Molecular Plant Pathology

MOLECULAR PLANT PATHOLOGY (2016) 17(6), 985–995 DOI: 10.1111/mpp.12352

Characterization of the *Sclerotinia sclerotiorum* cell wall proteome

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SUMMARY

We used a proteomic analysis to identify cell wall proteins released from *Sclerotinia sclerotiorum* hyphal and sclerotial cell walls via a trifluoromethanesulfonic acid (TFMS) digestion. Cell walls from hyphae grown in Vogel's glucose medium (a synthetic medium lacking plant materials), from hyphae grown in potato dextrose broth and from sclerotia produced on potato dextrose agar were used in the analysis. Under the conditions used, TFMS digests the glycosidic linkages in the cell walls to release intact cell wall proteins. The analysis identified 24 glycosylphosphatidylinositol (GPI)-anchored cell wall proteins and 30 non-GPI-anchored cell wall proteins. We found that the cell walls contained an array of cell wall biosynthetic enzymes similar to those found in the cell walls of other fungi. When comparing the proteins in hyphal cell walls grown in potato dextrose broth with those in hyphal cell walls grown in the absence of plant material, it was found that a core group of cell wall biosynthetic proteins and some proteins associated with pathogenicity (secreted cellulases, pectin lyases, glucosidases and proteases) were expressed in both types of hyphae. The hyphae grown in potato dextrose broth contained a number of additional proteins (laccases, oxalate decarboxylase, peroxidase, polysaccharide deacetylase and several proteins unique to *Sclerotinia* and *Botrytis*) that might facilitate growth on a plant host. A comparison of the proteins in the sclerotial cell wall with the proteins in the hyphal cell wall demonstrated that sclerotia formation is not marked by a major shift in the composition of cell wall protein. We found that the *S. sclerotiorum* cell walls contained 11 cell wall proteins that were encoded only in *Sclerotinia* and *Botrytis* genomes.

Keywords: cell wall proteins, glycosyl hydrolases, melanin, sclerotia, *Sclerotinia*.

INTRODUCTION

The ascomycete *Sclerotinia sclerotiorum* (lib.) de Bary is a major necrotrophic plant pathogen with a broad host range. The fungus has been shown to infect over 400 plant species and to cause an estimated $200 million of crop loss annually in the USA (Bolton *et al.*, 2006; Purdy, 1979). The fungus is characterized by the production of sclerotia, which are hard, resting structures composed of an interwoven network of hyphae (Bolton *et al.*, 2006; Purdy, 1979; Willetts and Bullock, 1992). The sclerotia can survive for many years in soil. Sclerotia are capable of producing vegetative hyphae which infect plant hosts, or can produce apothecia, ascospore-containing fruiting bodies. The apothecia forcibly eject ascospores, which can germinate to produce vegetative hyphae capable of infecting hosts. The fungus produces oxalic acid, a known pathogenicity factor (Godoy *et al.*, 1990; Guimaraes and Stotz, 2004; Liang *et al.*, 2015a), and an array of lytic enzymes to attack plant hosts (Riou *et al.*, 1991). The mechanisms involved in host cell penetration and pathogenicity have been examined (Bolton *et al.*, 2006; Erental *et al.*, 2007; Liang *et al.*, 2015a; Liberti *et al.*, 2013; Rollins, 2003).

Although a number of studies have focused on the pathogenicity of *S. sclerotiorum* and the production of lytic enzymes and oxalic acid, very little is known about the cell wall of the fungus. The cell wall is a vital organelle for the pathology, growth, survival and morphology of fungal cells (Chaffin, 2008; Free, 2013; Klis *et al.*, 2006; Latge, 2007; Latge *et al.*, 2005; Lesage and Bussey, 2006; Ruiz-Herrera *et al.*, 2006). It protects the cell from a wide range of environmental conditions, such as heat, freezing, desiccation, osmotic stress and other microbes. It plays a key role in pathogen–host interactions and in defining the morphology of the fungal cell. It contains sensors that allow the cell to assess environmental conditions and adhesion proteins that allow fungi to interact with host tissues and other cells, and to form biofilms.

Fungal cell walls consist of an interconnected network of chitin, glucans and cell wall proteins. The cell wall chitin and glucans are synthesized by chitin and glucan synthases. These synthases are located in the plasma membrane and extrude the chitin and glucan polymers into the cell wall space as the polymers are synthesized. The cell wall proteins have a signal peptide at their N-terminus which directs them into the endoplasmic reticulum (ER) during their synthesis. N-linked glycosylation occurs on cell wall proteins as they are being translated. Approximately one-half of the cell wall proteins are glycosylphosphatidylinositol (GPI)-anchored proteins. These proteins contain a GPI anchor addition signal at their carboxyl terminus. The addition of GPI anchors to these proteins occurs soon after the proteins are released into the

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ER lumen. The cell wall proteins then travel through the secretory pathway, where *O*-linked glycosylation takes place, and are then secreted into the cell wall space. Soon after arriving in the cell wall space, the chitin, glucans and cell wall proteins are cross-linked together to create a functional cell wall.

To facilitate the identification of cell wall proteins, we have recently developed a method to obtain cell wall proteins free from the glucan/chitin matrix to which they are covalently cross-linked (Maddi *et al.*, 2009). The method uses trifluoromethanesulfonic acid (TFMS), which digests glycosidic linkages without digesting peptide bonds. The proteins released by TFMS digestion of a cell wall can be used directly for proteomic analyses. The method has been used to identify cell wall proteins from cell walls of *Neurospora crassa*, *Saccharomyces cerevisiae* and *Candida albicans* (Birkaya *et al.*, 2009; Bowman *et al.*, 2006; Maddi and Free, 2010; Maddi *et al.*, 2009). The TFMS digestion removes all of the *O*-linked oligosaccharides and all of the *N*-linked oligosaccharides except for the *N*-acetylglucosamine residue which is attached to the asparagine residue. By including asparagine with an attached *N*-acetylglucosamine as a modified form of asparginine in the mass spectrometric (MS) analysis used in the identification of peptides, an investigator can identify sites of *N*-linked glycosylation in the proteomic analysis. We report herein a TFMS analysis of the *S. sclerotiorum* cell wall proteome. To assess whether the protein composition of the cell wall might be influenced by the presence or absence of plant-derived signals, we analysed the cell walls of hyphae that had been grown in either Vogel's salts medium with glucose as a carbon/energy source or potato dextrose broth (PDB) medium. We also compared the cell walls from sclerotia and vegetative hyphae to determine whether there were major changes in the cell wall protein composition as the fungus formed sclerotia. We found that all three cell wall types contained a core group of cell wall biosynthetic proteins and secreted proteins that were directed against the host plant cell wall (cellulases, pectin lyases, glucosidases and proteases). However, the PDB-grown hyphae contained a number of additional cell wall proteins that probably function to facilitate growth on the host (laccases, peroxidase, oxalate decarboxylase and cell wall proteins unique to *Sclerotinia* and *Botrytis*). An examination of the proteins in the sclerotial cell walls showed that most of the proteins were identical to those found in the vegetative hyphal cell walls. Our results demonstrate that the *S. sclerotiorum* cell wall does not undergo major changes in protein composition during the formation of sclerotia.

RESULTS

Proteomic analysis of cell wall proteins

As described in Experimental procedures, we analysed the cell walls from hyphal cells grown in Vogel's salts medium supplemented with 2% glucose and hyphal cells grown in PDB. Vogel's glucose medium contains a defined set of salts, 2% glucose and biotin as its only components. PDB medium contains potato infusion (the liquid that contains the material released into boiling water from 200 g of unpeeled potatoes/litre of medium) and 2% glucose. The pH values of the two media were similar (pH 5.5 for Vogel's medium and pH 5.1 for PDB). The potatoes release biotin during the boiling process, and so the two media will have the 2% glucose and biotin in common. The differences between the media are in their salt compositions and the presence of material released from unpeeled potatoes into boiling water in PDB medium. We used the TFMS digestion procedure to release cell wall proteins from the purified *Sclerotinia* cell walls, and subjected the released proteins to a proteomic analysis. Most of the proteins identified by several peptide fragments in the proteomic analysis of hyphal cell walls grown in PDB and Vogel's glucose medium were *S. sclerotiorum* homologues of cell wall biosynthetic proteins that had been identified previously in the cell walls of *Sa. cerevisiae*, *Schizosaccharomyces pombe*, *C. albicans*, *N. crassa* and *Aspergillus fumigatus* (Chaffin, 2008; Costachel *et al.*, 2005; Free, 2013; de Groot *et al.*, 2005, 2007; Klis *et al.*, 2001, 2010; Latge, 2007; Lesage and Bussey, 2006), which suggests that there was minimum contamination from cytosolic proteins in the hyphal cell wall samples.

We also analysed the cell wall proteins from sclerotia grown on potato dextrose agar (PDA). In this analysis, we found that a larger number of cytosolic proteins were identified than in the hyphal cell wall analyses. This was not unexpected, because it was difficult to grind the highly melanized sclerotia into a very fine powder in the mortar and pestle. However, we identified a number of cell wall proteins in the sclerotial cell wall sample. Tables S1, S2 and S3 (see Supporting Information) provide lists of the cell wall and secreted proteins identified in our analysis and the peptides used in their identification (236 peptides from GPI-anchored proteins, 171 peptides from non-GPI-anchored proteins and 142 peptides from secreted proteins). Most of these peptides contained serines and/or threonines. Some of these serines and threonines would have contained *O*-linked oligosaccharides, which would have precluded their identification without TFMS digestion. We identified 41 peptides containing an *N*-linked glycosylation site as defined by the presence of an asparagine residue modified by the presence of *N*-acetylglucosamine (7% of the identified peptides) (Tables S1, S2 and S3). All of these modified asparagine residues were found in the context of N-X-S/T, the known target for the addition of *N*-linked oligosaccharides. As shown in Tables 1, 2 and 3, the analyses identified 54 cell wall proteins and 19 secreted proteins. Five of these proteins were identified based solely on the presence of peptides containing modified asparagine residues (Tables S1 and S2).

Table 1 List of glycosylphosphatidylinositol (GPI)-anchored cell wall proteins identified in *Sclerotinia sclerotiorum* cell wall samples.

Protein name and GI no.	Identified in hyphae from PDB medium (no. of peptides)	Identified in hyphae from Vogel's glucose medium (no. of peptides)	Identified in sclerotia from PDA medium (no. of peptides)
Gas1 (GH72) β-1,3-Glucanase SS1G_12017 156039761	Yes (6)	Yes (5)	Yes (3)
Gas1 (GH72) β-1,3-Glucanase SS1G_10353 156044498	Yes (6)	Yes (7)	Yes (5)
Gas1 (GH72) β-1,3-Glucanase SS1G_12294 156040555	Yes (5)	Yes (5)	No
Gas1 (GH72) β-1,3-Glucanase SS1G_02370 156060463	Yes (8)	Yes (6)	Yes (1)
Gas1 (GH72) β-1,3-Glucanase SS1G_14460 156030472	Yes (1)	Yes (1)	No
EglC (GH17) β-1,3-Glucanase SS1G_04852 156055002	Yes (5)	Yes (5)	Yes (5)
Crh1 (GH16) β-1,3-Glucanase SS1G_04497 156057531	Yes (9)	Yes (5)	No
Dfg5 (GH76) α-1,6-Mannanase SS1G_14497 156030546	Yes (11)	Yes (7)	No
Dfg5 (GH76) α-1,6-Mannanase SS1G_11579 156041200	Yes (5)	Yes (3)	Yes (2)
Dfg5 (GH76) α-1,6-Mannanase SS1G_04850 156054998	Yes (2)	Yes (2)	No
Putative laccase SS1G_02155 156060037	Yes (2)	No	No
Putative laccase SS1G_07784 156050395	Yes (1)	No	No
Putative laccase SS1G_00809 156065597	Yes (1)	No	No
SAP-like Protease SS1G_12969 156035531	Yes (4)	Yes (2)	No
Ecm33 SS1G_10572 156043139	Yes (1)	Yes (1)	No
Cfem SS1G_07295 156051776	Yes (1)	Yes (2)	Yes (2)
Mad1 adhesin SS1G_03268 156058512	Yes (13)	Yes (11)	Yes (2)
Proline-rich cell wall protein SSIG_07359 156051904	Yes (7)	Yes (5)	Yes (4)
GPI-anchored cell wall protein SS1G_10827 156043647	Yes (1)	Yes (1)	No
Cell wall protein specific for *Sclerotinia* & *Botrytis* SS1G_02872 156061455	Yes (21)	Yes (17)	No
Cell wall protein specific for *Sclerotinia* & *Botrytis* SS1G_04213 156056965	Yes (5)	Yes (4)	No
Cell wall protein specific for *Sclerotinia* & *Botrytis* SS1G_04429 156057397	Yes (1)	No	No
Cell wall protein specific for *Sclerotinia* & *Botrytis* SSIG_11413 156040870	No	Yes (1)	No
Cell wall protein specific for *Sclerotinia* & *Botrytis* SSIG_07183 156051552	No	No	Yes (2)

List of GPI-anchored proteins. The first column lists each of the GPI-anchored cell wall proteins identified in the proteomic analysis, together with its identification number. The second, third and fourth columns show which sample(s) contained the proteins (PDB-grown hyphae, Vogel's glucose-grown hyphae and sclerotia), and the number of peptides that were identified in the cell wall samples. PDA, potato dextrose agar; PDB, potato dextrose broth.

GPI-anchored cell wall proteins

In analysing the cell wall proteins, we divided them into two types: GPI-anchored proteins and non-GPI-anchored proteins. The GPI anchor has been demonstrated to be an important element in directing GPI-anchored proteins to the plasma membrane and in attaching the proteins to the cell wall. Approximately one-half of

Table 2 List of non-glycosylphosphatidylinositol (GPI)-anchored and secreted proteins identified in *Sclerotinia sclerotiorum* cell walls.

Protein name and GI no.	Identified in hyphae from PDB medium (no. of peptides)	Identified in hyphae from Vogel's glucose medium (no. of peptides)	Identified in sclerotia from PDA medium (no. of peptides)
Exo-β-1,3-glucanase (GH17) SS1G_04264 156057067	Yes (4)	Yes (4)	Yes (1)
Exo-β-1,3-glucanase (GH17) SS1G_12024 156039775	Yes (4)	Yes (2)	Yes (2)
Exo-β-1,3-glucanase (GH17) SS1G_12930 156036482	No	No	Yes (1)
Exo-β-glucanase (GH17) SS1G_03606 156059188	Yes (1)	No	No
Chitinase (GH18) SS1G_08695 156047928	Yes (4)	Yes (1)	No
Chitinase (GH18) SS1G_00773 156065525	Yes (4)	Yes (2)	No
Endoglucanase (GH45) SS1G_13860 156032908	Yes (2)	Yes (2)	No
Chitin deacetylase SS1G_00642 156065263	No	No	Yes (1)
Polysaccharide deacetylase SS1G_04473 156057483	Yes (1)	No	Yes (5)
Exo-α-1,6-mannosidase (GH125) SS1G_04152 156056843	Yes (5)	No	Yes (7)
WSC protein SS1G_11239 156042880	Yes (4)	Yes (2)	Yes (4)
HetC-like protein SS1G_00446 156064873	No	No	Yes (3)
NCW-2 cell wall protein SS1G_10923 156043839	Yes (6)	Yes (6)	Yes (3)
Allergen cell wall protein SS1G_12262 156040491	No	Yes (1)	Yes (1)
Odc2 oxalate decarboxylase SS1G_10796 156043585	Yes (2)	No	Yes (3)
HsaA hydrophobin SS1G_09270 156045988	Yes (2)	Yes (1)	Yes (1)
Laccase SS1G_04196 156056931	Yes (9)	Yes (2)	Yes (7)
Putative cell wall protein SS1G_13599 156033047	Yes (7)	Yes (3)	Yes (6)
Putative cell wall protein SS1G_05917 156054138	Yes (4)	No	Yes (4)
Putative cell wall protein SS1G_08907 156048352	Yes (1)	No	Yes (1)
Putative cell wall protein SS1G_05337 156055970	Yes (2)	Yes (2)	Yes (2)
Putative cell wall protein with FG-GAP repeat SS1G_14133 156031050	Yes (2)	No	No
Putative cell wall protein SS1G_08110 156051043	Yes (1)	No	Yes (1)
Putative cell wall α/β hydrolase SS1G_07093 156051372	No	No	Yes (5)
Putative S/T-rich cell wall protein, unique to *Sclerotinia* & *Botrytis* SS1G_06942 156051070	Yes (8)	Yes (6)	No
Putative cell wall protein, unique to *Sclerotinia* & *Botrytis* SS1G_02714 156061143	Yes (2)	No	No
Putative cell wall protein, unique to *Sclerotinia* & *Botrytis* SS1G_04429 156057397	Yes (1)	No	No

Table 2 *Continued*

Protein name and GI no.	Identified in hyphae from PDB medium (no. of peptides)	Identified in hyphae from Vogel's glucose medium (no. of peptides)	Identified in sclerotia from PDA medium (no. of peptides)
Putative cell wall protein, unique to *Sclerotinia* & *Botrytis* SS1G_00263 156064511	Yes (1)	No	No
Putative cell wall protein, unique to *Sclerotinia* & *Botrytis* SS1G_09150 156045748	Yes (1)	No	No
Putative cell wall protein, unique to *Sclerotinia* & *Botrytis* SS1G_11120 156042644	Yes (1)	No	No

List of non-GPI-anchored proteins. The first column lists each of the non-GPI-anchored cell wall proteins identified in the proteomic analysis, together with its identification number. The second, third and fourth columns show which sample(s) contained the proteins (PDB-grown hyphae, Vogel's glucose-grown hyphae and sclerotia), and the number of peptides that were identified in the cell wall samples. PDA, potato dextrose agar; PDB, potato dextrose broth.

the cell wall proteins identified in other ascomycetes are GPI-anchored proteins (Free, 2013; de Groot *et al.*, 2005). In our analyses, we found 24 GPI-anchored proteins (Table 1). Many of these proteins were homologues of GPI-anchored proteins that have been identified previously in the cell walls of other ascomycetes. These included five homologues of the Gas/GEL family of glycosyl hydrolases (CAZy family GH72), an EglC β-1,3-glucanase (CAZy family GH17), a Crh1 homologue (CAZy family GH16) and three homologs of the Dfg5 family of α-1,6-mannanases (CAZy family GH76). All of these enzymes have been shown to be involved in cross-linking the cell wall glucans, chitin and glycoproteins together to generate the cell wall matrix (Gastebois *et al.*, 2010a; Hartl *et al.*, 2011; Maddi *et al.*, 2012b; Mouyna *et al.*, 2000; Pardini *et al.*, 2006; Ragni *et al.*, 2007). The cell wall analysis also identified four other proteins that are routinely found in other ascomycete cell walls: a homologue of the Ecm33 protein, a Cfem homologue, a SAP protease and a Mad1 adhesin. The Ecm33 and Cfem proteins have been found as 'structural' proteins in other cell walls. GPI-anchored SAP proteases have been found in *Sa. cerevisiae* and *C. albicans* cell walls, and adhesins are routinely found in fungal cell wall preparations (Chabane *et al.*, 2006; Dranginis *et al.*, 2007; Krysan *et al.*, 2005; Martinez-Lopez *et al.*, 2006; Pardo *et al.*, 2004; Romano *et al.*, 2006). In addition to these well-known cell wall proteins, the analysis also identified three putative GPI-anchored laccase enzymes, which might be involved in melanin biosynthesis. Although melanin is generally

Table 3 List of secreted proteins identified in a proteomic analysis of *Sclerotinia sclerotiorum* cell walls.

Protein name and GI no.	PDB	Vogel's glucose	Sclerotia
Cellulase (GH5) SS1G_00458 156064897	Yes (4)	Yes (4)	No
Cellulase SS1G_04085 156056709	Yes (2)	Yes (2)	Yes (2)
Cellulase SS1G_07554 156049937	Yes (1)	Yes (1)	Yes (1)
Cellobiohydrolase (GH7) SS1G_04945 156055188	Yes (3)	Yes (4)	No
Glucoamylase (GH15) SS1G_13809 156032806	Yes (2)	No	Yes (2)
Invertase (GH32) SS1G_07184 156051554	Yes (5)	Yes (1)	No
Maltase (GH31) SS1G_01083 156061938	Yes (2)	Yes (2)	Yes (2)
Pectate lyase SS1G_09216 156045880	Yes (3)	Yes (1)	No
Pectate lyase SS1G_07393 156051972	No	Yes (4)	Yes (8)
Glycosyl hydrolase (GH2) (galactosidase, mannosidase or arabinofuranosidase) SS1G_01262 156062292	Yes (4)	Yes (3)	Yes (1)
β-Glucosidase (GH3) SS1G_13255 156034641	Yes (5)	Yes (1)	Yes (12)
β-Glucosidase (GH3) glycosyl hydrolase SS1G_05368 156056032	Yes (1)	No	No
β-Glucosidase (GH3) SS1G_07146 156051478	No	Yes (3)	Yes (4)
Aspartyl protease SS1G_03941 156059856	Yes (4)	Yes (1)	No
Aspartyl protease SS1G_11818 156039363	Yes (1)	Yes (3)	Yes (5)
Secreted protease, chymotrypsin-like SS1G_09169 156045786	Yes (14)	Yes (7)	No
Catalase SS1G_01081 156061934	Yes (9)	No	Yes (2)

Table 3 *Continued*

Protein name and GI no.	PDB	Vogel's glucose	Sclerotia
Peroxidase-like SS1G_02495 156060705	Yes (5)	No	Yes (2)
Alkaline phosphatase SS1G_07639 156050107	Yes (1)	No	No

List of secreted proteins. The first column lists each of the secreted proteins identified in the proteomic analysis, together with its identification number. The second, third and fourth columns show which sample(s) contained the proteins (PDB-grown hyphae, Vogel's glucose-grown hyphae and sclerotia), and the number of peptides that were identified in the cell wall samples. PDB, potato dextrose broth.

thought to be made in melanosomes and exported into the cell wall, a cell wall laccase has been identified in *Cryptococcus neoformans* and has been shown to be involved in melanin biosynthesis (Waterman *et al.*, 2007). We also identified seven additional GPI-anchored cell wall proteins in our cell wall samples. These included two cell wall proteins with a number of homologues in other fungi and five GPI-anchored proteins that were found to be uniquely encoded in the *Sclerotinia* and *Botrytis* genomes.

Non-GPI-anchored cell wall proteins

We identified 30 proteins that were included in our list of non-GPI-anchored cell wall proteins (Table 2). Our non-GPI-anchored cell wall protein list was generated by identifying proteins with a signal peptide and then excluding transmembrane proteins, proteins with ER retention signals, proteins known to reside in the Golgi apparatus and proteins that are targeted to the vacuole (lysosome). Proteins that are thought to be secreted because they function in host carbohydrate and cell wall degradation and proteins that have been routinely identified in growth media were placed in a secreted protein category (see below) and were not included in the list of cell wall proteins. The proteins included in our cell wall protein list all have a signal peptide, and many have homologues that have been identified in the cell walls of other fungi. As shown in Table 2, the non-GPI-anchored proteins include seven glycosyl hydrolases (β-1,3-glucanases and chitinases) that are likely to function in cell wall biogenesis by cross-linking the glucan and chitin cell wall components together. The analysis also identified a chitin deacetylase and a polysaccharide deacetylase that may function to generate chitosan from the cell wall chitin. Chitin deacetylase has been shown to be an important cell wall enzyme during *Cr. neoformans* infections (Baker *et al.*, 2007, 2011). The cell wall contained an α-1,6-mannosidase that might act on *O*-linked and *N*-linked galactomannans commonly found in fungal cell walls. Among our non-GPI-anchored cell wall proteins, we identified a WSC protein, a homologue of the *N. crassa* NCW-2 (non-GPI-anchored cell wall protein), a homologue of the *A. fumigatus* cell wall allergen Asp F4, a laccase and a HetC-like protein. Homologues of these proteins have been seen in the cell walls of other ascomycetes (Hall *et al.*, 2010; Levin, 2011; Maddi *et al.*, 2009, 2012a; Wu *et al.*, 1998). WSC proteins are used as receptors for cell wall integrity signal transduction pathways and the laccase might be involved in melanin biosynthesis (Levin, 2011; Waterman *et al.*, 2007). HetC proteins have been found to function in the process of cell to cell recognition (Hall *et al.*, 2010; Wu *et al.*, 1998). Cellular functions have not been assigned to the NCW-2 and allergen proteins, and these might function as cell wall 'structural proteins'. We found the odc2 oxalate decarboxylase among our non-GPI-anchored cell wall proteins. The *S. sclerotiorum* odc2 oxalate decarboxylase has been shown previously to be associated with the cell wall fraction (Liang *et al.*, 2015b). The non-GPI-anchored protein list also includes the hydrophobin HsaA. Hydrophobins are found as non-covalently attached cell wall proteins in other fungi (Bayry *et al.*, 2012). Our list of non-GPI-anchored proteins includes seven additional putative cell wall proteins that have homologues in other fungi, and six putative cell wall proteins that were found to be encoded uniquely in the *Sclerotinia* and *Botrytis* genomes. Although these 13 putative cell wall proteins have signal peptide sequences and are included in our non-GPI-anchored cell wall protein list, it is possible that some of these putative cell wall proteins might be secreted into the medium or targeted to an intracellular location.

Secreted proteins

In our proteomic analyses, we also identified 19 proteins that we believe are secreted by the fungus. Secreted proteins would be expected to be found in our analyses because secretory proteins pass through the wall and can be trapped in purified cell wall preparations. These secreted proteins are listed in Table 3. Our list of secreted proteins includes three cellulases, a cellobiohydrolase, a glucoamylase, an invertase, a maltase, two pectate lyases, three glucosidases and a probable arabinofuranosidase. All of these enzymes are expected to be secreted and to degrade plant materials to provide for the nutritional needs of the fungus. We also found two secreted aspartyl proteases and a chymotrypsin-like protease. These proteases could be important for the digestion of plant proteins during the fungal infection. Our list of secreted proteins also includes a catalase and a peroxidase-like enzyme that might protect the fungus from host reactive oxygen species. Although we placed the aspartyl proteases, catalase and peroxidase in our list of secreted proteins, these enzymes might be covalently attached to the cell wall.

Comparison of the protein composition present in different types of hyphal cell wall

To assess whether we could identify any cell wall proteins that might be induced by the presence of the plant materials in the PDB medium, we compared the cell wall proteins present in PDB-grown hyphae with the cell wall proteins present in hyphae that had been grown in Vogel's glucose medium. We identified 22 GPI-anchored proteins in the PDB cell wall sample and 19 GPI-anchored proteins in the Vogel's glucose cell wall sample (Table 1). Of these proteins, 18 were found in both samples. For the most part, these GPI-anchored proteins are cell wall biosynthetic enzymes and structural proteins, and we can conclude that the presence of plant materials in the PDB sample did not induce a major change in this core group of GPI-anchored cell wall proteins. The GPI-anchored proteins found only in the PDB-grown hyphae and not in Vogel's glucose-grown hyphae included all three of the GPI-anchored laccases and a GPI-anchored cell wall protein that was encoded only in the *Sclerotinia* and *Botrytis* genomes (Table 1).

An examination of the non-GPI-anchored cell wall proteins from the two hyphal samples showed that the cell wall biosynthetic and cell wall structural proteins were very similar, but that the cell walls from PDB-grown hyphae contained a number of additional cell wall proteins. As shown in Table 2, we found 25 non-GPI-anchored cell wall proteins in the PDB cell wall and 13 non-GPI-anchored cell wall proteins in Vogel's glucose cell walls. Of the 25 non-GPI-anchored cell wall proteins from the PDB-grown hyphae, 12 were found in Vogel's glucose-grown cells and 13 were found only in the cell walls of PDB-grown hyphae. The non-GPI-anchored cell wall proteins found in the PDB-grown hyphae, but not in Vogel's glucose-grown hyphae, included the odc2 oxalate decarboxylase, an exo-α-1,6-mannosidase, a β-glucanase, a polysaccharide deacetylase and nine putative cell wall proteins, including five that were encoded only in the *Sclerotinia* and *Botrytis* genomes (Table 2).

We also looked for differences in the secreted proteins found in the two types of hyphal cell walls. Both cell wall types contained cellulases, a cellobiohydrolase, invertase, maltase, a pectin lyase, some glucosidases and two aspartyl proteases (Table 3). These secreted enzymes function in digesting the host cell wall and obtaining host nutrients. There were five secreted proteins found in the PDB-grown hyphae, but not in the hyphae grown in Vogel's glucose medium. These were glucoamylase, a glucosidase, alkaline phosphatase, catalase and the peroxidase. We hypothesize that the starch in PDB was responsible for inducing glucoamylase. We hypothesize that the catalase and peroxidase might have been induced by other factors in PDB.

In summary, our findings demonstrate that the composition of a core group of cell wall proteins involved in fungal cell wall biogenesis, in attacking the host cell wall and in mobilizing nutrients from the host was nearly indistinguishable between the two types of hyphae. However, the PDB-grown hyphae produced a number of cell wall and secreted proteins not found in Vogel's glucose-grown hyphal cell walls. These included laccases, oxalate decarboxylase, polysaccharide deacetylase, catalase, peroxidase and 10 putative cell wall proteins, including six that were encoded only in the *Sclerotinia* and *Botrytis* genomes. We hypothesize that the presence of plant materials in PDB induced the synthesis of these cell wall and secreted proteins.

Comparison of the protein composition present in the cell walls from hyphae and sclerotia

We were interested in learning whether there were major changes in the proteins that were incorporated into the cell wall during the formation of sclerotia. The sclerotial cell is a highly melanized structure which allows the fungus to survive in the soil, and also functions to generate apothecia (Bolton *et al.*, 2006; Purdy, 1979; Willetts and Bullock, 1992). The apothecium is a reproductive structure which generates ascospores. The outer surface of the sclerotium is highly melanized, whereas the more interior regions of the sclerotium are not. The melanized sclerotium made it more difficult to isolate cell wall material, and the melanin itself appeared to be resistant to digestion with TFMS. However, TFMS did release cell wall proteins from the isolated sclerotial cell walls. We identified nine GPI-anchored proteins from our sclerotial cell walls, and found that eight of these were among the cell wall proteins present in the hyphal cell wall samples (Table 1). The only GPI-anchored protein identified in the sclerotial cell wall that was not present in the hyphal samples was a GPI-anchored cell wall protein that was encoded only in the *Sclerotinia* and *Botrytis* genomes (Table 1). We conclude that the GPI-anchored protein composition of the sclerotial cell wall is very similar to that of the hyphae.

We identified 19 non-GPI-anchored proteins in our sclerotial cell wall samples. Of these 19 proteins, 15 were present in the hyphal cell wall samples. The four non-GPI-anchored proteins that were unique to the sclerotial cell wall in our analysis were an exo-β-1,3-glucanase, a HetC-like protein, a chitin deacetylase and a putative cell wall protein. These results from the analysis of both GPI-anchored proteins and non-GPI-anchored proteins show that there may be a few cell wall proteins that are specific to sclerotia. However, most of the proteins found in the sclerotial cell wall are identical to those found in hyphae. We conclude that the formation of sclerotia is not marked by a major change in the composition of cell wall proteins.

DISCUSSION

We used a TFMS digestion protocol to identify cell wall proteins from *S. sclerotiorum* cell walls (Maddi *et al.*, 2009). The use of TFMS to digest the glycosidic bonds in the cell wall glucans and

chitin polymers has several advantages for proteomic analyses. First, it releases the proteins from the glucan/chitin matrix and makes them readily available for proteomic analysis. Second, it removes all of the *O*-linked glycosylation, which allows for the proteomic identification of peptides that would otherwise not be available because of the presence of *O*-linked glycosylation. As cell wall proteins often contain a large number of serines and threonines, which are *O*-glycosylated, the TFMS digestion significantly increases the number of peptides that can be identified from highly glycosylated cell wall proteins. Third, TFMS digestion removes all of the *N*-linked oligosaccharides, except for the *N*-acetylglucosamine residue attached to asparagine. By including asparagine with *N*-acetylglucosamine as a modified asparagine residue in the analysis, peptides with *N*-linked oligosaccharide addition sites can be identified. We were able to identify 24 GPI-anchored and 30 non-GPI-anchored proteins, and 19 secreted proteins. Many of these have homologues in the cell walls of other ascomycetes. We found that the maximization of the number of observable peptides by the removal of *O*-linked oligosaccharides and the inclusion of peptides with *N*-linked glycosylation sites were very helpful. We found that 7% of the identified peptides contained *N*-linked glycosylation sites, and five of the identified cell wall proteins were identified solely on the basis of a peptide with an *N*-linked glycosylation site. We conclude that the TFMS digestion procedure is particularly well suited for the identification of cell wall proteins.

The protein composition of the *S. sclerotiorum* cell wall included 10 GPI-anchored glycosyl hydrolases and eight non-GPI-anchored glycosyl hydrolases. These types of enzyme have been shown to be among the major proteins in ascomycete cell walls (Chaffin, 2008; Free, 2013; de Groot *et al.*, 2005; Klis *et al.*, 2010; Latge, 2007, Lesage and Bussey, 2006), and they were among the proteins identified by a larger number of peptides (Tables 1 and 2). There is strong evidence for these enzymes having a role in the cross-linking of the cell wall matrix together. In addition to catalysing the cross-linking reactions, the proteins themselves become a major part of the cross-linked matrix. In *S. sclerotiorum* cell walls, we identified five GPI-anchored Gas/GEL proteins (Table1). These have been shown to catalyse the cleavage and joining of β-1,3-glucans to shorten or lengthen the glucan polymers (Hartland *et al.*, 1996; Hurtado-Guerrero *et al.*, 2009; Mouyna *et al.*, 2000). We found a GPI-anchored Crh1 (GH16), an enzyme that has been implicated in the cross-linking of chitin and glucan polymers together (Cabib *et al.*, 2007; Pardini *et al.*, 2006). We also found a GPI-anchored EglC/Bgl2 (GH17) enzyme in our analysis. These enzymes have been shown to be involved in the cross-linking of glucans together (Gastebois *et al.*, 2010b; Goldman *et al.*, 1995). Altogether, we identified 12 enzymes that would be classified as glucanases and two chitinases among our cell wall proteins (Tables 1 and 2). We also identified three GPI-anchored DGF5-like α-1,6-mannanases (CAZy family GH76) in our analysis. We have shown recently that these enzymes function in the cross-linking of cell wall proteins into the cell wall matrix through the galactomannans or outer chain mannans attached to the *N*-linked oligosaccharides (Maddi *et al.*, 2012b). Thus, these enzymes, like the other glycosyl hydrolases, function in the cross-linking of the cell wall matrix together. The glycosyl hydrolases are expressed as multigene families in the cell walls of other fungi, and the *S. sclerotiorum* cell wall is typical of the situation in other fungi. We also identified four laccase homologues, chitin and polysaccharide deacetylases, an Ecm33 homologue, a Cfem homologue, a Mad1 adhesin, a HetC homologue, a WSC homologue, aspartyl protease homologues, an NCW-2 homologue, a cell wall allergen homologue, odc2 oxalate decarboxylase and a hydrophobin (HsaA) in our cell wall samples. Homologues of all of these have been found in the cell walls of other fungi. We also found a number of putative cell wall proteins, proteins that have not been catalogued previously as cell wall proteins. What was most interesting is that we found 11 of these putative cell wall proteins in our cell walls that were encoded only in the *Sclerotinia* and *Botrytis* genomes (Tables 1 and 2). Our results demonstrate that the *S. sclerotiorum* cell wall contains an array of typical cell wall biosynthetic proteins, as well as an array of secreted hydrolases to digest the host cell wall and obtain host nutrients (cellulases, cellobiohydrolase, pectin lyase, invertase, maltase, glucosidases and aspartyl proteases). The results also show that, in the presence of plant materials, the wall also contains a number of cell wall proteins that are unique to *Sclerotinia*.

To look for cell wall proteins that are induced by plant materials, we compared the cell walls of PDB-grown hyphae with the cell walls from hyphae grown in Vogel's glucose medium, a medium without plant-derived materials. We found a number of interesting proteins that were expressed in the presence of plant materials, but not in Vogel's medium, including three GPI-anchored laccases, odc2 oxalate decarboxylase, a catalase, a peroxidase and a number of putative cell wall proteins. We hypothesize that the laccases might be involved in the generation of cell wall melanin. A cell wall laccase has been found in *Cr. neoformans* and has been shown to be involved in melanin synthesis (Waterman *et al.*, 2007), and the *S. sclerotiorum* laccases might function similarly during growth in the plant host and during sclerotia formation. Melanization of *S. sclerotiorum* vegetative hyphae has been shown to occur recently (Butler *et al.*, 2009). Melanization of the fungal cell wall has been shown to help provide protection against reactive oxygen species and against host-generated glycosidases directed against the fungal cell wall polymers (Butler and Day, 1998; Eisenman and Casadevall, 2012). The catalase and peroxidase might function to protect the hyphae from host-produced reactive oxygen species. An *S. sclerotiorum* odc2 oxalate decarboxylase mutant has been

characterized, and the enzyme has been found to help regulate the amount of oxalic acid accumulating in the proximity of the hyphae (Liang *et al.*, 2015b). The production of these cell wall and secreted proteins in response to the presence of plant materials would help to facilitate host infection by protecting the fungus from host defences and by regulating the production of oxalic acid, a known pathogenicity factor. The putative cell wall proteins present in the PDB-grown hyphae are particularly interesting. Several of these proteins were found to be encoded only in the *Sclerotinia* and *Botrytis* genomes. These proteins may well represent cell wall proteins that have allowed the fungus to be an effective pathogen. It will be interesting to determine how these cell wall proteins function and whether they are important for the pathology of the fungus.

A comparison of the protein composition of the sclerotia with that of the vegetative hyphae revealed only five proteins that were identified in the sclerotia, but not in the vegetative hyphae. The sclerotia contained a β-1,3 glucanase not found in the vegetative hyphae, but the vegetative hyphae did contain other β-1,3-glucanases (Tables 1 and 2). The sclerotia also contained a HetC-like protein not found in the vegetative hyphae. The function of the HetC-like protein in *S. sclerotiorum* is unknown, but HetC proteins in other fungi function in cell-to-cell recognition (Hall *et al.*, 2010; Wu *et al.*, 1998). We also identified a chitin deacetylase, a GPI-anchored protein and a non-GPI-anchored protein in the sclerotia that were not seen in the vegetative hyphae. Most importantly, of the 28 cell wall proteins identified in the sclerotia, 23 (82%) were also found in the vegetative hyphae. Our results indicate that sclerotia formation is not marked by a major change in cell wall protein composition. We conclude that the protein composition of the cell wall is largely retained as hyphae participate in sclerotia formation.

Important insights into the *S. sclerotiorum* infection process have been gained from the analysis of the genome (Amselem *et al.*, 2011; Rollins *et al.*, 2014) and from transcriptomic analyses of the fungus during the course of plant infection (Guyon *et al.*, 2014; Muellenborn *et al.*, 2011; Oliveira *et al.*, 2015; Sexton *et al.*, 2006). An examination of the *S. sclerotiorum* expressed sequence tag (EST) libraries shows that mRNAs encoding most of the cell wall proteins identified in our proteomic analysis are expressed. More importantly, these transcriptomic studies clearly show that, during plant infection, there is a temporal sequence to the expression of the pathogen's genome, and that the transcription of the genome is regulated in a complex manner. Our study showing that plant material induces the expression of some cell wall and secreted proteins which may be important for the growth of the pathogen on plant materials should be considered in the context that plant infection is a complex process. The proteins identified here may be reflective of the early-stage changes in the cell wall during infection, and additional changes may occur during the course of an infection.

EXPERIMENTAL PROCEDURES

Strains and growth conditions

The wild-type *S. sclerotiorum* isolate (ATCC#18683) was obtained from the American Type Culture Collection (Manassas, VA, USA) and used throughout the experiments. The fungus was routinely maintained on PDA plates. Hyphal samples were grown in liquid PDB or in liquid Vogel's glucose medium (Davis and DeSerres, 1970). PDB-grown hyphae were produced by inoculating hyphae from a PDA plate into Petri dishes with 20 mL of PDB, and were allowed to grow for 5 days at 24 °C before harvesting. To produce hyphae that had been grown free of plant materials, the fungus was passaged once on a Vogel's glucose agar plate, and hyphae were used to inoculate Petri dishes containing 20 mL of Vogel's glucose liquid medium. The hyphae were allowed to grow for 6 days at 24 °C before harvesting. Sclerotia were produced by inoculating the fungus on Petri dishes with PDA and incubating the plates at 24 °C for 12 days with a 12-h/12-h light/dark cycle. The sclerotia were manually harvested and the adherent hyphae were removed from the sclerotia.

Isolation of cell wall material

The hyphal samples were harvested on a Büchner funnel and washed with sterile distilled water. The hyphae were then transferred to a mortar containing liquid nitrogen and were ground to a fine powder with a pestle. Liquid nitrogen was periodically added to the sample to keep the hyphae frozen during the grinding process. The harvested sclerotia were also transferred to a mortar containing liquid nitrogen and ground to a very fine powder. The ground samples were suspended in 10 mL of phosphate-buffered saline (PBS), transferred to a centrifuge tube and the cell walls were collected by a 10-min centrifugation at 6000 ***g*** in a refrigerated centrifuge. The samples were washed three times with ice-cold PBS and then suspended in PBS containing 1% sodium dodecyl sulfate (SDS). The centrifuge tube containing the samples was then placed in a boiling water bath for a 15-min incubation to release proteins that were not covalently attached to the cell wall. The samples were then washed twice with PBS and three times with distilled water. The cell wall pellets were then subjected to lyophilization to remove any water associated with the cell wall material.

TFMS digestion, SDS gel and proteomic analysis

Fifteen milligrams of the purified lyophilized cell walls were weighed out on an analytical balance and subjected to digestion with TFMS. The TFMS digestions were carried out as described previously (Bowman *et al.*, 2006; Maddi *et al.*, 2009). Briefly, the lyophilized samples were placed in glass test tubes and 1.25 mL of TFMS containing 16% anisole was added to each of the samples with a glass syringe. The samples were purged with N_2, covered with parafilm and placed in a chamber that was continually purged with N_2. The samples were subjected to TFMS digestion for 24 h. During this time, the samples were periodically purged with N_2 and were covered with parafilm. The digestion occurred at 4 °C and all of the glassware and syringes were dried in a vacuum prior to use to ensure that anhydrous conditions prevailed during the digestion. The hyphal cell wall samples were completely digested at the end of the 24-h incubation, as assessed by visual inspection, whereas the more melanized sclerotial cell

wall samples retained some undigested material. At the end of the digestion, 3.75 mL of a pyridine–methanol–water solution (3 : 1 : 1 ratio of pyridine, methanol and water) were added dropwise to each of the samples. The samples were swirled in a dry ice–ethanol bath during the addition of the pyridine–methanol–water solution to keep the samples cold. The samples were left in the dry ice–ethanol bath for 20 min and transferred to a −20 °C freezer for an additional 20 min. The samples were then removed from the freezer, allowed to thaw and 1 mL of 5% ammonium bicarbonate was added to each sample. The proteins released by TFMS digestion of the cell wall were then collected by trichloroacetic acid (TCA) precipitation. Six millilitres of 30% TCA in acetone were added to each sample and the proteins were allowed to precipitate during an overnight incubation at −20 °C. The precipitated proteins were then collected by a centrifugation step (10 000 ***g*** for 10 min) and washed three times with −20 °C acetone. Some salt precipitate is often seen when collecting the precipitated protein, but this dissolves during the washing steps.

The precipitated protein was resuspended in SDS-gel loading buffer and subjected to a 5-min electrophoresis in a NuPage 4–12% bis-tris acrylamide gel (Life Technologies, Grand Island, NY, USA), so that the protein entered the top of the gel. The gel was then stained with Coomassie blue to delineate how far into the gel the proteins had migrated. The region of the gel containing the proteins (the upper 0.5 mm) was cut from the gel and sent to the Fred Hutchinson Cancer Research Center shared facility proteomics laboratory (Seattle, WA, USA) for trypsin digestion and proteomic analysis. The identified peptides were matched to the *S. sclerotiorum* proteome by a search of the annotated *S. sclerotiorum* genome in the National Center for Biotechnology Information (NCBI) database. Each of the proteins identified in the samples was then examined for the presence of a signal peptide using the Signal P predictor program and for the presence of a GPI-anchoring site using the Big P program (Eisenhaber *et al.*, 2004; Petersen *et al.*, 2011). Any proteins that were thought to be lysosomal, ER or Golgi proteins were removed from the list of potential cell wall proteins.

ACKNOWLEDGEMENT

This research was supported by grant R03-AI103897 from the National Institutes of Health and funds from the University at Buffalo Foundation.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1. Glycosylphosphatidylinositol (GPI)-anchored proteins identified in the proteomic analysis of the *Sclerotinia sclerotiorum* cell wall from hyphae and sclerotia.

Table S2. List of non-glycosylphosphatidylinositol (GPI)-anchored cell wall proteins identified in the proteomic analysis of the *Sclerotinia sclerotiorum* cell wall from hyphae and sclerotia.

Table S3. List of secreted proteins identified from *Sclerotinia sclerotiorum* cell walls.