Genetic and biochemical characterization of the GH72 family of cell wall transglycosylases in *Neurospora crassa*.

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Abstract

The *Neurospora crassa* genome encodes five GH72 family transglycosylases, and four of these enzymes (GEL-1, GEL-2, GEL-3 and GEL-5) have been found to be present in the cell wall proteome. We carried out an extensive genetic analysis on the role of these four transglycosylases in cell wall biogenesis and demonstrated that the transglycosylases are required for the formation of a normal cell wall. As suggested by the proteomic analysis, we found that multiple transglycosylases were being expressed in *N. crassa* cells and that different combinations of the enzymes are required in different cell types. The combination of GEL-1, GEL-2 and GEL-5 is required for the growth of vegetative hyphae, while the GEL-1, GEL-2, GEL-3 combination is needed for the production of aerial hyphae and conidia. Our data demonstrates that the enzymes are redundant with partially overlapping enzymatic activities, which provides the fungus with a robust cell wall biosynthetic system. Characterization of the transglycosylase-deficient mutants demonstrated that the incorporation of cell wall proteins was severely compromised. Interestingly, we found that the transglycosylase-deficient mutant cell walls contained more β -1,3-glucan than the wild type cell wall. Our results demonstrate that the GH72 transglycosylases are not needed for the incorporation of β -1,3-glucan into the cell wall, but they are required for the incorporation of cell wall glycoprotein into the cell wall.

1.Introduction

The fungal cell wall is composed of glucan, chitin, and glycoproteins which have been cross-linked together in a strong three-dimensional matrix (Latge, 2007, Chaffin, 2008, Klis et al., 2006, Free, 2013). It plays a vital role in hyphal elongation and branching and in defining fungal morphology. The wall protects the fungus from environmental stresses and prevents cell lysis. For pathogenic fungi, the cell wall plays an important role in host/pathogen interactions and represents an important pathogenicity factor.

The major polymers in the fungal cell wall are β -1,3-glucan, chitin, and glycoproteins. The β -1,3-glucan and chitin polymers are made by plasma membrane-associated glucan and chitin synthases and extruded as linear polymers into the cell wall space during their synthesis. The cell wall glycoproteins are made on ER-associated ribosomes and the proteins are extensively modified by the addition of both N-linked and O-linked oligosaccharides. During their passage through the secretory pathway, the basic GlucNAC 2/Mannose 9 N-linked oligosaccharides found on fungal cell wall proteins are further modified by the addition of galactomannans or outer chain mannans. These outer mannan and galactomannan structures contain a linear α -1,6-mannose polymer with mannose and galactose-containing side chains. Once the glucans, chitins, and glycoproteins are released into the cell wall space, they are cross-linked together to create the cell wall matrix.

A number of cell wall cross-linking enzymes have been identified by proteomic analyses of purified fungal cell walls (Free, 2013, Klis et al., 2010, De Groot et al., 2005). These cross-linking enzymes function as transglycosylases and are thought to catalyze two related reactions. First, they function as glycosyl hydrolases to cleave the glucans, chitins, and cell wall protein-

associated oligosaccharides and generate a covalent bond between the polysaccharide and the enzyme (an enzyme-polysaccharide intermediate). Second, they function as glycosyl transferases to form new glycosidic bonds between the original cleaved polysaccharide and a second polysaccharide. In so doing, they can create cross-links between the cell wall glucans, chitins, and the oligosaccharides associated with cell wall glycoproteins and generate a cell wall matrix. These cross-linking enzymes are generally encoded by multi-gene families. Members of each of five families of these glycosyl hydrolase/glycosyl transferases (GH families) are commonly found in cell wall proteomic analyses. These are the GH16 family of β -1,3-glucanases, the GH17 family of β -1,3-glucanases, the GH18 family of chitinases, the GH72 family of β -1,3-glucanases, and the GH76 family of α -1,6-mannanases. The GH72 family of transglycosylases has been shown to be capable of changing the lengths of β -1,3-glucans (creating both shorter and longer molecules), and it has been proposed that these transglycosylases may play a role in “sizing” the β -1,3-glucan for insertion into the wall (Hartland et al., 1996, Hurtado-Guerrero et al., 2009, Mazan et al., 2011). The GH16 family of β -1,3-glucanases is thought to be involved in cross-linking the glucans together (Cabib et al., 2007, Pardini et al., 2006). The GH17 β -1,3-glucanase and the GH18 chitinase families are thought to function in cross-linking the glucan and chitin polymers together (Gastebois et al., 2010c, Goldman et al., 1995, Gastebois et al., 2009). The GH76 α -1,6-mannase family is thought to participate in cross-linking the cell wall glycoprotein-associated galactomannans and outer chain mannans into the cell wall matrix (Maddi et al., 2012). Together, these five families of commonly found cell wall enzymes may represent all of the cross-linking activity needed to cross-link the cell wall components together to create the cell wall matrix.

We have previously carried out proteomic analyses of *Neurospora crassa* vegetative hyphae and conidia cell walls and demonstrated the existence of cross-linking enzymes from all five families of commonly found cross-linking enzymes (Ao et al., 2016, Maddi et al., 2009, Maddi and Free, 2010, Maddi et al., 2012). The *N. crassa* genome has 13 genes from the GH16 family, 3 genes from the GH17 family, 10 genes from the GH18 family, 5 genes from the GH72 family and 9 genes from the GH76 family (Colot et al., 2006). To further examine the role that these cross-linking enzymes play in cell wall biogenesis and to determine whether different cell types in the *Neurospora* life cycle utilize different members of these gene families, we carried out an extensive genetic analysis of the GH72 family and characterized how the cell wall was affected by loss of the cross-linking enzyme. Our results demonstrate the importance of the GH72 family of cross-linking enzymes for generating the cell wall. We found that multiple enzymes from the GH72 gene family are expressed in each of the cell types examined and that these enzymes have partially overlapping activities, which provides a redundant and robust system for cross-linking the cell wall together. We demonstrate that the cell wall glycoproteins are released into the growth medium from transglycosylase-deficient mutant cells. We also demonstrate that different combinations of GH72 enzymes are being expressed in different cell types. Our results demonstrate that one reason for the presence of these multigene families of cross-linking enzymes is to provide for cell type-specific cross-linking enzymes during the various stages of the fungal life cycle.

Our analyses of the carbohydrate composition and linkages present in the cell walls from wild type and transglycosylase-deficient mutant cells showed that the transglycosylase-deficient mutant cell walls were enriched in β -1,3-glucan and deficient in cell wall glycoprotein. We found that the material released by the mutants into the growth medium contained large amounts of cell wall glycoprotein and a small amount of glucan. These results demonstrate that the transglycosylases are not required for the incorporation of β -1,3-glucan into the wall, but they are needed for the incorporation of cell wall protein. The mechanism whereby the transglycosylases facilitate glycoprotein incorporation into the cell wall remains to be elucidated.

Materials and Methods

2.1. Strains, Growth, and Genetics

Single deletion mutants for members of the GH72 family *gel-1* (NCU08909), *gel-2* (NCU07253), *gel-3* (NCU01162), and *gel-5* (NCU06781) were obtained from the Fungal Genetics Stock Center (Manhattan, KS, USA). Strains carrying multiple deletions within a family of cross-linking enzymes were generated via standard mating procedures (Davis and DeSerres, 1970). Individual ascospore progeny from these matings were tested for the presence of the gene deletions by isolating DNA from the individual progeny (Colot et al., 2006) and doing PCR reactions to assess the presence of the wild type and deletion alleles. All isolates were maintained on Vogel's sucrose agar medium.

2.2. PCR amplification and primers

PCR amplification of wild type and mutant alleles of the GH72 genes was done using Taq polymerase. The GH72 deletion alleles were generated by the Neurospora genome project using homologous recombination between the genomic DNA and a DNA construct containing sequences upstream of the coding region, the hygromycin-resistance cassette, and regions downstream of the coding region. The resultant gene knock out mutations are marked by the presence of the hygromycin resistance cassette (Colot et al., 2006). The various GH72 deletion mutants were mated to create double, triple, and quadruple mutants. Two sets of PCR primers were used for testing the status of GH72 gene family members within the progeny genomes from these matings. One set of primers contained a primer sequence 5' of the site into which the hygromycin-resistance gene insertion occurred in the gene being tested and a primer from within the gene coding sequence. These primers were 8909F (TAGAGGTCTTTCGCTACTGCC) and 8909R (GTGGAATCGAGTGTGGTTAGC) for *gel-1*, 7253F (TCTTGATGGTGGCTATGCAC) and 7253R (GTGATGGAAGCACGCTTGGTG) for *gel-2*, 1162F (ATTCGACGTCTGTGGTGACAC) and 1162R (GCGTATTGCGATGTGTAAGCG) for *gel-3*, and 6781F (ATTCCTGACACTCGTTCGTCC) and 6781R (CAACGTTACATGCGTGCCAC) for *gel-5*. PCR amplification with these primers gave PCR products from the wild type alleles of the genes. A second set of PCR amplification primers was used to identify the presence of the knock out gene alleles. These PCR amplifications used the same primer sequence 5' of the site of the hygromycin insertion site

(8909F, 7523F, 1162F and 6781F) and a second primer from within the hygromycin-resistance gene (GGCTGTGTAGAAGTACTCGCC).

2.3. Phenotype screening morphology and cell wall stress tests

Mutant phenotypes were assessed by visual inspection of the gross colony morphology of mutants growing on Vogel's sucrose agar medium and in Vogel's sucrose liquid medium. Microscopic inspection of the growing edge of a colony was used to examine hyphae morphology. The hyphae linear growth rate was assessed by inoculating conidia on Vogel's sucrose agar medium and measuring extension of the growth front as a function of time. To directly determine whether the mutants produced weakened cell walls, a series of stress tests were performed. These tests were performed by inoculating WT and mutant conidia onto Vogel's sucrose agar medium containing cell wall stress reagents. The stress reagents used included high salt (10% NaCl), SDS (0.01% SDS), the chitin synthase inhibitor Calcofluor white (10 mg/ml) and the β -1,3-glucan synthase inhibitor caspofungin (10 mg/ml). The sensitivity of mutant and WT cells to the reagents was assessed by examining the growth of the cells in the presence of the stress reagents as described previously (Maddi and Free, 2010). Osmotically stabilized medium contained Vogel's salts, 2% sucrose, and 1.5 M sorbitol.

2.4. Purification of wild type and mutant cell walls

Wild type and mutant hyphae were harvested from liquid grown cultures on a Buchner funnel, and the cells were ground to a fine powder under liquid nitrogen in a mortar and pestle, and a volume of extract buffer (1 X PBS, 1% SDS) was added to the sample. The cell walls were collected by centrifugation (6,000 xg for 10 minutes). The cell walls were washed four times with extract buffer, resuspended in extract buffer, and subjected to a 15 minute incubation in a boiling water bath to help release any material not covalently attached to the cell wall. The cell walls were then collected by centrifugation, washed twice with 1 X PBS, and washed twice with distilled water. The cell walls were then lyophilized and weighed. Samples of lyophilized cell wall were sent to the Complex Carbohydrate Research Center (Athens, GA) for carbohydrate analyses.

2.5. Characterization of cell wall defects

To examine the release of cell wall proteins and polysaccharides into the growth medium, wild type and mutant cells were grown in Vogel's sucrose liquid medium and the cells and growth medium were separated by collecting the hyphae on a Buchner funnel. The cells were washed with water and ground to a fine powder under liquid nitrogen in a mortar and pestle. A volume of extraction buffer (1 X PBS with 1% SDS) was added and a cytosolic extract was prepared by centrifugation. The concentration of protein in the cytosolic extract was determined with the DC Bradford assay (BioRad). The proteins and polysaccharides in the growth medium were precipitated by the addition of an equal volume of 25% trichloroacetic acid (TCA) in acetone and incubating at -20°C for 24 hours. The precipitated material was collected by centrifugation (10,000 xg for 15 minutes), washed with acetone, dried, and weighed. An aliquot of the TCA-precipitated material was sent to the Complex Carbohydrate Research Center for carbohydrate composition and linkage analyses. A second aliquot of the material was

resuspended in PBS and the amount of protein in the medium assessed by the Bradford protein assay. To further characterize the proteins being released into the medium, aliquots of glycoprotein released from cells containing 1 mg of cytosolic protein were subjected to SDS PAGE. These SDS PAGE gels were stained with Coomassie Brilliant Blue to give an indication of the pattern of the released glycoprotein. SDS PAGE gels were also processed for Western blot analysis with antibodies directed against ACW-1, a major GPI-anchored cell wall protein (Bowman et al., 2006), with monoclonal antibodies directed against laminarin (β -1,3-glucan) (Biosupplies Australia, Victoria, Australia), and with monoclonal antibodies directed against lichenin (β -1,3/1,4-glucan) (Biosupplies Australia) to assess the presence of the ACW-1 cell wall protein and cell wall glucans.

2.6. Carbohydrate analyses of purified cell wall preparations

All carbohydrate analyses were carried out by the Complex Carbohydrate Research Center (Athens, GA). Cell wall samples and samples of polysaccharides released into the medium were subjected to carbohydrate composition and glycosyl linkage analyses. The carbohydrate composition of the samples was analyzed by combined gas chromatography/mass spectrometry (GC/MS) of the per-*O*-trimethylsilyl (TMS) derivatives of the monosaccharide methyl glycosides produced from the sample by acidic methanolysis. For glycosyl linkage analysis, the samples were permethylated, depolymerized, reduced, and acetylated, and the partially methylated alditol acetates (PMAAs) were analyzed at the Complex Carbohydrate Research Center by GC/MS. The full data sets for these analyses are found in Tables S1 through S4.

Results

3.1. The GH-72 family of glucanoyl hydrolases/glucanoyl transferases (Gas/Gel glucanases)

The *N. crassa* genome contains five members of the GH-72 family of β -1,3-glucan hydrolase/glucanoyl transferase genes. Our previous proteomic analyses have identified four of the encoded gene products (GEL-1 (NCU08909), GEL-2 (NCU07253), GEL-3 (NCU01162), and GEL-5 (NCU06781)) as being cell wall proteins (Maddi et al., 2009, Maddi and Free, 2010, Maddi et al., 2012, Ao et al., 2016). We decided to focus on these four GH-72 enzymes and determine if they were important for cell wall biosynthesis in *N. crassa*. Deletion mutants for all four of these genes were available in the *N. crassa* single gene deletion library (Colot et al., 2006). To determine whether these enzymes were important for cell wall formation in *N. crassa*, we generated isolates with all combinations of double, triple, and quadruple mutations by matings between the four deletion mutants. The presence or absence of the wild type and mutant alleles in the progeny from the various matings used in generating these isolates was assessed by PCR analyses as described in Materials and Methods.

The morphology and cell wall stress phenotypes of all of the single, double, triple and quadruple isolates were characterized as described in Materials and Methods. We found that all of the single and double mutants had normal gross colonial morphologies, but that two of the triple mutants had dramatically altered colonial morphologies. The *gel-1*, *gel-2*, *gel-5* triple

mutant formed very tight colonial colonies on agar medium (Fig. 1) and in liquid medium. This mutant phenotype was partially corrected when we tested the mutant for growth in the 10% NaCl cell wall stress medium (Table 1). Growth of the mutant on an agar plate with 1.5 M sorbitol provided for a nearly complete restoration of a wild type morphology. This suggests that the partial correction seen on 10% NaCl may be due to osmotic stabilization. These results demonstrate that the GEL-1, GEL-2, and GEL-5 transglycosylases have redundant and overlapping enzymatic activities and are essential for the formation of a normal cell wall in vegetative hyphae. Interestingly, these three cross-linking enzymes were all found in hyphae cell wall proteomic analyses and are the only GH72 family transglycosylases that have been identified in vegetative hyphae (Maddi et al., 2009, Maddi and Free, 2010, Maddi et al., 2012), so there was a complete correspondence between the cell wall cross-linking enzymes being expressed in the hyphae cell wall and the mutant phenotype.

The second triple mutant that had a dramatic morphological phenotype was the *gel-1, gel-2, gel-3* mutant, which grew slower than the wild type hyphae on agar medium and was unable to elaborate aerial hyphae and conidia (Fig. 1). When grown in liquid medium this triple mutant grew in tight balls instead of generating a loosely interwoven hyphal network. The triple mutant also grew in a tight colonial manner when grown in the cell wall stress reagents caspofungin (10 mg/ml) or 0.01% SDS, and was unable to grow in the 10% NaCl cell wall stress test (Table 1). When growing on an agar medium with 1.5 M sorbitol as an osmotic stabilizer, the mutant produced abundant aerial hyphae and conidia. These results indicate that GEL-1, GEL-2 and GEL-3 are being expressed during asexual development (production of aerial hyphae and conidia) and during vegetative growth. It demonstrates that these cross-linking enzymes have overlapping and redundant activities. It was particularly interesting that GEL-3 was among the cross-linking enzymes needed to make cell wall during conidiation. We have previously shown that GEL-3 is found in the conidia cell wall and has a conidia-specific expression pattern (Ao et al., 2016). What the two most severely affected triple mutants have in common is the loss of both *gel-1* and *gel-2*, a tight colonial morphology in liquid growth conditions, and a restoration of normal morphology in the presence of the osmotic stabilizer 1.5 M sorbitol.

A third triple mutant, the *gel-1, gel-3, gel-5* mutant, had a more subtle mutant phenotype. This triple mutant had normal morphology but was unable to grow in the 10% NaCl cell wall stress test (Table 1). We were unable to define a phenotype for the fourth triple mutant (*gel-2, gel-3, gel-5*). We also generated the quadruple *gel-1, gel-2, gel-3, gel-5* mutant, and found that it was viable and morphologically indistinguishable from the *gel-1, gel-2, gel-5* triple mutant when grown on agar plates. This result may reflect the fact that *gel-3* has a conidia-specific expression pattern and may not contribute to the β -glucan cross-linking activity in the vegetative hyphae.

3.2. Characterization of the glycoprotein present in the cell walls and secretomes from transglycosylase-deficient mutants

Since cell wall protein is thought to be cross-linked to the cell wall β -1,3-glucan, we would predict that mutants affected in cross-linking glucan together might be releasing large amounts of cell wall protein into the medium. We tested this prediction by using the TCA precipitation procedure described in Materials and Methods to collect the protein being released

from mutant and wild type cells grown in Vogel's sucrose liquid medium. Fig. 2 shows a Coomassie blue stained gel of the proteins released from the wild type and triple mutants. As is evident in Fig. 2, the triple mutants are releasing large amounts of protein into the medium, while a minimum of proteins are being released from the wild type hyphae. The patterns of proteins being released into the medium by the two triple mutants were clearly similar, but some differences are obvious (Fig. 2). This suggests that the enzymes are not totally redundant. We hypothesize that the differences we see between the proteins released from the two triple mutants reflect slight differences in the ability of the cross-linking enzymes to incorporate specific proteins into the wall structure.

To directly ask whether a known cell wall protein was being released into the medium by the transglycosylase-deficient mutants, a Western blot assay experiment was done. ACW-1/CCG-15 (homolog of the *S. cerevisiae* EMC33 cell wall protein) has been shown to be a major *N. crassa* cell wall protein (Bowman et al., 2006, Maddi et al., 2009). Antibody directed against ACW-1 was used in a Western blot assay to determine whether ACW-1 was among the proteins released into the medium by the *gel-1*, *gel-2*, *gel-5* triple mutant. As shown in Fig. 3, the *gel-1*, *gel-2*, *gel-5* mutant released large amounts of the cell wall protein into the medium (lane 1), while we were unable to detect the release of any ACW-1 by the wild type cells (lane 3). Western blot analysis of the cytosolic proteins showed that the wild type and mutant cells were synthesizing similar amounts of ACW-1 (lanes 2 and 4). Western blot experiments with the *gel-1*, *gel-2*, *gel-3* triple mutant showed that it too releases ACW-1 into the growth medium. We conclude that the ACW-1 cell wall protein is not able to be efficiently incorporated into the cell wall in the two transglycosylase-deficient mutants.

To quantify the amounts of proteins being released into the medium by the *gel-1*, *gel-2*, *gel-5* and *gel-1*, *gel-2*, *gel-3* mutants, we carried out protein determination assays on the TCA-precipitated material. The protein determination for the TCA-precipitated material from the wild type cells was below the limit of what we could detect with our assay. Protein analysis on the TCA precipitated material from the *gel-1*, *gel-2*, *gel-5* triple mutant indicated that the mutant had released 33 ug of protein into the medium per mg of cellular protein. Similarly, our analysis showed that the *gel-1*, *gel-2*, *gel-3* mutant released 55 ug of protein into the medium per mg of cellular protein. Our analysis of the amount of released protein was in agreement with the data in Fig. 2, and demonstrated that the mutants released large amounts of cell wall glycoprotein into the medium.

3.3. Carbohydrate analysis of the wild type and mutant cell walls and of the polysaccharides recovered from the medium by TCA and ethanol precipitations

To determine if the carbohydrate composition of the cell wall was altered in the deletion mutants, cell walls from liquid-grown cultures were isolated as described in Materials and Methods and sent to the Complex Carbohydrate Research Center for compositional analysis and linkage analysis. The results for the compositional analysis are given in Table 2 and the linkage analysis results are found in Table 3. As is clear in Table 2, there was a dramatic difference between the wild type cell wall and the cell walls from the *gel-1*, *gel-2*, *gel-5* and *gel-1*, *gel-2*, *gel-3* mutants. The mutant cell walls had significantly less mannose and galactose and more

glucose than the wild type cell wall. In *N. crassa*, most of the cell wall mannose and galactose present in the cell wall are associated with the post-translational modifications on cell wall glycoprotein (Maddi and Free, 2010, Maddi et al., 2012). The decreased levels of mannose and galactose in the mutant cell walls demonstrates that there is a significant decrease in the amounts of cell wall glycoproteins. The mutant cell walls had an increase in the amounts of glucose present. We found that 83.4% and 92.9% of the cell wall carbohydrate in the glycosylase-deficient mutants was glucose as compared to a value of 74.9% glucose in the wild type cell wall. The linkage analysis in Table 3 for both the wild type and mutant cell walls samples showed a number of different types of glucose, mannose, and galactose residues. The mannose and galactose residues are representative of what has been seen in other *N. crassa* cell wall preparations (Maddi and Free, 2010, Maddi et al., 2012, Fu et al., 2014a) and typical for mannose and galactose residues found in N-linked oligosaccharides, the galactomannans which are attached to the N-linked oligosaccharides, and O-linked oligosaccharides. As expected, the levels of these mannose and galactose residues are reduced in the mutant cell wall preparations. What is most interesting about the cell wall carbohydrate linkage analysis shown in Table 3 is that the β -1,3-transglycosylase-deficient mutants have cell walls with an increased amount of 1,3 linked glucose (56.1% and 54% 1,3-linked glucoses in the mutant cell walls as compared to the wild type value of 35.4% 1,3-linked glucose). We have previously demonstrated that in *N. crassa* α -1,3-glucan is a conidiation-specific glucan, and that β -1,3-glucan is the only 1,3-linked glucan in the cell walls of vegetative hyphae (Fu et al., 2014b). Table 3 also shows that the mutant cell walls contain much more 1,4-linked N-acetylglucosamine than the wild type cell wall (8.4% and 8.5% for the mutants as compared to 0.3% for the wild type). This suggests that the mutant cell wall may contain more chitin than the wild type cell wall. Our results clearly demonstrate that the cell walls from the mutants have an altered composition and that GH72 β -1,3-transglycosylases are not required for the incorporation of β -1,3-glucan into the *N. crassa* cell wall.

To assess whether the transglycosylase-deficient mutants released cell wall polysaccharides into the cell wall, we carried out carbohydrate composition and linkage analyses on the TCA precipitated material. Table 4 shows the compositional analysis data and the linkage analyses are provided in Table 5. The wild type sample of TCA-precipitated material lacked polysaccharide and so no data for the wild type could be included in Tables 4 and 5. In marked contrast with the wild type sample, 44% of the dry weight of the *gel-1*, *gel-2*, *gel-5* sample and 18% of the dry weight of the *gel-1*, *gel-2*, *gel-3* sample was found to be carbohydrate. The compositional analyses for the material released into the medium by the mutants showed that the major sugars present were mannose and galactose with much smaller amounts of glucose (10.4% and 7.1% of the total carbohydrate being glucose for the two mutant samples). The linkage analysis in Table 5 shows that the types of linkages on the mannose and galactose residues are consistent with what we would expect for *N. crassa* cell wall glycoproteins. What is most striking about these analyses of cell wall material being released into the medium is that glucans were present in rather low amounts. These analyses provide additional evidence that the *gel-1*, *gel-2*, *gel-5* and *gel-1*, *gel-2*, *gel-3* mutants are releasing large amounts of cell wall glycoproteins into the medium and strongly suggests that the mutants are not releasing large amounts of cell wall glucans into the growth medium.

In doing Western blot assays, we found that both β -1,3-glucan (laminarin) and mixed β -1,3-/-1,4-glucan (lichenin) enter polyacrylamide gels during SDS-PAGE and can be detected in Western blots using anti-laminarin and anti-lichenin antibodies. These glucans are precipitated by the TCA-precipitation procedure used to precipitate proteins. To determine if these glucans were being released by the *gel-1*, *gel-2*, *gel-5* and *gel-1*, *gel-2*, *gel-3* triple mutants, Western blot experiments using anti-laminarin and anti-lichenin antibodies were used to look for the glucans in cytosolic protein and TCA-precipitated medium samples. Commercially available laminarin and lichenin were included as positive controls in these experiments. As can be seen in Fig. 4A (anti-laminarin) and Fig. 4B (anti-lichenin), laminarin and lichenin were not detected in the medium from wild type cells. In contrast, the Western blots show that β -1,3-glucan and a mixed β -1,3-/-1,4-glucan are both being released by both of the triple mutants. We hypothesize that both β -1,3-glucan and mixed β -1,3-/-1,4-glucan are found among the small amount of glucan being released by the mutant cells. This is the first report demonstrating the presence of a mixed β -1,3-/-1,4-glucan in *N. crassa*, although prior linkage analyses had demonstrated the presence of 1-4-linked glucose in the cell wall (Maddi and Free, 2010, Maddi et al., 2012, Fu et al., 2014b).

4. Discussion

Genetic and biochemical analyses have established that the GH72 family transglycosylases play a critical role in cell wall biogenesis (Free, 2013, Klis et al., 2006, Latge, 2007). These enzymes are among the most abundant proteins found in fungal cell wall proteomes. The GH72 transglycosylases have been shown to be capable of shortening and lengthening β -1,3-glucans (Hartland et al., 1996, Mouyna et al., 2000). The crystal structure of *S. cerevisiae* Gas2p has been elucidated and the enzyme has been shown to have an extended binding pocket capable of accommodating up to 10 β -1,3-linked glucose residues (Hurtado-Guerrero et al., 2009). The GH72 transglycosylases have been shown to be important for cell wall formation in *Saccharomyces cerevisiae*, *Candida albicans*, and *Aspergillus fumigatus*. The *S. cerevisiae* and *C. albicans* genomes contain five GH72 genes denoted as GAS or PHR genes, respectively (Calderon et al., 2010, Ragni et al., 2007b). The *A. fumigatus* genome contains seven GH72 genes denoted as GEL genes (Gastebois et al., 2010a, Gastebois et al., 2010c). In *S. cerevisiae*, the GAS1 and GAS5 genes are expressed during vegetative growth, and loss of GAS1 results in a reduction in cell wall glucan (Rolli et al., 2011, Ram et al., 1998). The *S. cerevisiae* GAS2 and GAS4 genes are expressed during sporulation, and the GAS2/GAS4 double mutant is affected in sporulation (Rolli et al., 2011, Ragni et al., 2007a). In *C. albicans*, Phr1p and Phr2p are required for normal morphology and virulence (Fonzi, 1999). In *A. fumigatus*, GEL4 is essential for vegetative growth (Gastebois et al., 2010a) while GEL2 is important for virulence (Mouyna et al., 2005). These previous studies show that the GH72 transglycosylases are important for fungal cell wall biogenesis, have redundant or partially overlapping activities, and are often expressed in a cell-type specific manner.

Our research on the *N. crassa* GH72 family of transglycosylases corroborates the research done in other fungi, extends our knowledge of how the GH72 enzymes function, and provides

insights into fungal cell wall biogenesis and how the cell wall is affected when the GH72 cross-linking enzymes are absent. We have characterized mutants affected in all possible combinations of the four GH72 transglycosylases found in the *N. crassa* cell wall proteome (Ao et al., 2016, Maddi et al., 2009, Maddi and Free, 2010, Maddi et al., 2012). Our results demonstrate that different combinations of these transglycosylases are being used in different cell types. We found that the combination of GEL-1, GEL-2, and GEL-5 is important for vegetative growth while the combination of GEL-1, GEL-2 and the conidia-specific GEL-3 is needed for asexual development. Our results show that growth from the hyphal tip is compromised in the *gel-1, gel-2, gel-5* mutant, which grows with a tight colonial morphology. Growth of aerial hyphae and the production of conidia were compromised in the *gel-1, gel-2, gel-3* triple mutant. We demonstrated that the two triple mutants were sensitive to cell wall stress conditions and that the addition of 1.5 M sorbitol as an osmotic stabilizer to the medium partially corrects the mutant morphological phenotypes. The high concentration of sorbitol in the medium may have activated the HOG MAP kinase signal transduction pathway, which could upregulate the synthesis of cell wall components and compensate for the loss of the GEL proteins. Analysis of the protein released into the liquid growth medium by the wild type, the *gel-1, gel-2, gel-5* triple mutant, and the *gel-1, gel-2, gel-3* triple mutant showed that the wild type cell efficiently incorporates its cell wall proteins into the wall, while the mutants released large amounts of cell wall proteins into the growth medium. Differences in the pattern of cell wall proteins being released into the medium by the two triple mutants suggests that the different GELs have partially overlapping specificities for cross-linking cell wall proteins into the cell wall, and are not totally redundant (Fig. 2). Our stress tests on several examples of each single, double, and triple mutant strongly suggested that the mutants were affected in cell wall biosynthesis and provide some further suggestive evidence for the idea that the GELs are not totally redundant in their activities (Table 1). In evaluating the data from these cell wall stress tests, we considered two different interpretations. One possibility is that GEL-1, GEL-2, GEL-3, and GEL-5 are fully redundant in their activities and that the differences we see in their phenotypes simply represent differences in the amount of “remaining” activity in each of the triple mutants. In this interpretation, the morphological consequences and sensitivity of cell wall stress reagents to the amount of remaining GEL activity in vegetative cells (Table 1) would have to be arranged in the following declining order: 1) inability to grow in 10% NaCl, 2) sensitivity to 0.01% SDS, 3) sensitivity to 10mg/ml caspofungin, and 4) tight colonial growth. The other interpretation is that there are some subtle differences in the activities of the different GELs (that is the enzymes are not fully overlapping) and the differences in the observed phenotypes reflect these subtle differences in enzymatic activity. We favor the second interpretation because the most severely affected triple mutant, *gel-1, gel-2, gel-5*, while having a tight colonial phenotype, is not “sensitive” to 10% NaCl, which would have been expected if the enzymes were fully redundant.

One interesting observation we made in characterizing the transglycosylase-deficient mutants was that the tight colonial morphology shown by the *gel-1, gel-2, gel-5* mutant in agar and liquid media and by the *gel-1, gel-2, gel-3* mutant in liquid medium is virtually identical to the morphology of the *och-1* mutant and the *dfg-5, dcw-2* mutant (Maddi and Free, 2010, Maddi et al., 2012). These mutants are affected in the synthesis of the galactomannan that is attached to

the N-linked oligosaccharides and in the GH76 family of α -1,6-mannases that are thought to be involved in cross-linking the galactomannan into the cell wall matrix. Similar to what we found with the *gel-1*, *gel-2*, *gel-5* mutant, the cell walls from the *och-1* mutant and the *dfg-5*, *dcw-1* mutant have been previously shown to be deficient in cell wall proteins and these mutants release cell wall proteins into their growth medium (Maddi and Free, 2010, Maddi et al., 2012). Thus, the morphology of the transglycosylase mutants is consistent with a deficiency in the incorporation of cell wall glycoprotein into the cell wall.

Our characterization of the GH72 transglycosylase-deficient mutants demonstrated that the mutants are defective in the incorporation of cell wall glycoproteins into the cell wall matrix. This was demonstrated in a number of different ways, including directly measuring the level of proteins released into the growth medium, SDS-PAGE gel analysis of proteins that were released into the growth medium (Fig. 2), Western blot experiments for ACW-1, a known cell wall protein (Fig. 3), an analysis of the composition and linkages of carbohydrates present in the cell wall (Tables 2 and 3), and an analysis of the composition and linkages of carbohydrates present in the material released into the medium (Tables 4 and 5). Each of these analyses clearly demonstrates that cell wall glycoproteins are not being efficiently incorporated into the cell walls of the glycosylase-deficient mutants.

What was most striking about the results of our analyses characterizing the transglycosylase-deficient mutants were the observations that the mutant cell walls had an increase in the amounts of β -1,3-glucan (Tables 2 and 3) and that the material released into the growth medium only had a small amount of glucan (Tables 4 and 5). We were able to demonstrate that the released material did contain β -1,3-glucan and mixed β -1,3-/1-4-glucan (Fig. 4), but the amounts of these glucans in the material released into the growth medium was much lower than the amounts of released cell wall glycoprotein. These observations clearly demonstrate that the GH72 family of β -1,3-glucan transglycosylases are not required for the incorporation of β -1,3-glucan into the cell wall matrix. Our data suggests that the transglycosylases play a role in the incorporation of cell wall glycoprotein into the cell wall. The exact role of the transglycosylases in this process remains to be elucidated. One likely possibility is that the transglycosylases could work in concert with the GH76 family of α -1,6-mannanases to create glycosidic bond cross-linking β -1,3-glucans with the cell wall glycoproteins. For example, the GH76 family of α -1,6-mannanases could provide the GH72 family of transglycosylases with a processed galactomannan, which the transglycosylases then use as an acceptor in a glycosyl transferase reaction. This type of reaction would incorporate cell wall glycoproteins into the cell wall by the creation of glycosidic linkages between β -1,3-glucans and the galactomannans associated with N-linked oligosaccharides present on cell wall glycoproteins.

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WT

$\Delta gel - 1,$
 $\Delta gel - 2,$
 $\Delta gel - 5$

$\Delta gel - 1,$
 $\Delta gel - 2,$
 $\Delta gel - 3$

WT	$\Delta gel - 1,$ $\Delta gel - 2,$ $\Delta gel - 3$	$\Delta gel - 1,$ $\Delta gel - 2,$ $\Delta gel - 5,$
----	--	---

188 –

97 –

62 –

49 –

38 –

29 –

17 –

$\Delta gel - 1, \Delta gel - 2,$ $\Delta gel - 5$	$\Delta gel\ 1, \Delta gel - 2,$ $\Delta gel - 5$	WT	WT
Secreted Protein	Cytostolic Protein	Secreted Protein	Cytostolic Protein

A **B**

Figure legends

Fig. 1 Morphologies of GH72 family mutants. Wild type and mutant isolates were inoculated onto Vogel's sucrose agar slants and allowed to grow for 5 days. Image 1 shows the colony morphology for wild type cells. Image 2 shows the morphology of a *gel-1*, *gel-2*, *gel-5* mutant isolate. Image 3 shows the morphology of a *gel-1*, *gel-2*, *gel-3* mutant isolate.

Fig. 2 GH72 family mutants release cell wall proteins into their growth medium. Wild type and mutant cells were grown in Vogel's sucrose liquid medium and the protein released into the medium were collected by TCA-precipitation. Aliquots representing the amount of protein released from cells with 200 µg of cytosolic protein were subjected to SDS PAGE and stained with Commassie Blue. The first lane contains the protein released from wild type cells. The second lane contains the protein released from a *gel-1*, *gel-2*, *gel-3* mutant. The third lane contains the protein released from a *gel-1*, *gel-2*, *gel-5* isolate.

Fig. 3 GH72 family mutants release ACW-1, a known cell wall protein into the growth medium. The presence of the GPI-anchored cell wall protein ACW-1 in the growth medium and in the cytosol of wild type and GH72 family mutants was assessed by Western blot analysis. The first lane contains the proteins released into the growth medium from *gel-1*, *gel-2*, *gel-5* mutant cells containing 0.1 µg of cytosolic protein. The second lane contains 0.1 µg of cytosolic protein from the *gel-1*, *gel-2*, *gel-5* mutant. The third lane contains the proteins released into the growth medium from wild type cells containing 0.1 µg of cytosolic protein. The fourth lane contains 0.1 µg of wild type cytosolic protein. Note that the wild type and mutant cytosols contain similar amounts of ACW-1, and that the mutant is releasing large amounts of ACW-1 into the growth medium.

Fig. 4 GH72 family mutants release glucans into their growth medium. Wild type and mutant cells were grown in Vogel's sucrose liquid medium and materials released into the medium were collected by TCA-precipitation. The amount of material released from cells with 200 µg of cytosolic protein was subjected to SDS PAGE. Western blot experiments were done using monoclonal antibodies directed against laminarin (β-1,3-glucan) in panel A and monoclonal antibodies directed against lichenin (β-1,3/β-1,4-glucan) in panel B. In both panels the first lane contains material released from wild type cells, the second lane contains material released from *gel-1*, *gel-2*, *gel-3* mutant cells, and the third lane contains material released from *gel-1*, *gel-2*, *gel-5* mutant cells. Note that mutant cells are releasing both types of glucan.

Table 1
Mutant sensitivities to cell wall perturbation reagents

Strain	Sensitivity to 10% NaCl	Sensitivity to 0.01% SDS	Sensitivity to 10 µg/ml caspofungin
Wild type	Not sensitive	Not sensitive	Not sensitive
<i>gel-1</i> (ΔNCU8909)	Not sensitive	Not sensitive	Not sensitive
<i>gel-2</i> (ΔNCU7253)	Not sensitive	Not sensitive	Not sensitive
<i>gel-3</i> (ΔNCU1162)	Not sensitive	Not sensitive	Not sensitive
<i>gel-5</i> (ΔNCU6781)	Not sensitive	Not sensitive	Not sensitive
<i>gel-1, gel-2</i>	Not sensitive	Not sensitive	Not sensitive
<i>gel-1, gel-3</i>	Not sensitive	Not sensitive	Not sensitive
<i>gel-1, gel-5</i>	Not sensitive	Not sensitive	Not sensitive
<i>gel-2, gel-3</i>	Not sensitive	Not sensitive	Not sensitive
<i>gel-2, gel-5</i>	Not sensitive	Not sensitive	Not sensitive
<i>gel-3, gel-5</i>	Not sensitive	Not sensitive	Not sensitive
<i>gel-2, gel-3, gel-5</i>	Not sensitive	Not sensitive	Not sensitive
<i>gel-1, gel-3, gel-5</i>	Sensitive	Not sensitive	Not sensitive
<i>gel-1, gel-2, gel-3</i>	Sensitive	Sensitive	Sensitive
<i>gel-1, gel-2, gel-5</i>	Partial rescue	Sensitive	Sensitive

Conidia from the various strains were used to inoculate Vogel's sucrose slants containing 10% NaCl, 0.01% SDS, or 10µg caspofungin. Growth was assessed by visual inspection of the slants after 72 hours.

Table 2
Cell Wall Carbohydrate Composition

residue	Wild type cell wall	<i>gel-1, gel-2, gel-5</i>	<i>gel-1, gel-2, gel-3</i>
Glucose	74.9%	92.9%	83.4%
Mannose	13.6%	2.9%	6.6%
Galactose	10.5%	2.9%	7.5%
Xylose	0.6%	0.0%	0.2%
N-acetylgalactosamine	0.2%	0.6%	1.6%
N-acetylglucosamine	0.1%	0.2%	0.3%

Cell wall samples from the wild type and mutant isolates were subjected to a neutral sugar compositional analysis at the Complex Carbohydrate Research Center.

Table 3
Cell Wall Carbohydrate linkage analysis

Residue	Wild type cell wall	<i>gel-1, gel-2, gel-5</i>	<i>gel-1, gel-2, gel-3</i>
3 linked glucopyranosyl	35.4%	54.0%	56.1%
4 linked glucopyranosyl	11.6%	8.8%	9.7%
Terminal glucopyranosyl	3.8%	1.2%	1.2%
3,6 linked glucopyranosyl	1.1%	1.5%	1.4%
4,6 linked glucopyranosyl	0.0%	0.0%	0.4%
3,4,6 linked glucopyranosyl	0.0%	0.4%	0.4%
2,6-linked mannopyranosyl	12.2%	3.9%	3.7%
2 linked mannopyranosyl	7.8%	3.1%	2.6%
Terminal mannopyranosyl	5.5%	1.5%	1.6%
6 linked mannopyranosyl	3.3%	1.2%	1.3%
2,3 linked mannopyranosyl	0.9%	0.8%	1.0%
4 linked mannopyranosyl	0.5%	1.7%	0.7%
Terminal galactofuranosyl	7.7%	3.3%	2.5%
Terminal galactopyranosyl	3.7%	2.0%	1.3%
4 linked galactopyranosyl	2.4%	2.9%	2.4%
2,3 linked galactopyranosyl	1.1%	3.2%	3.5%
3,4 linked galactopyranosyl	0.9%	1.1%	1.1%
3 linked hexofuranosyl	1.3%	0.8%	0.7%
2 linked hexofuranosyl	0.4%	0.0%	0.2%
4-linked N-acetylglucosamine	0.3%	8.4%	8.5%
4-linked N-acetylgalactosamine	0.0%	0.1%	0.1%

Cell wall samples from the wild type and mutant isolates were subjected to glycosyl linkage analysis. The samples were permethylated, depolymerized, reduced, and acetylated before being analyzed by gas chromatography-mass spectrometry.

Table 4
Carbohydrate composition of material released into the growth medium

residue	<i>gel-1, gel-2, gel-5</i>	<i>gel-1, gel-2, gel-3</i>
Glucose	15.4%	10.4%
Mannose	55.5%	58.8%
Galactose	37.1%	30.5%
Xylose	0.2%	0.1%
N-acetylgalactosamine	0.0%	0.0%
N-acetylglucosamine	0.1%	0.2%

Samples of the TCA-precipitated material from the growth medium in which wild type and mutant isolates had been grown were subjected to a neutral sugar compositional analysis at the Complex Carbohydrate Research Center.

Table 5

Carbohydrate linkage analysis on material released into the growth medium

Residue	<i>gel-1, gel-2, gel-5</i>	<i>gel-1, gel-2, gel-3</i>
3 linked glucopyranosyl	0.2%	0.0%
4 linked glucopyranosyl	9.5%	4.3%
Terminal glucopyranosyl	0.5%	1.6%
3,6 linked glucopyranosyl	0.3%	0.0%
4,6 linked glucopyranosyl	1.0%	0.9%
3,4,6 linked glucopyranosyl	0.0%	0.0%
2,6-linked mannopyranosyl	33.6%	30.7%
2 linked mannopyranosyl	11.7%	16.9%
Terminal mannopyranosyl	6.9%	8.7%
6 linked mannopyranosyl	8.8%	8.3%
2,3 linked mannopyranosyl	0.7%	0.0%
4 linked mannopyranosyl	5.2%	6.5%
Terminal galactofuranosyl	6.1%	3.4%
Terminal galactopyranosyl	4.3%	7.2%
4 linked galactopyranosyl	1.0%	0.0%
2,3 linked galactopyranosyl	0.0%	0.0%
3,4 linked galactopyranosyl	0.0%	0.0%
3 linked hexofuranosyl	1.4%	0.4%
2 linked hexofuranosyl	0.5%	0.0%
4-linked N-acetylglucosamine	1.8%	5.3%
4-linked N-acetylgalactosamine	0.0%	0.0%

Samples of the TCA-precipitated material from the growth medium in which wild type and mutant isolates had been grown were subjected to glycosyl linkage analysis. The samples were permethylated, depolymerized, reduced, and acetylated before being analyzed by gas chromatography-mass spectrometry.