; 9- *Coccidioides posadasii*; 10- *Cryptococcus neoformans*