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Discovery of Fungal Cell Wall Components Using Evolutionary and Functional Genomics

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ABSTRACT OF THE DISSERTATION

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Dr. Jason E. Stajich, Chairperson

The cell wall is an essential component of fungi that provides shape and protection to cells. As the composition of the wall differentiates pathogenic fungi from the plants and animals they infect, it is a common target for anti-fungal compounds. The goal of the research presented in this dissertation is to make a significant contribution in furthering our understanding of the fungal cell wall and its various components. I have identified and characterized genes involved in cell wall growth and maintenance in the model fungus *Neurospora crassa* by employing a functional screen based on expression data of vegetative colony development. 14 novel cell wall stress sensitive mutants having multiple phenotypic defects in the categories cell wall morphogenesis, MAPK signaling, Rho-signaling and transport were identified. Taking a broader approach, a combination of phylogenetics, comparative genomic and biochemical approaches was used to extend the understanding of the cell wall components to the early fungal species by reconstructing the phylogenetic history of the fungal cell wall across the evolution of the kingdom. Profile HMMs constructed for proteins involved cell wall biosynthesis,

maintenance and remodeling were searched against a collection of fully sequenced genomes to reveal a new class of chitin synthase (CHS) and chitinase (CTS) gene families and the timing of gain of 1,3-beta glucan metabolism machinery in the fungal kingdom. Biochemical characterization of fungal cell walls from early diverging lineages uncovered the existence of noticeably distinct cell walls within the fungal kingdom. The Blastocladiomycota cell walls were found to be similar to Ascomycota i.e. glucan-rich and poor in chitin. Whereas a Chytridomycota fungus had a chitin-rich and glucan-poor cell wall, with mannose being the most substantial component. The Mucormycotina were the most unique having the heteropolymer 'mucoran' as the largest fraction, followed by chitin and very less glucan. This study is the first to report that the Chytridomycete cell walls contain no 1,3 beta-glucan. The presented research should lead to development of better drug targets for designing anti-fungal drugs and enhance our understanding of how cell walls of differing fungal lineages have evolved.

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INTRODUCTION

Characterizing and cataloguing the fungal cell wall components is of great importance as the cell wall is one of the important features that differentiates the fungi from the plants and animals that they infect. This makes it an excellent target for anti-fungal drug development. Components of the cell wall, β-1,3-glucan synthase and chitin synthase, are targeted by the currently available anti-fungals such as echinocandins and nikkomycin respectively. Identification of additional cell wall components and the proteins necessary for their production will enable development of additional potential drug targets for combating the serious disease-causing fungi in humans and plants. In the presented study a multidisciplinary approach has been taken to identify novel genes important in cell wall processes in a model filamentous fungus and to catalogue the genomic and biochemical repertoire of the cell wall across the fungal kingdom including early diverging lineages. This approach involves a combination of functional genomics, evolutionary biology and comparative genomics and has proven to be very powerful. I anticipate that the knowledge gleaned from this research will lead to development of additional targets for anti-fungal drugs that can be useful against pathogenic fungi. In addition an insight on the range of variation present in cell wall composition can advance our understanding of the evolutionary history of the fungal kingdom. In the following sections the concepts important for this study are discussed.

Fungi

Overview

By general definition, fungi are multicellular, eukaryotic organisms dependent on absorptive nutrition and reproduce by means of spores. The Fungi constitute a monophyletic group of organisms and have been classified as the taxonomic kingdom (also known as Mycota). Fungi are the sister clade to the animal and Choanoflagellate kingdom (James, Kauff et al. 2006) and represent one of the three kingdoms that contain multicellular organisms (Ruiz-Trillo, Burger et al. 2007) . There are estimated to be at least 1.5 million different species of fungi (Hawksworth 2001), however a recent study states there may be over 5 million species (Blackwell 2011). For comparison there are estimated to be 4.9 million animal species (arthropods) and about 420,000 seed plants (Hawksworth 2001).

The fungi can influence human environments in beneficial and detrimental ways. Among the advantageous roles are the one the fungi play as decomposers and recyclers of organic matter, including the degradation of cellulose and wood by the specialized enzymes unique to fungi. Also fungi are important sources of secondary metabolites that are used in medicine, for example the antibiotic, penicillin (from *Penicillium notatum*) and the immunosuppressant, cyclosporin A (from *Trichoderma polysporum*); in textile industry as lytic enzymes for example cellulases from *Trichoderma reesei* give jeans a stonewashed effect and also in food industry for the production of citric acid

(using *Aspergillus niger*). Fungi are also major sources of food, they are used for production of cheese, bread, beer, as mushrooms and in manufacture of fermented foods like yogurt, soy sauce, miso and koji. Additionally fungi are among the few organisms that can be genetically manipulated for producing hetrologous gene products, for eg. Yeast (*Saccharomyces cerevisiae*) has been used for the production of hepatitis vaccine similar to the way the bacterium (*Escherichia coli*) is used for producing insulin. Moreover fungi are increasingly being used as commercial biological agents providing alternatives to chemical pesticides for combating insect pests, nematodes and pathogenic fungi.

Although the fungi have quite a few beneficial uses, yet they have managed to gain notoriety of being a bane to mankind. Fungi are the most important cause of crop diseases, responsible for billions of dollars worth of damage each year and for periodic devastating disease epidemics like corn smut, wilt, black root rot etc. They also cause many lethal and acute diseases in animal and humans like HIV, hepatitis, aspergillosis, oral thrush, athlete's foot, yeast vaginitis and meningitis among others.

There are specific characteristics that clearly separate fungi from other organisms and thus serve to define the fungal kingdom. Fungi are generally eukaryotic which means they have membrane bound nuclei containing chromosomes and other cytoplasmic organelles (mitochondria, vacuoles etc.). Fungi typically grow as filaments (hyphae) that branch repeatedly to give rise to a network called mycelium. Some fungi however grow as single celled yeasts (For e.g. *S. cerevisiae*), which reproduce by budding.

Dimorphic fungi are that can switch between yeast and hyphal phases in response to environmental conditions. Fungi are hetrotrophs, which means they need preformed organic compounds as energy sources. This is achieved in the fungi by secreting enzymes to degrade complex polymers and then absorbing the simple soluble nutrients released by the degrading enzymes. Most fungi have a distinctive cell wall composition typically including chitin, glucans, glycoproteins and other sugars in proportions that vary among species. Fungi mostly exist as haploid (unlike other eukaryotes); however many fungi have multiple nuclei within each hyphal compartment and also many budding yeasts exist as diploid. Fungi reproduce by both sexual and asexual means and typically produce spores to propagate themselves. Fungi can live in modes as a parasite (or pathogen) of another living organism, by growing as a symbiont (in association) with another organism or as a free-living saprotroph on organic matter.

Evolution and diversification

Fungi diverged from the ancestor of Animals & Fungi around 1,500 million years ago (Wang, Kumar et al. 1999; Taylor and Berbee 2006). Fungi likely colonized the land during the Cambrian era, over 500 million years ago (Taylor and Osborn 1996) but fossils only become uncontroversial and common during the Devonian, 400 million years ago (Taylor, Hass et al. 1992). The Early Devonian Rhynie chert is host to a plethora of fossil fungi including various endotrophic mycorrhizae, the earliest ascomycetes, several chytridiomycetes and various other undescribed fungi (Taylor and Berbee 2006). Until recently, Chytridiomycota, were widely believed to be among the

most ancient of the present known fungi (Hibbett, Binder et al. 2007). However genome sequencing of five species of Microsporidia (Capella-Gutierrez, Marcet-Houben et al. 2012) and the Rozella species of Cryptomycota (Jones, Forn et al. 2011) and subsequent phylogenetic analysis (James, Pelin et al. 2013) concluded that Microsporidia and Cryptomycota were sister clades that were the earliest to diverge in the fungal kingdom. The major fungal phyla described presently Microsporidia, Cryptomycota, Neocallimastigomycota, Chytridiomycota, Blastocladiomycota, Zygomycota, Ascomycota and Basidiomycota.

Microsporidia are unicellular parasitic fungi with chitin containing walls. They lack true mitochondria but they have reduced structures known as mitosomes that are derived from mitochondria but don't contain any DNA (Corradi and Keeling 2009). DNA sequence analysis has shown that Microsporidia possess a highly reduced genome (Keeling and Corradi 2011). Microsporidia are obligate parasites of animals, especially of insects, crustaceans and fish. Some species are also known to infect mammals including humans. A polar tube of the microsporidian spore penetrates the host cell and the contents of the spore are injected into the host. The sporangium then replicates within the host cell and produces new infective spores. Cryptomycota is the most recently discovered phylum of fungal kingdom (Jones, Forn et al. 2011). Its only known member, *Rozella allomycis* is an intracellular parasite of 'water mold'- *Allomyces*. The infection begins with uniflagellate, wall-less zoospores of the parasite that swim to the host, attach to the surface and form a penetration tube through which the parasite

cytoplasm is injected into the host. Once inside the host, *R. allomycis* then grows as a wall-less form that feeds on the host cytoplasm (James and Berbee 2012).

Neocallimastigomycota possess hydrogenosomes (hydrogen- and ATP-producing organelles), lack mitochondria, and grow as commensal organisms in the gut of a variety of herbivores (Griffith, Baker et al. 2010). These obligate anaerobic symbionts play a key role in the digestion of plant cell wall material and provision of hydrogen to the herbivore digestive tract ecosystem. They have been found in virtually all herbivorous mammals studied so far, including elephants, llamas, donkeys, horses, capybaras, and ruminants. *Piromyces* species are the only known members of this phylum till date. They produce uniflagellate zoospores that develop into a monocentric thallus consisting of a zoosporangium and an extensively branched rhizoidal system (Orpin 1977). The Chytridiomycetes are flagellated, zoosporic fungi that live in water or moist soil (James, Kauff et al. 2006). They are mostly saprobic or parasitic on pollen, algae and invertebrates and play an important ecological role in the degradation of recalcitrant materials, such as chitin, keratin, and cellulose. Most species are unicellular, protected by a chitnous cell wall that matures into to a sporangium, which then gives rise to posteriorly-uniflagellate zoospores. The zoospores are cleaved from the sporangial cytoplasm and swim to a fresh substrate, retract the flagellum, and secret a new cell wall to encyst. The cyst germinates to start the life cycle anew (Stajich, Berbee et al. 2009). *Batrachochytrium dendrobatidis* is the most well known member of this phylum, a parasitic fungus that causes the disease chytridiomycosis in amphibians and

has been responsible for global declines in frog populations. Blastocladiomycetes are posteriorly flagellated like the Chytridiomycetes but grow as hyphae (that often sprout rhizoids) (James, Kauff et al. 2006). They are unique in possessing alternating haploid and diploid generations. In *Allomyces macrogynus*, the most well studied blastocladiomycete, the two gametes fuse to form a diploid zygote which germinates to form a diploid mycelium that in turn produces numerous flagellated zoospores, which are all diploid. These diploid organisms also produce a zoosporangium that is a thick walled resting spore capable of surviving unfavorable conditions such as dry weather or freezing. Nuclei in the resting sporangium undergo meiosis, giving rise to haploid zoospores that are released into water and begin the haploid stage of the life cycle. The haploid mycelium is similar to that of the diploid mycelium but it matures to produce female and male gametes (Porter, Martin et al. 2011).

Zygomycota is the phylum that has formerly been known to encompass four subphyla- Mucoromycotina, Entomophthoromycotina, Zoopagomycotina and Kickxellomycotina (White, James et al. 2006). As of today, Entomophthoromycota has been raised to a phylum level (Gryganskyi, Humber et al. 2013) and the subphylum- Glomeromycota has been added to Zygomycota (F. Martin, unpublished results). These fungi exist as saprobes on soil/animal dung, as parasites on insects or as mutualists of other fungi and invertebrate animals. These fungi produce cell walls containing chitin. They grow primarily as mycelia, or filaments of long cells called hyphae, which are generally coenocytic (multinucleate) because they lack cross walls or septa. The unique character

(synapomorphy) of the Zygomycota is the 'zygospore'. Zygospores are formed within a zygosporangium after the fusion of specialized hyphae called gametangia during the sexual cycle. A single zygospore is formed per zygosporangium. The mature zygospore is often thick-walled, and undergoes an obligatory dormant period before germination. Most zygomycota are thought to have a zygotic or haplontic life cycle, thus the only diploid phase takes place within the zygospore. The sub-phylum Mucormycotina consists of fungi of the genera *Mucor, Rhizopus, Phycomyces,* which grow mainly as saprotrophs in soil/dung or over-ripe fruits (James, Kauff et al. 2006). The second subphylum Zoopagomycotina consists of fungi like *Zoophagus*, which traps rotifers, amoebae or nematodes by attracting the tiny animals to feed on short, lateral hyphae that are covered with adhesives (Whisler and Travland 1974). These fungi are parasitic on animals or other fungi and that form haustoria, hyphae that are specialized to promote nutrient transfer from host to fungus. The third subphylum is the Kickxellomycotina consisting of fungi like *Kickxella* sp. and *Coemansia* sp. that are typically saprobes, mycoparasites of Mucoromycotina and animal parasites. Kickxellomycotina produce septa containing central pores by means of mitosis in their hyphae that become plugged with time (Tretter, Johnson et al. 2013). Glomeromycotina the newly added sub-phylum to Zygomycota comprises of arbuscular mycorrhizal fungal species like *Acaulospora* sp. and *Geosiphon* sp. The Glomeromycotina have generally coenocytic (occasionally sparsely septate) mycelia and reproduce asexually through blastic development of the hyphal tip to produce spores (Schubler, Schwarzott

et al. 2001). Entomophthoromycota phylum contains fungal species like *Conidiobolus* sp. and *Entomophthora* sp. that are primarily obligate arthropod parasites that are found in diverse habitats worldwide. These fungi can grow as a well-developed, multinucleate or septate mycelium that is either walled or protoplastic, depending on the species and the stage of growth. Most of the entomopathogenic species develop inside their arthropod hosts as multinucleate hyphal bodies. Asexual reproduction in species of Entomophthoromycota is characterized by the production of forcibly discharged conidia on distinctive conidiophores (Gryganskyi, Humber et al. 2013).

The most recently diverged fungi belong to the sub-kingdom Dikarya comprising of the phyla Ascomycota and Basidiomycota, both of which in general produce dikaryons (cells with two nuclei) and may be filamentous or unicellular (Hibbett, Binder et al. 2007). Ascomycota is one of the most important phyla and contains about 75% of the fungi known to date. The feature that characterizes all members of this phylum is the ascus- a cell in which two compatible haploid nuclei of different mating types come together and fuse to form a diploid nucleus, followed by meiosis to produce haploid sexual spores, termed 'ascospores' (Alexopoulos, C.W. et al. 1996). Some notable Ascomycete species are unicellular yeasts like *S. cerevisiae* and *Schizosaccharomyces pombe* that reproduce asexually by budding or fission. Sexual reproduction takes place when two adjacent haploid cells of opposite mating types fuse. Most ascomycete fungi are filamentous like *Neurospora sp*. and *Aspergillus sp*., which reproduce asexually by means of conidia that form at the tips of specialized hyphae and sexually through

formation of a dikaryon as a result of fusion of two opposite mating type conidia. Basidiomycota is the second phyla in the Dikarya, which comprises of fungi like *Ustilago maydis* and *Puccinia graminis* that are parasitic on plants and *Coprinopsis cinerea* that grow as mushrooms. The hyphae of basidiomycete fungi have septa with small, distinctive pores. The 'basidium', a swollen cell at the tip of a specialized hypha is the charateristic sexual reproductive structure of the club fungi. The basidium is the site of nuclear fusion and meiosis and in mushroom forming fungi, the basidia form on specialized structures called gills or pores (Alexopoulos, C.W. et al. 1996).

Model fungal systems

Saccharomyces cerevisiae

S. cerevisiae is a single-celled eukaryotic budding yeast belonging to the Ascomycete phylum. The most important commercial use for *S. cerevisiae* is in food production. In the presence of oxygen, yeast cells can reproduce and carbon by ways of the Citric Acid Cycle. When oxygen is not abundant, *S. cerevisiae* can also metabolize and reproduce, producing carbon dioxide and alcohol by ways of fermentative glycolysis (Dombek and Ingram 1988). Used as the primary fermenter in brewing and as a leavening agent in baking, *S. cerevisiae* ferments sugars into carbon dioxide and alcohol during the brewing and baking processes. Because of these functions, *S. cerevisiae* is commonly referred to as 'Baker's yeast' or 'Brewer's yeast'. In addition, the *S. cerevisiae* genome can be engineered to overproduce certain metabolites and commercially important

compounds for the use in pharmaceutical agents. Examples are naringenin, coumarate, artemisinin, taxol, amorphadiene and vitamin C.

The life cycle of *S. cerevisiae* begins as a unicellular diploid that reproduces through budding. Under times of environmental stress, meiosis occurs and results in four haploid spores. These spores are called ascus. The haploid spores can be one of two "mating types", a or α, which is determined by the MAT genetic locus (Herskowitz 1988). The combination of two ascospores of different mating types will result in a diploid vegetative cell. *S. cerevisiae* also has an HO gene, which allows cells to switch mating types. Strains with this gene are referred to as homothallic.

Neurospora crassa

N. crassa is a multicellular filamentous Ascomycete, which is a model organism in fungal biology. *N. crassa* has been successful for research in the laboratory because it has a very tractable life cycle and spends most of its life as a haploid organism. This makes it easy to study the function of genes without any concern about dominant or recessive alleles (Davis 2000). Also its life cycle is quite rapid (around 2 weeks), allowing scientists to rapidly conduct experiments and lastly it requires a very simple chemical diet so we can screen for mutants by their inability to grow on 'minimal medium' (Davis and Perkins 2002). *N. crassa* has an asexual as well as sexual growth cycle (Borkovich, Alex et al. 2004). The asexual growth occurs in the form of haploid mycelia by extending hyphae which are long tubular structures containing multiple

nuclei. The asexual spores- macroconidia are formed from the aerial hyphae and usually consist of several haploid nuclei whereas the uninucleate microconidia are formed from structures called the microconidiophores. The sexual cycle occurs under reduced carbon and nitrogen conditions and requires parents of two mating types- *mat a* and *mat A*. Either of the two strains can act as a female by forming the protoperithecia with hyphae that grow toward the pheromones produced by conidia of the opposite mating type (male). The two parental nuclei fuse together in the ascus, which is a long tubular sac inside the perithecium. The mature asci then shoot haploid ascospores through the beak (ostiole) of the perithecium. These ascospores can then germinate upon heat activation or remain dormant for long periods (Davis 2000).

Colot et al., (Colot, Park et al. 2006) developed a high throughput gene knockout procedure to create the null deletion mutants of *N. crassa* genes. Using this approach a large library of knock out mutants has been built and is a great asset to the field of *N. crassa* genetics. A few other valuable tools include expression and tiling microarrays (Kasuga and Glass 2008; Lambreghts, Shi et al. 2009; Greenwald, Kasuga et al. 2010), single nucleotide polymorphism data for widely used strains (Lambreghts, Shi et al. 2009) and successfully developed Fluorescent protein (FP) labelling for *N. crassa* (Fuchs, Prokisch et al. 2002). These advancements have augmented our capability to study the cell biology of *N. crassa*.

Fungal Cell wall

A typical fungal cell wall consists of two layers, the inner layer is composed of polysaccharides such as chitin and β-1,3- & 1,6-glucans whereas the outer layer is comprised of covalently linked glycoproteins. Chitin is a fibrillar polysaccharide that is present just above the plasma membrane and serves as a skeleton for the cell wall. Beta-glucans are the branched polysaccharides which form the central core whereas the Glycoproteins occur on the outside, forming the amorphous cement for the cell wall. The chitin and glucan cell wall components are synthesized on the plasma membrane and extruded into the cell wall space during their synthesis. Glycoproteins are synthesized in the endoplasmic reticulum, modified in the Golgi and secreted into the cell wall space where they are integrated into the cell wall structure. The various components of the cell wall are cross-linked together in the cell wall space by cell-wall-associated glycosylhydrolases and glycosyltransferases.

Chitin

Chitin is an unbranched polysaccharide made of beta-1,4-linked N acetylglucosamine. It is a relatively minor, yet structurally important, component of the fungal cell wall. Yeast cell walls are known to contain 1-2% chitin by dry weight, whereas the cell walls of filamentous fungi, such as *Neurospora* and *Aspergillus*, are reported to contain 10–

20% chitin. An integral membrane enzyme called chitin synthase, catalyzes the transfer of N-acetylglucosamine from uridine diphosphate (UDP)-Nacetylglucosamine to a growing chitin chain which emerges through the plasma membrane as it is made, resulting in elongation of the chitin polymers. Chitin synthesis primarily occurs at sites of active growth and cell wall remodeling such as growing bud tip in yeast and the hyphal apex in filamentous fungi (Bowman and Free 2006). Chitin synthases are transported from the ER in an inactive form through special membrane bound microvesicles called chitosomes, which merge with the plasma membrane depositing the enzyme at their active sites.

Three chitin synthases (CHS1–3) are responsible for the synthesis of chitin in *S. cerevisiae*. Chs1p acts as a repair enzyme during cytokinesis whereas Chs2p is responsible for synthesizing septal chitin within the dividing yeast cell. Chs3p synthesizes the majority of cell wall chitin, so CHS3 mutants have greatly reduced chitin levels, diminished growth rates and defects in cell wall integrity (Latge 2007). Whereas *S. cerevisiae* has only three CHS genes, the higher fungi possess additional copies suggesting additional roles in the chitin biosynthesis. These have been grouped into seven different classes and three major divisions (for details refer to (Riquelme and Bartnicki-GarcÌa 2008)). *N. crassa* has one copy in each of the seven classes but the Class I gene, *chs-3* has been shown to be essential for survival, suggesting that it synthesizes the bulk of chitin (Bowman and Free 2006). Additional enzymes are

involved in the chitin synthesis and remodeling in *S. cerevisiae*, like Crh1p, Gfa1p and Pcm1p. Chs4p, Chs6p, and Chs7p are responsible for the export and transport of chitin synthase enzyme. Cda1p and Cda2p deactelyate chitin to produce chitosan in the cell wall whereas the chitinases Cts1p and Cts2p are responsible for degrading chitin. Special membrane bound microvesicles called chitosomes, which merge with the plasma membrane depositing the enzyme at their active sites.

Glucan

Glucan is the major structural polysaccharide of the fungal cell wall, constituting approximately 50– 60% of the wall by dry weight. The β-1,3-glucan serves as the main structural constituent to which other cell wall components are covalently attached. Other glucans such as β-1,6-, α-1,3 and α-1,4 linked glucans are also found in varying amounts in other species. Various studies have shown that yeast cell walls contain both β-1,3- and β-1,6- glucans whereas most of the filamentous fungi like *N. crassa* and *A. nidulans* don't contain β-1,6-glucan (Bowman and Free 2006). β-1,3-glucans, like chitin are synthesized by a plasma membrane-bound protein complex that uses UDP glucose on its intracellular side as a substrate and pushes out linear β-1,3-glucan chains through the membrane into the cell wall space. These oligosaccharide chains are branched by currently UG enzymes and are then cross-linked together and to chitin and mannoproteins to provide the cell wall with mechanical strength and integrity. In yeast,

the glucan synthase machinery consists of two catalytic subunits, FKS1 and FKS2 and a regulatory subunit Rho1. Disruption of either the FKS1 or FKS2 gene yields mutants with slow growth rates and cell wall defects whereas the simultaneous deletion of FKS1 and FKS2 is lethal, indicating that beta-1,3-glucan is important for yeast survival. Disruptions of the *S. cerevisiae* RHO1 gene have demonstrated that it is essential for survival (Latge 2007). The exo-1,3-beta-glucanase genes EXG1 and EXG2 and sporulation specific exo-1,3-betaglucanase SPR1 are the major glucan processing/remodeling enzymes. In *N. crassa*, homologs of FKS1 (called GLS1) and RHO1 are involved in glucan synthesis and an addition enzyme called GS1 is essential for regulation (Verdin, Bartnicki-Garcia et al. 2009).

Glycoproteins

Wall bound glycoproteins serve as the matrix in which the fibrillar polysaccharides are immersed and also act as recognition molecules. They mediate adhesion for cell migration and fusion as well as participate in cell wall remodeling. They account for 30-50% of the yeast cell walls and around 20-30% of the cell wall of filamentous fungi (Bowman and Free 2006). These glycoproteins are extensively modified with N-linked and O-linked oligosaccharides. The structures of these oligosaccharide chains vary amongst different fungi. The cell walls of yeasts like *S. cerevisiae* and *C. albicans* contain mannoproteins, which are glycosylated with chains rich in mannose, known as

mannans. In contrast, the glycoproteins of filamentous fungi like *N. crassa* and *A. fumigatus* contain galactomannan structures composed of both mannose and galactose residues. In addition to these modifications, some cell wall proteins contain glycosylphosphatidylinositol (GPI) anchor. The GPI anchor, a lipid and oligosaccharide-containing structure, is added to select proteins that contain a C-terminal signal sequence. This GPI anchor aids in directing and localizing these proteins to plasma membrane and cell wall (Latge 2007). In yeast, the most common genes coding for glycoproteins are the flocculation (FLO) genes, responsible for cell wall adhesion and AGA1/ SAG1 genes that are instrumental in agglutination. GAS and GEL genes are involved in glucan component remodeling, whereas the PIR genes are important for the strengthening of cell wall.

Importance of studying the fungal cell wall

Fungi cause a great majority, an estimated two-thirds, of infectious plant diseases. One or more fungi attack a large percentage of economically important plants and often many different fungi cause disease in a single plant species. Each year a large portion of the crop yield is destroyed because of fungi like *Ustilago maydis* (causes smut in maize), *Puccinia graminis* (causes rust in wheat), *Magnaporthae grisea* (causes blast in rice) and *Fusarium oxysporum* (causes wilt in tomato). The fungicides being used to kill these fungi are not very effective as they result in damaging the crops as well and also

because the fungi become tolerant to them after a while. Thus other approaches such as antifungal drugs that specifically kill the fungi without harming the plants are a better option.

Similarly the fungi like *Trichophyton rubrum* (causes athlete's foot), *Candida albicans* (cause of oral thrush) and *Rhizopus oryzae* (causes mucormycosis) are a menace to humans. Infections caused by human pathogenic fungi threaten increasing numbers of patients with suppressed immune systems, such as patients undergoing organ transplants or chemotherapy, and patients infected with the human immunodeficiency virus. *A. fumigatus* causes life-threatening mycosis, and due to the absence of adequate medication, death rates can reach up to 80% of infected patients (Beauvais and Latge 2001). *C. albicans*, on the contrary, is a dimorphic fungus that requires the ability to switch from a yeast form to a mycelial form for virulence (Lo, Kohler et al. 1997; Mitchell 1998). Current medication against fungal infections includes polyenes (fungicidal) and azoles (fungistatic) that both act on the plasma membrane of the fungal cell. The polyenes, such as amphotericin B, form complexes with membrane sterol, namely ergosterol, resulting in the formation of transmembrane pores that leak vital cellular ions, such as K^+ and Mg^{2+}. Since the polyenes also bind to mammalian cholesterol, toxicity is common (Georgopapadakou 1998). A second drawback is the increasing fungal resistance to polyenes, that is based on alterations in fungal membrane lipids, and is found especially in *Fusarium* and *Trichosporon* species

(Georgopapadakou 1998). The second group of antifungals, the azoles, also act on fungal ergosterol, but rather than binding to it like the polyenes, it affects ergosterol biosynthesis by inhibiting a demethylation step catalyzed by cytochrome P-450 leading to perturbation of the plasma membrane function (Georgopapadakou 1998). The toxicity of azoles to humans is low, but since azoles are fungistatic rather than fungicidal, lifetime medication is necessary. In addition, resistance is emerging in *Candida* species and in *C. neoformans* (Kelly, Lamb et al. 1996; Perea, Lopez-Ribot et al. 2001).

The fungal cell wall is unique among eukaryotes and therefore enzymes that are involved in its biosynthesis form ideal targets for the development of highly specific antifungal drugs (reviewed in(Kurtz and Rex 2001)). β-1,3-Glucan and chitin are cell-wall polymers that are both essential for cell viability and occur in most fungi. Accordingly, their synthases have been chosen for the development of novel cell wall targeted fungicides (Georgopapadakou and Tkacz 1995; Radding, Heidler et al. 1998) (Ohyama, Kurihara et al. 2000; Onishi, Meinz et al. 2000). For example, polyoxins and nikkomycins inhibit chitin synthesis, whereas echinocandins, the related pneumocandins, and papulacandins inhibit β-1,3-glucan synthesis. Additionally, pradimicins and benanomicins are a unique group of antifungal antibiotics isolated from *Actinomadura hibisca* that bind to mannans in the fungal cell wall by a mode of action that is still not fully understood (Debono and Gordee 1994).

Unfortunately, *C. neoformans* is not vulnerable towards inhibitors of β-1,3-glucan and chitin synthesis (Georgopapadakou 2001). In addition, fungal strains resistant towards candins have already been identified in *C. albicans* and the model-yeast *S. cerevisiae* (Douglas, Marrinan et al. 1994; el-Sherbeini and Clemas 1995; Kurtz, Abruzzo et al. 1996) indicating that the search for new targets has to continue. Hence other cell wall components that are essential for cell morphogenesis would be potentially interesting targets for future antifungals.

Hyphal morphogenesis and cell wall growth

Filamentous fungi grow by extending hyphae. The hyphal growth takes place almost exclusively at the tips, though how this growth actually occurs is not fully understood. One of the hypotheses to explain this was put forward by (Bartnicki-Garcia 1973), which suggests that for the cell wall growth to occur there must be a permanent balance between cell wall synthesis-lysis and the pushing out and mending of the cell wall. Studies have shown that the apex of a growing hypha is packed with two types of vesicles- microvesicles (less than 100 nm) and macrovesicles (greater than 100 nm). In most of the true fungi these vesicles are clustered with some other bodies to form a unique and dynamic structure called the Spitzenkörper (Bracker, Ruiz-Herrera et al. 1976). (Bartnicki-Garcia, Hergert et al. 1989) suggested that the Spitzenkorper acts as a

supply center for vesicles involved in tip growth and proposed a novel mathematical model to explain this phenomenon. The macrovesicles present in the hyphal tips are secretory vesicles that contain enzymes and preformed polymers that are used to form the amorphous matrix component. The microvesicles, on the other hand carry the chitin synthase enzyme to the plasma membrane where it catalyzes the formation of chitin in the cell wall (Riquelme, Yarden et al. 2011). A protein complex called polarisome is located at the growing hyphal tip close to the cell membrane, which complex has a crucial role in determining the direction of hyphal growth and in organizing the actin microfilaments in the hyphal tip.

More recently, fluorescent tagging has assisted in the identification of some of the predicted components of the Spitzenkörper and discovered the presence of new components, providing clues as to the mode of operation of this structure during polarized growth. Four of the seven predicted chitin synthases in *N. crassa* localize in the core of the Spitzenkörper in mature hyphae using fluorescent-protein tags (Riquelme, Bartnicki-Garcia et al. 2007) where microvesicles had been earlier seen by transmission electron microscopy. In contrast, GS-1, a protein needed for glucan synthase activity, was found in the most outer layer of the Spitzenkörper (Verdin, Bartnicki-Garcia et al. 2009), where mainly macrovesicles were observed by transmission electron microscopy (Grove and Bracker 1970; Riquelme, Roberson et al. 2002). These findings demonstrate that the Spitzenkorper is part of the apparatus that builds the hyphal cell wall.

Aims of this study

As discussed previously, understanding the various processes/pathways necessary for the biogenesis and maintenance of the cell wall is of immense value as that knowledge can be used for developing antifungals. Hence this dissertation attempts to make a significant contribution in furthering our understanding of the fungal cell wall and its various components. In chapter 1, I have identified and characterized genes involved in cell wall growth and maintenance in the model fungus *Neurospora crassa* by employing a functional screen. In chapter 2, I utilized comparative genomics approaches to reconstruct the evolutionary history of cell wall polysaccharides chitin and glucan in the early diverging fungal clades- Microsporidia, Cryptomycota, Chytridiomycota, Blastocladiomycota and Mucormycota. In chapter 3, using a combination of biochemical and comparative genomics I sought to compare and contrast the cell wall composition of the early diverging fungal clades Chytridiomycota, Blastocladiomycota and Mucormycota to the recently diverged Dikarya fungi.

Chapter 1 attempts to study the cell wall genes in *N. crassa* by utilizing the available collection of null deletion knock out strains of the *N. crassa* genome (Colot, Park et al. 2006). Our dataset for this screen included genes highly expressed in the tip of the vegetative growing culture of *N. crassa* (from a RNA-seq of and a previous similar study (Kasuga and Glass 2008)) and some hand-picked homologs of known cell wall genes in yeast. A systematic screen was employed to phenotype knockouts of corresponding genes. This screen consisted of basic growth and development assays to

identify the genes showing abnormal growth as compared to wild type (less or greater), defects in asexual and/or sexual cycle and overall abnormalities in morphology such as hyperbranching, swollen tips, irregular sepatation and other defects. This set of genes was tested further using cell wall stress assays such as sensitivity to oxidative stress, hyperosmolarity and membrane perturbatio to validate a role in the cell wall growth and/or regulation.

Chapter 2 seeks to reconstruct the evolutionary history of cell wall polysaccharides-chitin and glucan in early fungal lineages. Our study uses a comparative genomics approach to elucidate important differences between the cell wall composition of the species belonging to the early diverging (Microsporidia, Cryptomycota, Neocallimastigomycota, Chytridiomycota, Blastocladiomycota, Kickxellomycotina, Entomophthoromycota, Mucormycotina) and the later diverged clades (Ascomycota and Basidiomycota) of fungi. This was accomplished by employing fully sequenced representatives from each phylum and sensitive homology detection with profile HMMs trained on curated alignments of genes. I evaluated the history of these gene relationships in context of species relationships using phylogenetics to provide strong statistical support for the timing and patterns of duplication of these gene families. Our study sheds light on the evolutionary transitions of the cell wall in the fungal kingdom from the early flagellated form, which was may have been similar to the modern Chytridiomycota or Blastocladiomycota, to the filamentous hyphal growth form in the Dikarya fungi.

Chapter 3 explores the polysaccharide composition of cell walls of evolutionary distinct, early diverging fungal species by analyzing the species- *Spizellomyces punctatus, Allomyces macrogynus, Rhizopus oryzae* and *Phycomyces blakesleeanus* and correlating with the presence of genes responsible for cell wall biosynthesis and metabolism. Towards this end I utilized the biochemical characterization approach involving cell wall fractionation techniques followed by quantification of cell wall polysaccharides and genomic profiling done using a homology based pipeline, employing Hidden Markov Model profiles to identify genes involved in the synthesis and metabolism of the cell wall sugars in the afore mentioned species.

The research presented in this dissertation should lead to the characterization of novel cell wall genes in the filamentous fungi that eventually will lead to development of better drug targets for designing anti-fungal drugs. Also it will lead to better understanding of the cell walls of evolutionarily distinct fungi, which will enable us to combat the pathogenic fungi in a more powerful way. In terms of broader impact this research will be an important contribution to the knowledge of cell wall in the fungal community.

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CHAPTER I

A transcriptomics-directed functional genomics screen for discovery of novel cell wall genes in *Neurospora crassa*

ABSTRACT

A functional genomics screen was performed to identify cell wall mutants using the *Neurospora crassa* single gene deletion library. 64 mutants with sensitivity to cell wall integrity (CWI) stress were identified and 32 of them were newly associated with cell wall function (named as **c**ell **w**all **s**ensitive (cws)). A Random Forest approach enabled the placement of cell wall integrity mutants into processes/pathways that were suggested by their phenotypes and stress responses, which is important for future cell wall gene discovery studies. I was able to identify 14 novel cell CWI mutants having multiple phenotypic defects. These included five tip-expressed genes involved in cell wall morphogenesis: NCU05974 (*gh16-7*), NCU08560 (*cws-26*), NCU04145 (*cws-13*), NCU08132 (*gh13-10*) NCU00459 (*cws-2*), and NCU01393 (*gh18-10*); four genes that are likely to be targets of the MAPK signaling pathway: NCU04866 (ada6), NCU04663 (*cws-19*), NCU08131 (*gh13-1*) and NCU04475 *(cws-17);* and one gene, NCU08823 (*bd*), that might be involved in Rho-signaling pathway. Most of these genes have homologs in Pezizomycetes and hence may be useful as targets for anti-fungal

drug development against filamentous pathogenic fungi. This study represents a model approach to finding genes involved in a process by utilizing transcriptomic data to drive a functional genomics screen.

INTRODUCTION

The cell wall is one of the most important organelles of the fungal cell. It serves the dual purpose of maintaining the cell shape and protecting against physical damage. The cell wall also is responsible for maintenance of turgor pressure, stable osmotic conditions inside the cell and must be remodeled as part of morphogenesis. A typical fungal cell wall consists of two layers, the inner layer is composed of polysaccharides such as chitin and β-(1,3)- & β-1,6-glucans, whereas the outer layer is comprised of covalently linked glycoproteins. The *Neurospora crassa* cell wall is composed of 7-10% chitin (a polymer of N-acetyl-glucosamine (GlcNAc)), 25% β-(1,3)-glucans, 35% other glucans, and 10% proteins (Bartnicki-Garcia 1968). The synthesis of each carbohydrate polymer occurs at hyphal tips. The synthase responsible for production of each polymer is transported in an inactive form to hyphal tips in vesicles that fuse with the apical plasma membrane and then extrude the carbohydrate polymers through the membrane (Bowman and Free 2007). The *N. crassa* genome-sequencing project identified many genes by homology to *S. cerevisiae* genes that may play an important role in hyphal morphogenesis and cell wall synthesis and development to be present in the genome of *N. crassa* (Borkovich, Alex et al. 2004). However not all have been demonstrated to have the predicted function in *N. crassa* and other filamentous fungi.

Recent studies have confirmed some cell wall genes involved in polysaccharide metabolism (*chs*, *cts* etc), septation (*bud3*, *rho-4* etc) and polarity (*spa2, mak-2* etc) (Riquelme, Yarden et al. 2011) but there remain additional, potentially novel genes with no current association with cell wall biology in the *N. crassa* genome.

The cell wall is a dynamic structure and it responds to a multitude of environmental and chemical changes. Various stresses like cell wall integrity stress, oxidative and hyperosmolar stress reduce growth rates and affect cell viability. It is therefore essential for fungal cells to be able to respond and adapt rapidly to each stress condition for survival. Hence using cell wall stressors has been a successful approach in discovering new cell wall genes (de Groot, Ruiz et al. 2001; Auesukaree, Damnernsawad et al. 2009). The cell wall integrity (CWI) pathway allows the fungus to respond to cell wall stresses such as those caused by caspofungin, SDS and calcafluor white, so as to maintain cell wall integrity (Ram and Klis 2006). This pathway is composed of cell wall sensors, the plasma membrane-associated small GTPase Rho1, protein kinase C (Pkc1), and a mitogen-activated protein (MAP) kinase cascade. In *Saccharomyces cerevisiae*, a family of five membrane-spanning sensors (Wsc1p, Wsc2p, Wsc3p, Mid1p and Mtl1p) activates the CWI pathway (Levin 2011). In *N. crassa*, the *mak-1*/*mik-1*/*mek-1* MAP kinase cascade functions as the cell wall integrity signaling system, and is required for normal hyphal growth, colony morphology, and sexual development (Park, Pan et al. 2008), (Maerz, Ziv et al. 2008). The hyper osmotic stress response, triggered by agents such as glycerol and sodium chloride in yeast, is controlled by the High Osmolarity

Glycerol (HOG) pathway mediated by Sln1p and consisting of a MAP kinase (Hog1p), a MAP kinase kinase (Pbs2p), and three MAP kinase kinase kinases (Ste11p, Ssk2p, and Ssk22p) (Mager and Siderius 2002). In *Neurospora crassa*, the *os-1*, *os-2*, *os-4*, and *os-5* osmotic sensitive mutants have abnormal colony morphology, reduced aerial hyphae formation and conidiation, and altered cell wall composition (Livingston 1969; Mays 1969; Grindle and Temple 1982). The reactive oxidative species (ROS) generating agents such as hydrogen peroxide and paraquat elicit an oxidative stress response in *N. crassa* (Angelova, Pashova et al. 2005) which is regulated by two superoxide dismutases (*sod-1* and *sod-2*) (Chary, Dillon et al. 1994) and three catalases (*cat-1, cat-2, cat-3*) (Chary and Natvig 1989).

Components of cell wall biosynthesis, including β-(1,3)-glucan synthase and chitin synthase, are targets of clinically successful antifungals of the echinocandin and nikkomycin classes respectively (Latge 2007). In addition, signaling pathways such as Tor, calcineurin, Hsp60 and Hsp90 that can control cell wall processes have been targeted for developing antifungals (Cowen, Carpenter et al. 2006; Bastidas, Reedy et al. 2008). Identifying additional cell wall components and determining their role in growth and development could reveal new drug targets that impact growth or stability of fungal cell walls. Previous studies have been focused on identification and characterization of the cell wall genes of the model system, *S. cerevisiae*. But since yeast is a unicellular budding fungus, it may not be representative of the cell walls of diverse fungi that grow with filamentous and multicellular forms. Prior studies to

discover cell genes in the filamentous fungus *N. crassa* identified GPI-anchored proteins using proteomic and bioinformatics approaches and identified genes important for cell wall integrity maintenance (*wsc-1*), cell-to-cell hyphal fusion (*ham-7*) and crosslinking glycoproteins to the cell wall (*dfw-5* and *dcw-1*) (Maddi, Dettman et al. 2012; Maddi, Fu et al. 2012).

Studies in filamentous fungi have shown that the cell wall activity (such as synthesis and degradation) is the highest at the growing hyphal tip (Bartnicki-Garcia and Lippman 1969) and correspond to the aggregation of cell wall biosynthesis machinery in the Spizenkörper (Girbardt 1969). Other genetic screens have identified mutants with cell wall defects as having mutations in genes expressed in the tip (Seiler and Plamann 2003). A recent study employed microarray analysis to examine gene expression at six distinct stages of colony development in *N. crassa* and found that genes up-regulated in the tip timepoint (1 hr of growth) were enriched for the functional categories related to cell wall growth and biosynthesis (Kasuga and Glass 2008). It was noted that the genes highly expressed in the tip also include those involved in membrane biogenesis, cellular communication/signal transduction and cytoskeleton reconstruction, components that are essential for tip growth. Finally, the study reported that the tip-expressed genes were highly conserved between *Aspergilus niger* and *N. crassa* and the lineage specificity analysis showed that this cluster of genes contained a higher number of Pezizomycete and *N. crassa* orphan genes, suggesting that this set of genes are crucial for the hyphal morphogenesis in the filamentous fungal species.

This study takes a unique approach of utilizing expression data to select the best candidates and then using cell wall stressing agents and other hyphal morphological assays to identify genes involved in cell wall biosynthesis and development. Toward this end, I identified upregulated genes in the hyphal tip based on mRNA expression at the tip (1 hr growth) and colony interior (20 hr growth) of vegetative growing cultures of *N. crassa* along with homologs of known cell wall genes, as candidates for a phenotypic screen. The screen was conducted on an available collection of single deletion gene knockout mutants generated by the *Neurospora* knockout project (Colot, Park et al. 2006), using assays testing for growth and developmental defects as well as various stress pathway defects. Using this approach, I identified a group of genes not previously implicated in cell wall integrity that may be good targets for antifungal drug development. This set includes transcription factors, glycoside hydrolase family proteins, and several genes with no previously annotated functions. All of these genes possess homologs in other Peziomycotina fungi, improving their utility as possible targets for future drug targeting. This approach of enriching for genes potentially involved in growth by screening for highly expressed genes in hyphal tips resulted in a candidate gene set that impacts growth processes.

MATERIALS AND METHODS

Strains and culturing conditions

Neurospora crassa wild-type strain 74-OR23-IV A (FGSC2489) and the knockout strains were maintained in Vogel's minimal medium with 1.5% sucrose at room temperature (Davis and Deserres 1970). The gene knockout mutants were generated as a part of the *Neurospora* genome project by replacing the gene ORF with a hygromycin resistance cassette (Colot, Park et al. 2006) and the knockout mutant strains were obtained from the Fungal Genetics Stock Center (Kansas City, MO).

Processing of Microarray results

Gene expression data from a time course of *N. crassa* colony development from a previously published microarray study was used to identify genes differentially expressed in the hyphal tip (Kasuga and Glass 2008). As a part of the study, the hybridization intensity values of the different time points was processed and expressed as BAGEL values, which correspond to the relative expression (Supplementary Table 1, (Kasuga and Glass 2008)). The fold difference in expression values between the 1 hour and 21 hour samples were compared to identify genes upregulated in the tip (GEO GSE50205).

RNA isolation and sequencing

Dr John Abramyan, a former postdoc at UC Riverside, performed this procedure. See Appendix 1 for details.

RNA-Seq analysis

RNA sequencing data from the hyphal tip (1 hour timepoint) was obtained from the Glass laboratory at UC Berkeley sampling the hyphal tip expression (SRA accession SRA099094; GEO GSE50205). The FASTQ files containing the raw reads of the time points from RNA sequencing were trimmed using Sickle (https://github.com/najoshi/sickle), discarding read-pairs when both were not of quality cutoff. These were analyzed for read quality using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) to high quality data. The reads were aligned to the *N. crassa* genome using Tophat (ver 2.0.5) (Trapnell, Roberts et al. 2012) which identifies splice sites *ab initio* by large-scale mapping of RNA-Seq reads using the Burrows Wheeler transform and seed and extend algorithms. Transcript expression differences between timepoints was determined using Cuffdiff from the Cufflinks suite (ver 2.0.2) (Roberts, Pimentel et al. 2011). Cuffdiff assesses significance using a model of variability in the log-fold-change under the null hypothesis and obtains an estimate of the number of reads that originated from each gene and transcript, along with variances in those estimates. Genes that were significantly differentially expressed (p value <0.05) were extracted and the genes upregulated in the 1 hr and 20 hr time

points were obtained. Comparison between the 1 hr (Glass data) and 20 hrs of growth on VM was focused on as the most relevant to this finding highly expressed hyphal tip transcripts.

Identification of N. crassa homologs of yeast cell wall genes

The gene sequences of characterized cell wall genes in *S. cerevisiae* (based on literature search) were obtained from Saccharomyces Genome Database (http://yeastgenome.org) and searched by BLAST against the *N. crassa* annotated proteins (Galagan, Calvo et al. 2003) to generate 35 genes with unambiguous homologs.

Growth and development assays

Mycelial growth rate: Using a wet swab, conidia were collected from a VM slant of the mutant and swirled in a microcentrifuge tube containing 100 microliters sterile water. Approximately 0.5 microliters of the conidial suspension was pipetted in the center of the plate to inoculate. The plates were incubated at 30°C in light. Growth was measured at 24 hrs by measuring the radius of the colony from the center (taking 4 different measurements and averaging them). Also the plate image was captured at 24 hrs using a flatbed scanner. The hyphae at the edge of the colony were also imaged at 40X magnification using a steromicroscope (Olympus SZX10 stereomicroscope) at 24 hrs. Three replicates were performed for every mutant.

Aerial hyphae extension: 13x100mm test tubes containing 2 ml liquid VM were inoculated using 1 microliter of the conidial suspension described above. The tubes were incubated at 30°C in the dark for 24 hrs. A mycelial mat is formed at the top of the liquid medium after 24 hours. A mark was drawn on the outside of the tube to mark the top of the mycelial mat (if no mat formed then the medium meniscus top was marked). The cultures were incubated for 72 hours and at the end of 3 days the tubes were marked again and the aerial hyphae height was measured. Three replicates were performed for every mutant.

Conidial pigmentation: For this assay, 13x100mm test tubes containing 2 ml VM agar (VM slants) were inoculated using hyphae from mature *N. crassa* cultures. The tubes were incubated at 30°C in the dark for 3 days and then at room temp for 2 days. The conidial pigmentation was scored with the wild-type strains as a reference.

Sexual development: Synthetic Crossing media (SCM) slants were labeled and inoculated with conidia from knockouts. At the same time a VM slant was also inoculated with WT conidia for performing wild-type cross (which served as a positive control). The slants were incubated at room temp (light) for 7-10 days. Formation of protoperithecia was observed at this time using a stereomicroscope. SCM female cultures were fertilized (crossed) with conidia from the opposite mating type, FGSC 2489 (if knockouts are mat a) or FGSC 2356 (if knockouts are mat A). SCM tubes were

incubated for an additional week as before. Perithecial formation was evaluated at this two-week interval using the wild-type cross as a reference. Perithecia presence/absence was recorded and tubes were left for one remaining week at room temperature. At the end of a week, tubes were checked for ascospore development & ejection. Ascospores are ejected with such force they end up on the wall of the test tube opposite the agar surface. The ascospore presence/absence was reported.

Stress sensitivity assays

The sensitivity assays were adapted from (Maddi and Free 2010). Briefly, each of the knockout mutant and a wild-type control were inoculated on VM medium +/- a cell wall stress agent (referred to as chemical hereafter). The plates were incubated for 48 hours at 30°C dark. The colony growth radius was measured for all the plates and the ratio of growth in VM+chemical to growth in VM is calculated for both the wild-type and knockout. If this ratio of the mutant is significantly lower than that of the wild-type then the mutant strain is believed to be sensitive. If on the other hand it was found significantly higher than that of wild-type then the knockout is believed to be resistant. Three replicates were performed of each of the condition for the wild-type and all the mutants and a Student's t-test was used to find significance of sensitivity/resistance.

Cell wall stability assays were performed by inoculating mutants on 0.01% SDS in VM agar medium containing 1.5% sucrose. Hyperosmotic sensitivity was assessed by

measuring growth on VM medium containing 2M glycerol or 10% NaCl. Oxidative stress was assayed by measuring growth on 0.05 mM Hydrogen peroxide in VM liquid medium. To determine echinocandin fungicide-sensitivity, Caspofungin was used at a final concentration of 1.95 $x10^{-2}$ microgram/ml in VM liquid medium.

Genetic co-segregation analysis

Co-segregation experiments were carried out by mating each of the mutants to wild-type of the opposite mating type (Davis and Deserres 1970). At least 30 individual progeny from each of the crosses were tested for the presence of the deletion mutation by assessing the ability to grow in the presence of 200 mg/ml hygromycin. The resistant progeny were then tested for the presence or absence of the mutant phenotype using the same criteria used in the mutant screening procedures mentioned above. The finding that greater than 75% of the mutant progeny showed a mutant phenotype was taken as evidence that the deletion mutation co-segregated with, and was responsible for the mutant phenotype.

RESULTS

Candidate genes for phenotypic screening

I combined three sources of data to generate a candidate list of genes likely to be involved in processes of growth and the fungal cell wall. I re-analyzed published

microarray results of growth time points during *N. crassa* colony development and identified 56 genes with significantly higher expression in the hyphal tip (Kasuga and Glass 2008). Second, analysis of RNA-Seq from two time points, the hyphal tip (1 hr) and the colony interior (20 hrs), identified 77 genes expressed at least 5-fold higher in the tip. The total set of genes from these expression experiment totaled 107 as 26 overlapped between the two experiments. A third approach was taken to select *N. crassa* genes homologous to identified cell wall genes in yeast (*S. cerevisiae*). This resulted in identification of an additional 28 candidates for inclusion in the study (Table 1). Taken together, the three approaches yielded a total of 135 candidate genes, of which knockouts were available for 112 in the *N. crassa* deletion mutant collection (Colot, Park et al. 2006) (Table 2). An enrichment analysis of FunCat categories of the 112 candidate genes found categories for cell wall biogenesis, polysaccharide metabolism, directional cell growth, G-protein signaling and osmotic and salt stress response, suggesting a concentration of genes having functions in cell wall synthesis and regulation.

Identifying gene knockout strains with cell wall integrity defects

Knockout mutants were analyzed for phenotypes using a selection of stress and developmental growth assays. Out of the 112 candidates, 67 mutants (i.e. ~60%) showed sensitivity to the cell wall integrity stressors, antifungal caspofungin (35 mutants), SDS (15) or both (17). This proportion of strains with CWI sensitivity suggests that the selected gene set is indeed enriched for genes involved in cell wall

integrity. Henceforth, I refer to these genes as cell wall integrity or CWI mutants. Various previously known cell wall genes from *N. crassa* or homologs of *S. cerevisiae* were present in this list of CWI mutants. These include the chitin synthases *chs-1*, *chs-7, chs-7, chs-5,* the polarisome subunit *spa-2,* Calcinerun subunit *cnb-1,* the β-(1,3)-glucan synthase *do/fks,* and chitin degrading enzymes *ce4-4* and *cts*. The remaining genes are mostly uncharacterized, but are candidates for involvement in cell wall synthesis and development. I named these un-annotated genes as cws for Cell Wall Sensitive (Table 3).

Clustering phenotypes to classify CWI mutants

In order to explore the processes and pathways regulated by CWI genes, I clustered the mutants by the major phenotypes tested in this study using Unsupervised Random Forests (Liaw and Wiener 2002). The Unsupervised Random Forests approach generates classes of observations without a training set and assigns observations to each tree by majority voting and has been applied in several contexts to cluster genomic and phenotypic data (Shi and Horvath 2006; Dinsdale, Edwards et al. 2013). Because the distances in the map represent the similarity in the phenotypes, the CWI mutants that group with the well-characterized cell wall genes are likely to be involved in the process in which the gene is implicated. I observed that the mutants fell into three major clusters I have labeled Cluster A, B and C (Fig, 1 and Table 3). Cluster-A contains 20 CWI mutants, out of which 15 are cws genes. Characterized genes *do/fks-1* (β-(1,3)-glucan synthase), *cnb-1* (calcineurin subunit), *gh16-5* (glucanase), *gh18-6* (chitinase) are

members of the cluster many of which have demonstrated roles in cell wall synthesis, degradation and regulation. Cluster-B contains 15 CWI mutants, out of which 11 are cws genes. This cluster contains some likely cell wall genes *csc-1* (CHS chaperon), *ce4-4* (chitin deacetylase), *gla-1* (glucoamylase-1), *gel-1* (β-(1,3)-glucanosyltransferase), *gh18-3* and *gh18-8* (chitinases), also indicative of involvement in cell wall growth and degradation. Cluster C is composed of 9 CWI mutants, 5 of which are cws genes. This cluster contains the genes *chs-1*, *chs-5*, *chs-6*, *chs-7* (chitin synthases), *spa-2* (polarisome subunit), *bud-3* (septum formation) and *rdi-1* (sepatation regulation), all pathways known to be involved in cell wall growth and regulation.

CWI mutants with multiple defects

With an aim of finding cell wall genes involved in multiple cellular functions in *N. crassa*, I decided to focus on the CWI mutants having defects in at least two pathways. I compared the sets of mutants in the following categories: i) cell wall integrity stress sensitive ii) having hyphal growth slower than wild-type, iii) possessing abnormal hyphae and iv) having sexual defects (Figure 2). This yielded a set of 22 genes, of which, 14 genes are newly associated with cell wall biogenesis and development (Table 4).

Mutant strains of four of the seven chitin synthase genes (*chs-1, chs-5, chs-6* and *chs-7)* and the genes *bud-3, spa-2, cnb-1* and *rdi-1* showed morphological and growth defects. All these mutants are sensitive to cell wall stresses, grow more slowly than the wild-

type and have abnormal hyphal morphologies. All of these genes are implicated in cell wall processes in either *N. crassa* or their homologs in other species. The chitin synthase genes are expected members of this group as they are one of the most recognizable cell wall components. *chs-1*, *chs-5*, *chs-6* are involved in the biosynthesis of cell wall chitin and have been shown in separate studies to localize to the Spitzenkörper core of growing hyphae ((Sanchez-Leon, Verdin et al. 2011), M. Riquelme personal communication, (Riquelme, Bartnicki-Garcia et al. 2007)). *Spa-2* is a polarisome complex subunit, involved in cytoskeletal organization and it also localizes to the core of the Spitzenkörper (Araujo-Palomares, Riquelme et al. 2009). *chs-7* is involved in septal cell wall chitin synthesis and *bud-3* plays a role in septum formation; both localize at the cell wall during the septum formation (M. Riquelme personal communication, (Justa-Schuch, Heilig et al. 2010)). The *rdi-1* protein is a RHO protein GDP dissociation inhibitor that is important for septation regulation (Rasmussen and Glass 2007). *Cnb-1* is a regulatory subunit of calcineurin (a calcium and calmodulin-dependent protein phosphatase) that has been shown to be important for the macroconidiation pathway in *N. crassa* (Kothe and Free 1998). This set provides a positive control for the study as these tip-expressed genes (and the homologs of characterized cell wall genes in yeast) have been shown to be involved in the cell wall growth and development processes. This helps us to functionally characterize the other cell wall integrity mutants on the basis of traits similar to these.

The remaining 14 loci had no previously implicated cell wall activity or in some cases no known function at all. I first tested genetic co-segregation of the candidates to verify that the knockout was responsible for the phenotype. All strains showed greater than 75% co-segregation. Next, I compared the morphologies and stress sensitivity patterns of the CWI mutants to the previously characterized cell wall genes that enabled us to deduce the processes/pathways in which they might be involved.

Growth and Morphogenesis: The chitin synthase genes *chs-5* and *chs-7* show sensitivity to cell wall stress, grow slower and possess sparse and thin hyphae, characteristics that are shared by these candidate genes: NCU05974 (*gh16-7*), NCU08560 (*cws-26*) and NCU04145 (*cws-13*). *gh16-7* is a 365 amino acid protein putative glucanosyltransferase, conserved in fungi and plants (Figure 3). Its yeast homolog crh1p is a chitin transglycosylase that functions in the transfer of chitin to β-(1,6)- and β-(1,3)-glucans in the cell wall (Cabib, Blanco et al. 2007). *Cws-26* is a 482 amino acid long, dikarya-specific transmembrane protein containing an acyltransferase domain. *Cws-13* is a 577 amino acid long dikarya-specific protein and contains a DnaJ/Hsp40 domain. *Chs-1* and *chs-6* are sensitive to cell wall stress, grow sicker than wild-type and possess dense and hyperbranching hyphae. Two of the candidates, NCU08132 (*gh13-10*) and NCU00459 (*cws-2*) show similar morphology to these genes. *Gh13-10* is a 2375 amino acid long transmembrane domain containing alpha amylase and glycosyl transferase domains. The GH 13-10 CAZY enzymes are usually involved in β-(1,6)-glucosidase activity and FunCat classifies this as a polysaccharide metabolism gene. It is conserved

in the Fungal Dikarya and the Planta kingdom. *Cws-2* encodes a protein of 3055 amino acids containing RNA pol II promoter and Golgi localization protein domains. On the basis of these shared characteristics, I suggest these genes might be involved in the hyphal morphogenesis process essential for fungal growth. NCU01393 (*gh18-10*) is a member of the GH18 family that is implicated in chitin breakdown (Henrissat 1991). It is an ascomycete-mucormycete specific gene, which when mutated yields a strain that is sensitive to the cell wall stress and exhibits slow hyphal growth (20% less than wild-type). The mutant produces normal vegetative hyphae but its aerial hyphae are very unusual, displaying a pale orange color and fluffy appearance. This mutant fails to produce any sexual structures when grown on nitrogen-starved medium, indicating that the mutation affects sexual development in addition to the asexual growth and stress resistance.

Cell wall Integrity MAPK Signaling pathway: The MAPK signaling pathway genes *mak-1* (NCU09842), *mek-1* (NCU06419) and *mik-1* (NCU02234) are sensitive to cell wall integrity stress, fail to produce sexual structures, display slow growth and sparse, thin and curvy hyphae (Park, Pan et al. 2008). Four of the cell wall integrity mutant candidates, NCU04866 (*ada-6*), NCU04663 (*cws-19*), NCU08131 (*gh13-1*) and NCU04475 (*cws-17*), show similar characteristics (Figure 3). *Ada-6* encodes a 650 amino acid protein and is comprised of Fungal zinc-cysteine binuclear cluster domain and is homologous to the *S. cerevisiae* TEA1 protein, important for transcriptional regulation (Gray and Fassler 1996). This gene is conserved in Ascomycetes and

Zygomycetes and has been shown to be involved in cellulose degradation (Tian, Li et al. 2010). *Cws-19* is an 1184 amino acid ascomycete-specific protein containing a fungal zinc-cysteine binuclear cluster domain, suggesting it may have a role as a transcription factor. Its yeast homolog, Hap1 gene encodes for a transcription factor involved in heme signaling (Pfeifer, Kim et al. 1989). *Gh13-1* is a well-conserved gene in Fungal and Metazoan kingdoms containing an alpha-amylase domain and implicated in polysaccharide metabolism. The GH13 CAZYemes act on α-glucoside linkages and GH13-1 are usually alpha-amylases (Henrissat 1991). Motif analysis indicates it contains a signal peptide and is a GPI-anchored cell wall protein. It has been implicated in xylan degradation by *N. crassa* in a previous study (Sun, Tian et al. 2012). *Cws-17* is an unannotated gene with no known domains but is classified under the FunCat polysaccharide metabolism category. It is a Dikarya-specific protein that contains a signal peptide, suggesting that it is secreted out of the cell for its function. On the basis of these similar characteristics, I propose that these genes might be involved in regulating cell wall biogenesis through the MAPK CWI canonical pathway through an upstream/downstream interaction.

Other signaling pathways: The Rdi-1 gene is a GTPase that plays a role in regulation of the septation by negatively regulating Rho-4 (Rasmussen and Glass 2007). It has a growth rate approximately 50% of wild type, is sensitive to cell wall integrity stress and possesses very stunted, dense and hyper-branching hyphae. I observed that one of the candidates, NCU08823 (*bd*), exhibits the same characteristics, which is a strong

indication that it might be interacting with the Rdi-1 to regulate Rho-4. It is a widely conserved protein that is 214 amino acids long, possessing a Ras domain and FunCat classifies it as being involved in cAMP/cGMP mediated signal transduction, cell wall biogenesis, transcriptional control, cell aging, budding and cell polarity. It has been shown that this gene plays a role in light and circadian signaling in *N. crassa* (Belden, Larrondo et al. 2007). In *Aspergilus nidulans*, the gene RasB is found to be important for hyphal growth and virulence (Fortwendel, Zhao et al. 2005).

NCU00935 (*cws-3*) is a 582 amino acid protein that contains Pleckstrin homology domain suggesting that it may be involved in intracellular signaling. This gene shows rather high expression in the tip (15-fold up-regulation) and the mutant is sensitive to all the three stresses, grows 10% slower than the wild type and produces sparse, thin and hyperbranching hyphae. The mutant produces normal conidia and perithecia. This gene is conserved in most organisms across Metazoa, Plants, and Fungi, but appears to be absent from the Basidiomycetes.

Transport: The two mutants NCU01254 (*cws-4*) and NCU01911 (*cws-5*) show cell wall stress sensitivity, slow growth and defective sexual development and protein domains that implicate them in the transport processes; however only the former displays abnormal hyphal morphology (dense and hyperbranching hyphae). *Cws-4* is a 672 amino acid protein that contains a cation efflux family domain suggesting that it might be involved in integral membrane transport. It is an ascomycete-specific protein that is significantly similar to the yeast protein Zrg17p that is known to play a role in zinc

uptake in yeast (Toke, Bennett et al. 1998). *Cws-5* is a 689 amino acid protein that contains a GDP/GTP exchange factor Sec2p domain that has been shown in *S. cerevisiae* to be important for vesicular transport (Walch-Solimena, Collins et al. 1997). It is a well-conserved protein in the fungal kingdom. The mutant is sensitive to cell wall integrity stress and has a defective sexual cycle, as it produces very few ascospores after fertilization. It grows similar to wild-type and has a normal hyphal morphology. FunCat classification classifies this protein as being involved in cellular export and secretion. The exocyst, which is a multiprotein complex that enables docking of secretory vesicles before fusion with the plasma membrane, is known to be made of eight components, SEC-3, SEC-5, SEC-6, SEC-8, SEC-10, SEC-15, EXO-70, and EXO-84 (Hsu, TerBush et al. 2004); but no interaction with *cws-5* has been shown.

Overall growth patterns in CWI mutants set

The basal linear growth rate of the CWI sensitive mutants showed roughly equal numbers growing more slowly (70% or less relative to wild type; 30 mutants) or faster (20% or greater relative to wild type; 24 mutants) than wild type on simple VM medium (Table 3). To identify other pathways that might be affected, I assayed the mutants for oxidative (hydrogen peroxide) and hyperosmotic (sodium chloride and glycerol) stresses. Of the 67 mutants that were sensitive to cell wall integrity stress, 30 showed decreased resistance to oxidative stress, 35 were sensitive to hyperosmotic stress, 30 were slow growing, 34 had slow growing aerial hyphae; 14 had lighter conidial pigmentation, 21 possessed hyphal edge abnormalities and 8 had sexual developmental

defects (Figure 4). These findings suggest that many of the processes impacted in CWI mutants impact other growth and developmental pathways. I noted that hyperosmotic stress, aerial hyphal and oxidative stress pathways showed maximum overlap with the CWI pathway, whereas the sexual and conidiation pathways showed the least crosstalk. Additional combinations of the sensitivity to the three major stresses can be seen in Table 2 and Figure 5. It may be useful to understand whether the same mutants are sensitive to all the stresses or if each stress response requires independent genetic pathways.

The CWI mutants that exhibited morphological abnormalities were mainly found to be of two types: those having sparse, thin hyphae and those with dense, hyperbranching hyphal growth (Table 5a). A total of 23 out of 67 CWI mutants displayed morphological defects, out of which 12 were sparse and thin and 11 were dense and hyperbranching. I observed a general trend of the morphological phenotypes in the CWI mutant gene set- the mutants that grew more poorly than wild type and had dense and hyperbranching hyphae mostly fell under the 'Cell growth and morphogenesis' category (for eg: *bud-3, chs-1, gh13-10 and spa2*); whereas the genes in the mutants that grew better than wild type and possessed sparse and thin hyphae belong to the 'Degradation' category (e.g., *cws-17, gh18-8 and gh13-1*). There are other non- CWI sensitive mutants that also display abnormal morphologies, such as NCU00675 and NCU08822 that possess dense and hyperbranching hyphal growth, along with NCU02988, NCU04826 and NCU04500 that display sparse and thin hyphae (Table 2)

Several CWI mutants also exhibited a sexual developmental defect (Table 5b). Knockout mutants in genes such as *cws-17*, *gh13-1* and *gh18-10* were completely sterile producing no sexual structures, while others such as NCU00526 (*lcc1*) and *ada-6* produce defective structures, such as fewer ascospores or small protoperithecia, respectively. The *cws-19* mutant is interesting as it exhibits a great delay in production of protoperithecia (at least 20-25 days post fertilization) and very few perithecia thereafter.

DISCUSSION

The cell wall is an essential feature of fungal cells, as it shields and protects the cell from external stress and damage. The cell wall has proven a good target for designing anti-fungal drugs against pathogenic fungi, as it is a unique component that distinguishes the cells of the pathogen and the hosts (plants/animals) they infect. The knowledge gleaned from random mutagenesis screens (Seiler and Plamann 2003) and cell wall stress-based screens (Ram and Klis 2006) has contributed to our current understanding of the genes involved in the various processes leading to cell wall synthesis and hyphal growth in the model filamentous fungus *N. crassa*. In addition, expression studies (Kasuga and Glass 2008) have been instrumental in elucidating the growth and development pathways during colony establishment in *N. crassa*. I utilized this expression information as the motivation behind the functional genomics screen to

discover novel cell wall genes in *N. crassa*. By examining a tissue or developmental time point I expect to find an enrichment of genes responsible for the process. In order to include expression data from as many genes as possible I repeated the study using more sensitive and robust RNA sequencing to identify genes upregulated in the tip. About half of the genes were in common between (Kasuga and Glass 2008) microarray analysis and RNA sequencing datasets in addition to genes upregulated in the tip from the RNA sequencing study. This study represents a model approach to finding genes involved in a process.

More than half of the candidate gene-set corresponded to cell wall integrity mutants. In addition, this gene-set was also enriched in functional categories such as cell wall biogenesis, polysaccharide metabolism, and directional cell growth. This strengthens the premise that the tip-expressed genes are involved in cell wall related functions. It is worth noting that a similar study in *N. crassa* that tested GPI-anchored genes for cell wall function, did not find a substantial proportion (~15%) to have cell wall defects (Maddi, Dettman et al. 2012). I was able to functionally annotate 32 previously un-annotated genes as cws (**c**ell **w**all **s**ensitive) on the basis of their sensitivity to cell wall stress. This information will be submitted to the Fungidb database.

The random forest clustering approach helped us to place the cell wall integrity mutants into relevant cell wall pathways. This will be useful for future experimental studies focused on validating genes involved in cell wall processes in *N. crassa*. I chose to perform an overlap of the various defects in order to select the cell wall mutants that

were defective in multiple pathways because I wanted to focus on the mutants that had most drastic defects. I classified these candidates on the basis of the shared characteristics with known cell wall genes into pathways like the growth and morphogenesis, cell wall integrity signaling, Rho signaling and transport. This approach enabled us to identify genes in the various processes that come together to build, degrade and regulate the cell wall.

The Vesical Supply Center (VSC) model of hyphal morphogenesis suggests the hyphal growth requires the constant building and breaking of the cell wall (Bartnicki-Garcia, Hergert et al. 1989). There is substantial evidence that the various micro and macro–vesicles contains cell wall building enzymes accumulate at the hyphal tip (Spitzenkörper) before reaching the plasma membrane (Verdin, Bartnicki-Garcia et al. 2009; Sanchez-Leon, Verdin et al. 2011). I found five new tip-expressed genes involved in cell wall morphogenesis *gh16-7*, *cws-26*, *cws-13*, *gh13-10*, *cws-2*, *gh18-10*. The mutants of these genes have slower growth than wild-type and abnormal hyphal morphologies, which are similar to the known morphogenesis genes such as the chitin synthase genes *chs-1*, *chs-5*, *chs-6* and *chs-7*.

The cell wall integrity pathway is responsible for defending the cell against various physical and chemical onslaughts. The CWI MAPK pathway in *N. crassa* is comprised of *mak-1*, *mek-1*, and *mik-1* and involved in hyphal growth, sexual development and normal colony morphology (Park, Pan et al. 2008). In a recent study, the *wsc-1* gene has been identified as an upstream activator of the MAK-1 pathway (Maddi, Dettman et al.

2012). Four genes from the CWI mutant set, *ada6*, *cws-19*, *gh13-1* and *cws-17*, are strong candidates for being targets of the MAPK signaling pathway. The mutants of these genes phenotypically mimic the MAPK mutants (*mak-1*, *mek-1* and *mik-1*) by displaying slow growth, sensitivity to CWI stress, failure to produce any sexual structure, and sparse and curvy hyphae. It is worth noting that *wsc-1* also shows similar characteristics but has a normal sexual cycle (Maddi, Dettman et al. 2012).

Polarized hyphal growth in *N. crassa* requires a series of molecular switches in the form of Rho GTPases such as Rho, Rac, Cdc42 and others (Riquelme, Yarden et al. 2011). The *rdi-1* gene controls septation by negatively regulating Rho-4 (Rasmussen and Glass 2007). The gene *bd* is a candidate that shows similar characteristics as *rdi-1* i.e. slow growth, CWI sensitivity and very stunted, dense and hyperbranching hyphae. I hypothesize that this gene could be interacting with the Rho-signaling pathway to regulate septation.

Hyphal tip growth is a complex and highly regulated process that involves the continuous traffic of secretory vesicles to the apex and their fusion with the apical plasma membrane. Its known that an exocyst-mediated mechanism is necessary for the last steps of exocytosis to dock SPK vesicles to the apical plasma membrane (TerBush, Maurice et al. 1996), in *S. cerevisiae*, Sec2p associates with the exocyst via subunit Sec15p (Medkova, France et al. 2006). I hypothesize that *cws-5* plays a similar function in *N. crassa*. I was also able to find another putative transport gene *cws-4* possessing

domains important for integral membrane transport. These candidates could be new players in the vesicular transport pathways required for hyphal growth in *N. crassa*.

Lastly, there are some general observations gleaned from the present CWI screen. I saw a general trend of the morphological phenotypes in the CWI mutant gene set. The genes annotated in the 'Cell growth and morphogenesis' category grew more poorly than wild-type and had dense and hyperbranching hyphae, whereas the genes in the 'Degradation' category grew better than the wild-type and possessed sparse and thin hyphae. The finding of approximately equal numbers of slow or fast growing mutants strains that among the most sensitive to cell wall integrity assays suggests that cell wall integrity stress response and resistance is not necessarily coupled to growth rate in *N. crassa*. Also, I observed that out of all the defects, sensitivity to hyperosmotic stress showed maximum crosstalk with the cell wall integrity pathway, suggesting that these genes are necessary for both CWI and hyperosmotic stress response. This is in agreement with previous studies correlating sensitivity to CWI stress with oxidative and osmotic stresses (Maddi, Fu et al. 2012).

Using more than one type of assay criterion improves the sensitivity of a screen. The fact that I used hyphal morphology as an additional criterion enabled us to identify genes with mutants that were not sensitive to CWI stress, but Nonetheless were good cell wall candidates. For instance, two of the cell wall integrity candidates (NCU04826 and NCU02988), sensitive only to oxidative stress, show slow vegetative growth and sparse and thin hyphal morphology; not unlike mutants defective in the cell wall genes-

chs-5 and chs-7. Whereas NCU04826 contains a chromosome segregation domain and is defective in the production of ascospores, NCU02988 contains a methyltranferase domain and is unable to produce any sexual structure at all. Similarly, mutants lacking NCU00675 and NCU08822 are sensitive to oxidative and hyper-osmolar stress, but show characteristics similar to the chs-1 and chs-6 genes; slow compact growth and dense hyberbranching hyphal morphology.

In summary, I was able to place the cell wall integrity candidates under groups of processes/pathways that were suggested by their phenotypes and stress responses. However these assignments need to be checked for their interactions in the pathways by genetic interaction studies and molecular techniques to verify the exact function of a particular gene. Although focused on CWI, this list is also useful for mining mutants involved in other stress and development pathways. It is worth noting that this screen had very little overlap with the (Maddi, Dettman et al. 2012) study (12 candidates in common) and (Seiler and Plamann 2003) screen (1 candidate), which demonstrates that I were able to find novel genes involved in the cell wall related processes. The present study produced a collection of cell wall growth and development candidate genes most of which possess homologs in other Pezizomycotina fungi improving their utility as potential targets for future drug targeting. Also it provides a valuable addition to the repertoire of genes known to be involved in hyphal morphogensis and cell wall maintenance that can be followed up using molecular and genetic techniques.

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Figure 1 Random forest clustering of Cell wall integrity (CWI) mutant phenotypes gives rise to three major clusters- A, B and C. Each dot represent a gene and the colors represent the FunCat category it falls under. The un-named dots represent mutants that did not show sensitivity to CWI stress.

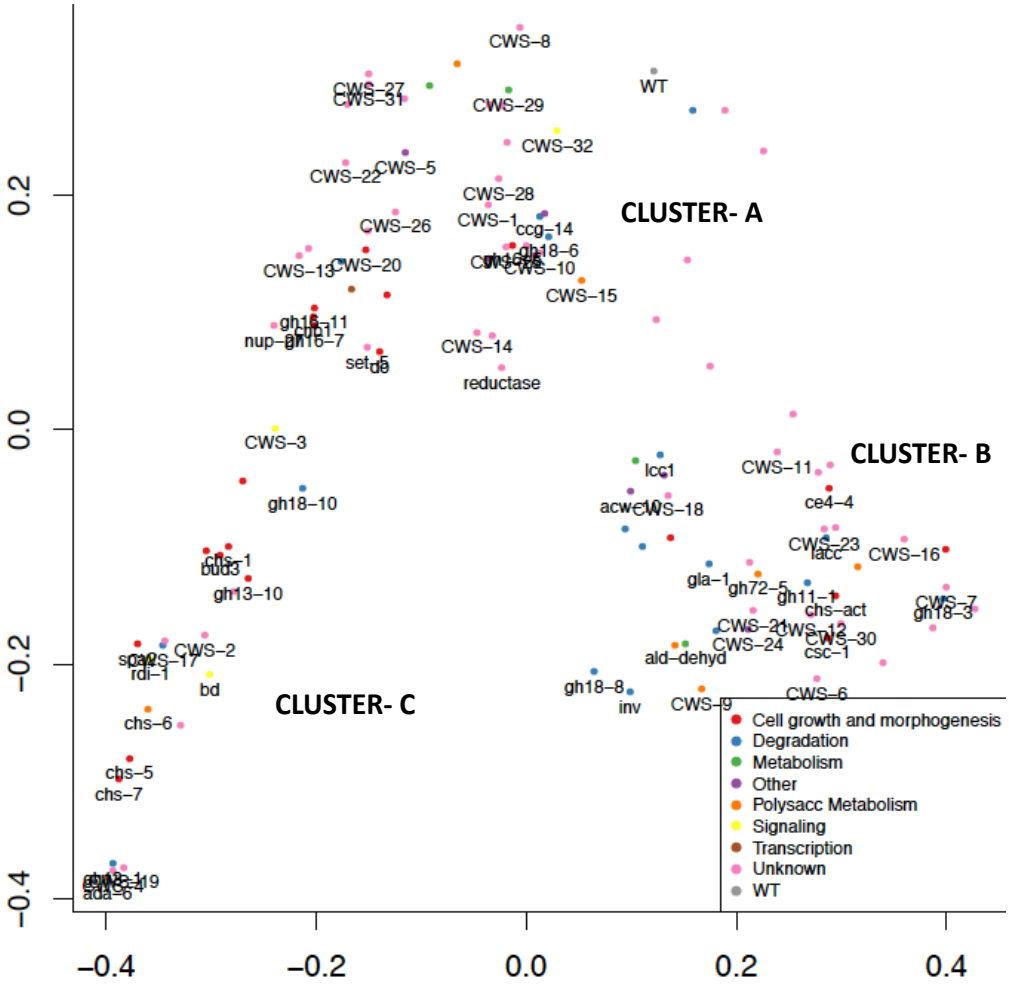

Figure 2 Overlap of mutants that possess CWI stress sensitivity, slow growth, abnormal hyphal edge and sexual defects. The genes in each of the four chosen defect overlap categories are shown.

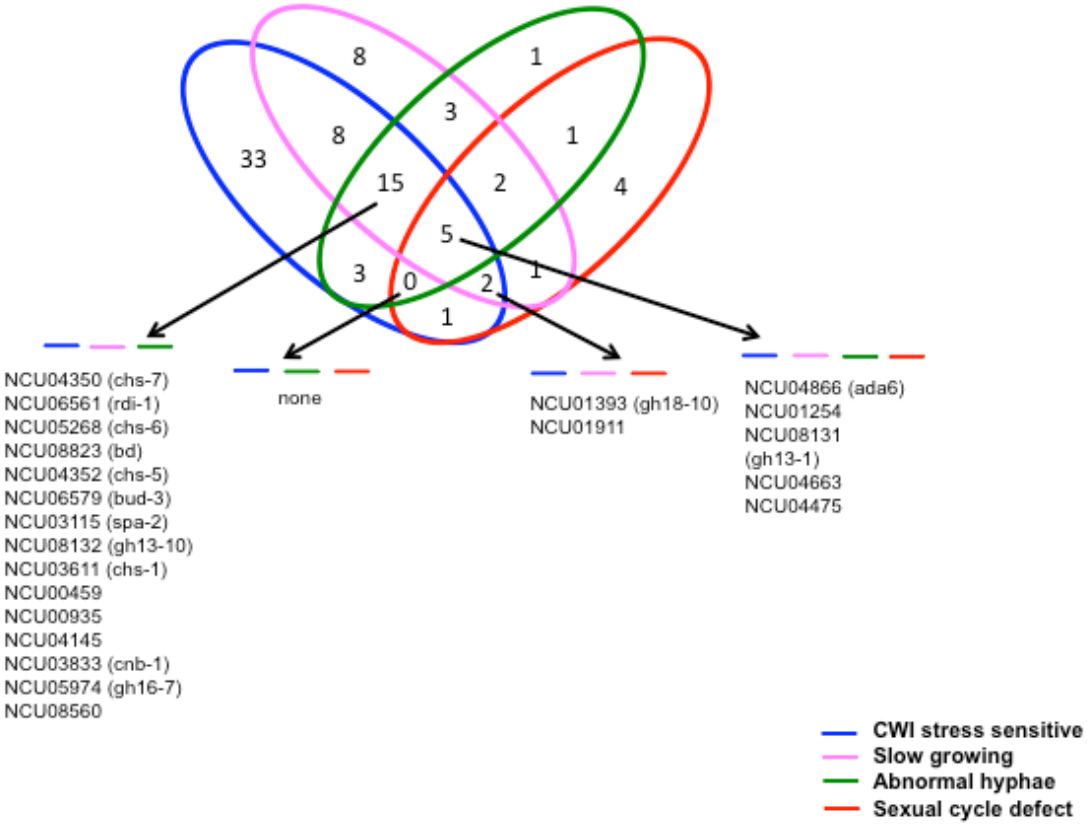

Figure 3 Hyphal growth and morphology of the chosen CWI candidates and the previously characterized mutants; grouped by similar morphology patterns.

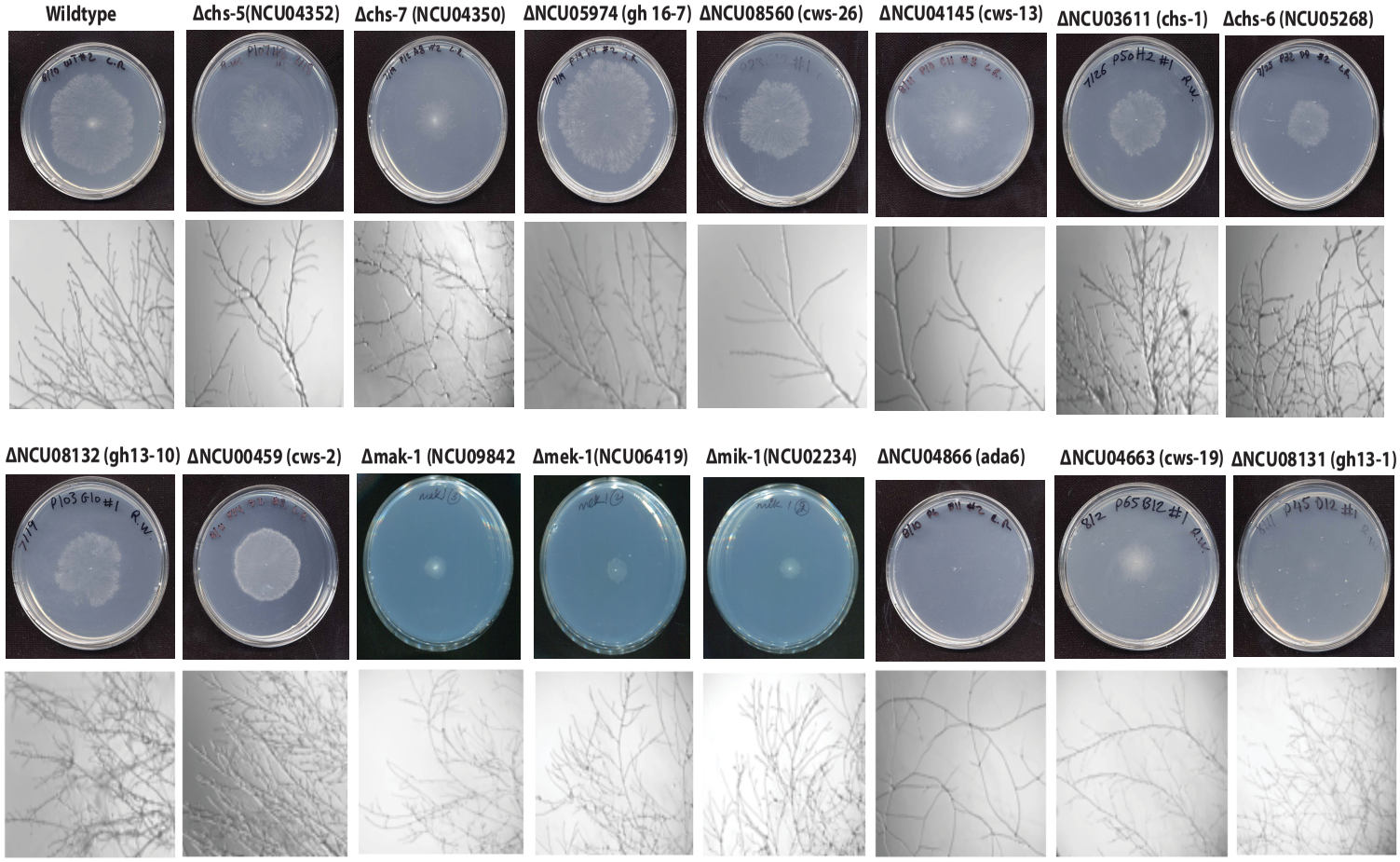

Figure 3 Hyphal growth and morphology of the chosen CWI candidates and the previously characterized mutants; grouped by similar morphology patterns. Continued from previous page.

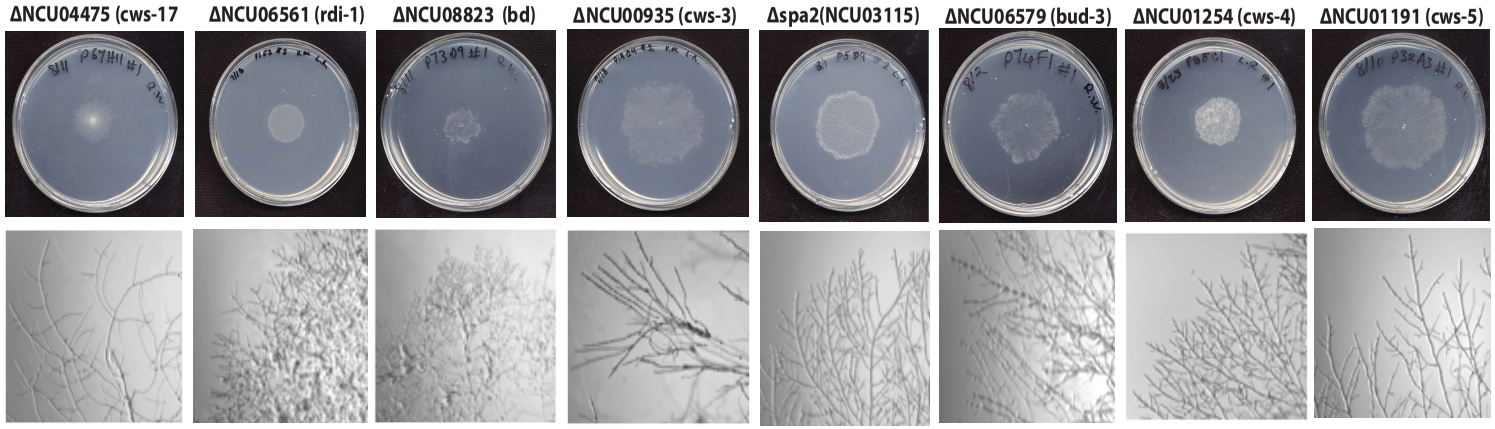

Figure 4 Distribution of cell wall integrity (CWI) mutants having defects in other pathways tested in this study. Hyper-osmotic (OSM) stress sensitive CWI mutants are the highest, showing the largest overlap between CWI and OSM stress pathways. Whereas Sexual defective CWI are the lowest in number, suggesting smallest overlap between the CWI stress and sexual development pathways.

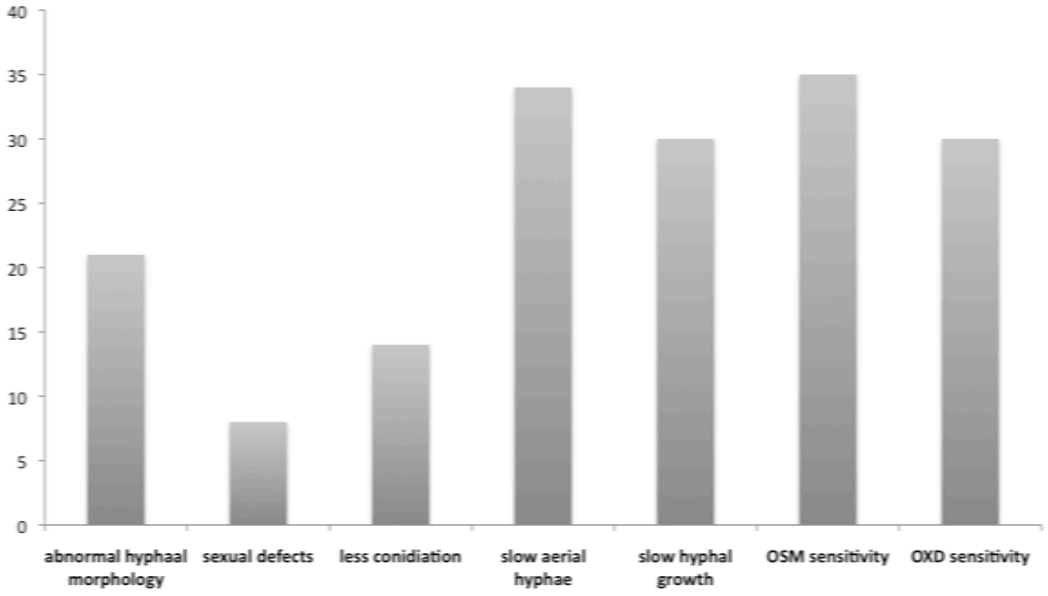

Figure 5 Distribution of stress sensitive mutants (89 genes) from the whole gene set (112 genes). The size of the circles signifies the number of mutants sensitive to a particular stress. CWI: Cell Wall Integrity stress; OSM: Hyperosmolar stress and OXD: Oxidative stress.

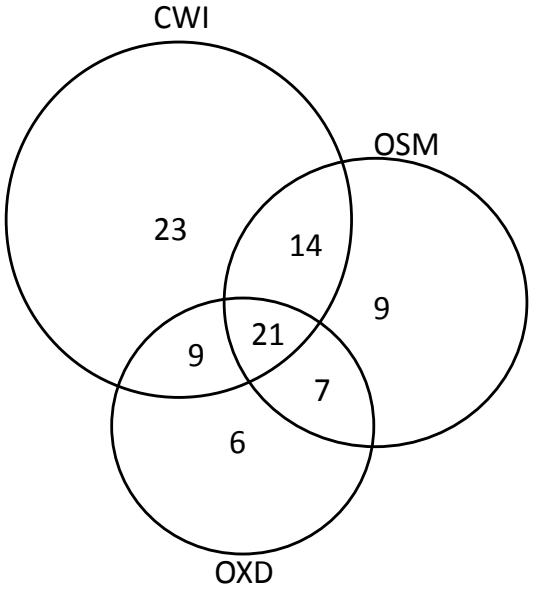

Table 1 Genes chosen to supplement the candidate set, identified by a homology search to the previously characterized cell wall genes in *S. cerevisiae*. * indicates a bidirectional homolog.

Gene accession	Name	Yeast homolog (evalue)
NCU06871	Dolly (gls1)	FKS1 (0)*
NCU09117	gh16-11	CRH1 (4.6e-67)*
NCU04592	3-oxoacyl-(acyl-carrier-protein) reductase	YMR226C (3.8e-11)
NCU04350	chs-7	CHS3 (7.8e-135)
NCU05268	chs-6	CHS3 (5.5e-13)
NCU05317	gh18-6	CTS2 (1.2e-05)
NCU05720	csc-1	CHS7 (1.6e-62)*
NCU03611	chs-1	CHS2 (5.9e-136)
NCU03833	cnb-1	CNB1 (1.7e-60)*
NCU03115	spa	SPA2 (7.1e-25)*
NCU04528	lacc	FET5 (3e-38)
NCU09508	ce4-4	CDA1 (5.3e-18)
NCU05686*	gh16-5	UTR2 (2.8e-90)*
NCU00526	laccase1	FET3 (3.5e-44)
NCU08909*	gh72-5	GAS1 (3e-111)*
NCU03026	gh18-3	CTS2 (3.1e-36)
NCU05785	FLC	FLC2 (2.8e-90)
NCU02592	chs activator	SKT5 (4.5e-40)

NCU07920	lcc2	FET5 (7.8e-38)
NCU03914*	gh5-4	EXG1 (3.3e-48)*
NCU04251	chs-3	CHS1 (1.1e-193)*
NCU09324	chs-4	CHS3 (7.2e-291)*
NCU02978	SLA1	SLA1 (1.8e-77)*
NCU02797	UGP1	UGP1 (9.2e-172)*
NCU04173	act	ACT1 (1.3e-190)*
NCU00090	pacc-1	RIM101 (2.5e-29)*
NCU04500	gh18-1	CTS1 (7.6e-58)*
NCU02216*	gh76-6	DCW1 (2.9e-81)

Table 2 Summary of the candidate genes and their phenotypes grouped by their sensitivity to stresses. UG- un-annotated gene, FC- Fold change, CWI- Cell wall integrity mutants (sensitive to caspofungin and/or SDS), OXD- Oxidative stress mutants (sensitive to hydrogen peroxide), OSM- Hyperosmolar stress mutants (sensitive to NaCl and/or Glycerol), HG- Hyphal growth (24 hr growth on Vogels Minimal agar plates), AH- Aerial hyphae extension (48 hr growth on Vogels minimal liquid tubes), L- less than wildtype, M- more than wildtype, S- same as wildtype, SD- Sexual development, PP- Protoperithecia defective, P- Perithecia defective, A- Ascospores defective, *- bidirectional homolog, ∞- RNA seq #- Kasuga Microarray.

Gene accession	Tip expression	Name	Yeast homolog (evalue)	Sensitivity to stress	HG	AH	C	SD defect	Hyphal morphology
i) Sensitive to cell wall integrity, hyperosmolar and oxidative stress (21)									
NCU08132	9 #	gh13-10	No significant hits	CWI OSM OXD	L	S	M	NONE	dense, hyperbranching
NCU03735	5 #	UG	No significant hits	CWI OSM OXD	M	S	L	NONE	
NCU04148	18.5 #	UG	No significant hits	CWI OSM OXD	S	M	S	NONE	
NCU00935	15 #	UG	No significant hits	CWI OSM OXD	L	S	S	NONE	sparse, hyperbranching

NCU01393	5 ∞	gh18-10	No significant hits	CWI OSM OXD	L	L	S	P, PP, A	
NCU07484	5 ∞	gh18-8	No significant hits	CWI OSM OXD	M	M	L	NONE	sparse, knotted hyphae
NCU06119	8.7 #	methyltransferase-5	SET2 (8.1e-09)	CWI OSM OXD	L	M	L	NONE	
NCU08131	5.3 #	gh13-1	IMA5 (2.6e-12)	CWI OSM OXD	L	L	L	P, PP, A	very sparse, thin hyphae
NCU06871	5	Dolly (gls1)	FKS1 (0)*	CWI OSM OXD	L	M	M	NONE	
NCU04866	17.1 ∞	ada-6	TEA1 (1.3e-11)	CWI OSM OXD	L	L	L	PP,A	very sparse, cruvy, thin hyphae
NCU04663	5.1 ∞	unknwn	No significant hits	CWI OSM OXD	L	L	L	P, PP, A	very sparse, stunted, thin hyphae
NCU04475	7 #	probable lipase B precursor	No significant hits	CWI OSM OXD	L	S	S	P, PP, A	very sparse, curvy, thin hyphae
NCU04265	22.5 ∞	inv	SUC2 (1.4e-35)*	CWI OSM OXD	M	L	L	NONE	knotted hyphae, sparse branch
NCU08823	5.1 ∞	bd	RAS2 (3.1e-52)	CWI OSM OXD	L	L	M	NONE	dense, hyperbranching, very stunted

NCU00459	5.7 ∞	UG	YPR117W (3.3e-125)*	CWI OSM OXD	L	L	S	NONE	dense, hyperbranching
NCU02922	10 #	short-chain dehydrogenase/reductase	AYR1 (1.9e-29)	CWI OSM OXD	M	L	S	NONE	
NCU07740	33.2 ∞	UG	TPO4 (1.7e-53)	CWI OSM OXD	M	S	L	NONE	
NCU07997	8.2 ∞	UG	No significant hits	CWI OSM OXD	S	M	S	NONE	
NCU01254	12.5 #	UG	ZRG17 (2e-13)*	CWI OSM OXD	L	L	L	P, PP, A	dense, hyperbranching, stunted
NCU09117	3	gh16-11	CRH1 (4.6e-67)*	CWI OSM OXD	L	M	S	NONE	
NCU04592	7.3	3-oxoacyl-(acyl-carrier-protein) reductase	YMR226C (3.8e-11)	CWI OSM OXD	M	S	NONE		

ii) Sensitive to Cell wall integrity and hyperosmolar stress (14)

NCU04352	5.3 ∞	chs-5	CHS3 (1.9e-129)	CWI OSM	L	L	S	NONE	sparse
NCU04350	4	chs-7	CHS3 (7.8e-135)	CWI OSM	L	L	L	NONE	sparse, stunted hyphae
NCU03238	10.2 ∞	UG	IZH3 (1.4e-39)*	CWI OSM	S	S	M	NONE	
NCU05268		chs-6	CHS3 (5.5e-13)	CWI OSM	L	L	L	NONE	hyperbranching, curvy, stunted

NCU05317		gh18-6	CTS2 (1.2e-05)	CWI OSM	S	S	S	NONE	
NCU02855	6.7 #	gh11-1	No significant hits	CWI OSM	M	L	S	NONE	
NCU05720	5	csc-1	CHS7 (1.6e-62)*	CWI OSM	M	L	S	NONE	
NCU01517	7 ∞	gla-1	SGA1 (7e-30)*	CWI OSM	M	L	S	NONE	
NCU02120	5.4 #	UG	No significant hits	CWI OSM	M	L	S	NONE	
NCU07141	6.2 ∞	UG	No significant hits	CWI OSM	M	S	S	NONE	
NCU03611	3	chs-1	CHS2 (5.9e-136)	CWI OSM	L	S	L	NONE	dense, hyperbranching
NCU09648	45 ∞	aldehyde dehydrogenase	ALD5 (3.5e-69)	CWI OSM	M	M	M	NONE	
NCU09003	5 #	UG	No significant hits	CWI OSM	S	M	S	NONE	
NCU06083	23.8 ∞	UG	No significant hits	CWI OSM	M	L	S	NONE	

iii) Sensitive to Cell wall integrity and oxidative stress (9)

NCU03013	7.8 #	acw-10	No significant hits	CWI OXD	M	L	S	NONE	sparse
NCU03428	5 #	UG	No significant hits	CWI OXD	M	S	S	NONE	
NCU03833	2	cnb-1	CNB1 (1.7e-60)*	CWI OXD	L	M	S	NONE	hyperbranching, long hyphae

NCU05974	26 ∞	gh16-7	CRH1 (5.1e-52)	CWI OXD	L	S	S	NONE	sparse branched, thin hyphae
NCU08560	5.5 #	UG	No significant hits	CWI OXD	L	S	M	NONE	sparse
NCU04160	5 ∞	UG	CSI2 (3.7e-05)*	CWI OXD	S	S	S	NONE	
NCU03115	4	spa	SPA2 (7.1e-25)*	CWI OXD	L	L	S	NONE	dense, hyperbranching, stunted
NCU04528		lacc	FET5 (3e-38)	CWI OXD	M	S	S	NONE	
NCU00265	935.5 ∞	UG	No significant hits	CWI OXD	S	L	S	NONE	

iv) Sensitive to Cell wall integrity stress (23)

NCU04145	5.3 ∞	UG	XDJ1 (1.9e-08)	CWI	L	L	S	NONE	sparse
NCU09508	2	ce4-4	CDA1 (5.3e-18)	CWI	M	S	S	NONE	
NCU10519	5 ∞	UG	No significant hits	CWI	L	S	S	NONE	
NCU10905	5.9 #	UG	HOF1 (1.4e-19)*	CWI	S	L	S	NONE	
NCU05686	4	gh16-5	UTR2 (2.8e-90)*	CWI	S	L	S	NONE	
NCU00526		laccase1	FET3 (3.5e-44)	CWI	M	L	L	NONE	
NCU08909	2.5	gh72-5	GAS1 (3e-111)*	CWI	M	L	S	NONE	
NCU03026		gh18-3	CTS2 (3.1e-36)	CWI	M	S	S	NONE	

NCU01911	6.8 #	Sec2	SEC2 (2.1e-21)*	CWI	L	L	M	NONE	
NCU05785	4	FLC	FLC2 (2.8e-90)	CWI	L	L	S	NONE	
NCU02592	3	chs activator	SKT5 (4.5e-40)	CWI	M	L	S	NONE	
NCU02172	5.3 ∞	UG	BBC1 (8.5e-05)	CWI	M	S	S	NONE	
NCU06561	9.2 ∞	rdi-1	RDI1 (2.3e-31)*	CWI	L	L	S	NONE	dense, hyperbranching, stunted
NCU09409	5.7 #	UG	No significant hits	CWI	S	L	S	NONE	
NCU09782	9.6 ∞	UG	No significant hits	CWI	M	M	S	NONE	
NCU02644	6.3 ∞	UG	No significant hits	CWI	S	S	S	NONE	
NCU06644	5 ∞	nup-27	MUC1 (1.7e-22)	CWI	L	S	S	NONE	
NCU04360	8.6 ∞	UG	No significant hits	CWI	M	S	S	NONE	
NCU04542	5.8 ∞	unknwn	No significant hits	CWI	M	L	S	NONE	
NCU06579	5 ∞	bud3	BUD3 (4.3e-07)*	CWI	L	L	S	NONE	dense, hyperbranching
NCU06560	5.9 ∞	UG	No significant hits	CWI	L	L	S	NONE	
NCU08681	5 ∞	UG	No significant hits	CWI	L	L	S	NONE	

NCU07787	5.5 #	ccg-14	No significant hits	CWI	S	S	L	NONE	

v) Sensitive to hyperosmolar and oxidative stress (7)

NCU01729	6.5 ∞	UG	No significant hits	OSM OXD	S	M	M	NONE	
NCU06911	7 ∞	UG	No significant hits	OSM OXD	M	M	S	NONE	
NCU00675	5 #	putative efr3	EFR3 (2.6e-25)*	OSM OXD	L	L	S	NONE	dense, hyperbranching, stunted
NCU08822	6. 8 ∞	UG	No significant hits	OSM OXD	L	S	L	NONE	dense, hyperbranching, stunted
NCU09175*	6.5 #	gh17-3	BGL2 (2e-17)	OSM OXD	M	M	L	NONE	
NCU07322	7.8 #	glyoxalase	No significant hits	OSM OXD	M	S	M	PP, P, A	sparse long one sided branched hyphae
NCU07920		lcc2	FET5 (7.8e-38)	OSM OXD	M	M	S	NONE	

f) Sensitive to hyperosmolar stress (9)

NCU03914*		gh5-4	EXG1 (3.3e-48)*	OSM	M	M	M	NONE	
NCU00729	17.3 ∞	UG	No significant hits	OSM	S	L	S	NONE	
NCU04467	11.9 ∞	UG	No significant hits	OSM	M	M	S	NONE	
NCU04251		chs-3	CHS1 (1.1e-193)*	OSM	L	L	M	NONE	

NCU03873	17.5 ∞	UG	No significant hits	OSM	M	S	S	NONE	
NCU05491	5.4 #	UG	No significant hits	OSM	M	S	S	NONE	
NCU05163	43 ∞	UG	No significant hits	OSM	S	S	S	NONE	
NCU03554	5 ∞	UG	No significant hits	OSM	M	S	S	NONE	
NCU09324	2	chs-4	CHS3 (7.2e-291)*	OSM	M	S	S	NONE	
vi) Sensitive to oxidative stress (6)									
NCU08720	20 ∞	UG	No significant hits	OXD	L	S	S	NONE	
NCU02988	5.8 ∞	UG	No significant hits	OXD	L	S	S	PP,P,A	sparse, thin hyphae
NCU09883	5 ∞	UG	No significant hits	OXD	S	S	S	NONE	
NCU02978	5	SLA1	SLA1 (1.8e-77)*	OXD	M	S	M	NONE	
NCU04826	5.6 ∞	UG	USO1 (3.7e-25)	OXD	L	S	L	PP,P, A	very sparse, thin hyphae
NCU08226	316 ∞	ces1 like	No significant hits	OXD	L	S	S	NONE	
vii) Sensitive to None (23)									
NCU02797	3	UGP1	UGP1 (9.2e-172)*		L	S	S	NONE	
NCU04173	3	act	ACT1 (1.3e-190)*		M	L	S	NONE	dense, knotted hyphae

NCU04272	5 ∞	UG	No significant hits	M	L	L	NONE	
NCU03667	9.3 ∞	UG	No significant hits	M	L	S	NONE	
NCU06351	5.5 #	pht-1	PHO11 (2.9e-12)	M	L	M	NONE	
NCU00090	2	pacc-1	RIM101 (2.5e-29)*	L	L	S	PP (big)	
NCU04500		gh18-1	CTS1 (7.6e-58)*	L	S	S	NONE	sparse, curvy, thin hyphae
NCU02216	3	gh76-6	DCW1 (2.9e-81)	S	S	S	NONE	
NCU02184	45.8 ∞	chit-1	CTS1 (1.3e-26)	S	L	S	NONE	
NCU04543	16.7 ∞	UG	No significant hits	S	S	S	NONE	
NCU09929	15 ∞	UG	No significant hits	S	L	M	NONE	
NCU06185	7 ∞	acw-9	No significant hits	L	L	S	NONE	
NCU02492	5.7 #	UG	No significant hits	S	L	S	NONE	
NCU08228	58 #	Ugd1	No significant hits	M	S	M	NONE	
NCU05694	16.6 ∞	UG	YEL023C (2.6e-15)	S	L	S	NONE	
NCU03605	5 ∞	amidohydrolase	No significant hits	S	S	S	NONE	
NCU07692	8 ∞	UG	No significant hits	M	S	S	NONE	
NCU07996	6 #	UG	No significant hits	S	L	S	NONE	
NCU08974	6.2 ∞	UG	PIB2 (1.5e-12)*	M	S	S	NONE	
NCU01855	11.4 ∞	UG	No significant hits	L	L	L	NONE	
NCU09812	7.4 ∞	hic-5	No significant hits	L	L	L	NONE	
NCU10521	17 ∞	UG	TEF4 (5.8e-07)	S	L	M	NONE	
NCU03079	6.2 ∞	UG	YGR266W (1.3e-74)	L	L	S	NONE	

Table 3 Cell wall integrity mutants (67) ordered by Random forest clusters. UG- un-annotated gene, FC- Fold change, CWI- Cell wall integrity mutants (sensitive to caspofungin and/or SDS).

Gene accession	Name	Sensitive to CWI stress
CLUSTER-A		
NCU07787	ccg-14	Casp
NCU03833	cnb-1	Casp
NCU00265	cws-1	Casp SDS
NCU03238	cws-10	Casp*
NCU04145	cws-13	Casp
NCU04148	cws-14	Casp
NCU04160	cws-15	Casp SDS
NCU05785	cws-20	SDS
NCU06560	cws-22	Casp
NCU07997	cws-25	Casp
NCU08560	cws-26	Casp
NCU08681	cws-27	Casp
NCU09003	cws-28	Casp
NCU09409	cws-29	Casp
NCU10519	cws-31	Casp
NCU10905	cws-32	Casp

NCU01911	cws-5	Casp
NCU02644	cws-8	Casp
NCU06871	do/fks	SDS
NCU09117	gh16-11	Casp
NCU05974	gh16-7	Casp* SDS
NCU05686	gh16-5	Casp SDS
NCU05317	gh18-6	SDS
NCU06644	nup-27	Casp
NCU04592	reductase	SDS
NCU06119	set-5	Casp SDS
CLUSTER-B		
NCU02120	cws-6	SDS
NCU02172	cws-7	SDS
NCU02922	cws-9	Casp SDS
NCU03428	cws-11	Casp
NCU03735	cws-12	SDS
NCU04360	cws-16	Casp
NCU04542	cws-18	Casp
NCU06083	cws-21	Casp
NCU07141	cws-23	SDS
NCU07740	cws-24	Casp
NCU09782	cws-30	Casp
NCU03013	acw-10	SDS
NCU09648	ald-hyd	Casp

NCU09508	ce4-4	Casp
NCU02592	chs-act	Casp*
NCU05720	csc-1	SDS
NCU01517	gla-1	Casp SDS
NCU04265	inv	SDS
NCU04528	lacc	Casp
NCU00526	lcc1	Casp
NCU02855	gh11-1	SDS
NCU03026	gh18-3	SDS
NCU07484	gh18-8	Casp SDS
NCU08909	gh72-5	Casp SDS
CLUSTER-C		
NCU00459	cws-2	Casp SDS
NCU00935	cws-3	Casp* SDS
NCU01254	cws-4	Casp SDS
NCU04475	cws-17	Casp
NCU04663	cws-19	Casp* SDS*
NCU03611	chs-1	Casp*
NCU04350	chs-7	Casp*
NCU04352	chs-5	Casp* SDS
NCU05268	chs-6	Casp*
NCU08131	gh13-1	Casp* SDS*
NCU08132	gh13-10	SDS
NCU01393	gh18-10	SDS

NCU04866	ada-6	Casp* SDS*
NCU08823	bd	Casp*
NCU06579	bud-3	Casp
NCU06561	rdi-1 (RHO protein GDP dissociation inhibitor-1)	Casp SDS
NCU03115	spa-2	Casp*

Table 4 Cell wall integrity mutants involved in multiple pathways (22), identified by overlapping the defects CWI stress sensitivity, slow growth, abnormal hyphal edge and sexual defects

Gene accession	Domains	Conservation	Hyphal Morphology
Known Cell wall genes			
NCU04350 (chs-7)	chitin synthase 2, Myosin domain	Fungal-Metazoa-Oomycota	Sparse, stunted
NCU04352 (chs-5)	chitin synthase 2, Myosin domain	Fungal-Metazoa-Oomycota-Planta	Sparse, stunted
NCU03611 (chs-1)	chitin synthase 1 domain	Fungal-Oomycota	dense, hyperbranching
NCU05268 (chs-6)	chitin synthase 1 domain	Fungal-Metazoa	dense, hyperbranching
NCU06579 (bud-3)	RhoGEF domain	Ascomycetes	dense, hyperbranching
NCU03115 (spa-2)	spa2 homology domain	Asco-Basidio-Zygo	dense, hyperbranching
NCU03833 (cnb-1)	EF-hand domain pair	Fungal-Metazoa-Oomycota-Planta	dense, hyperbranching, unusually long hyphae
NCU06561 (rdi-1)	RDI domain	Fungal-Metazoa-Oomycota-Planta	dense, hyperbranching
Growth and Morphogenesis			
NCU05974 (gh16-7)	GH16_fungal_CRH1_transglycosylase	Fungal-Planta	sparse branched, thin hyphae

NCU08560 (cws-26)	acyltransferase_3	Dikarya	sparse
NCU04145(cws-13)	DnaJ/Hsp40 domain	Dikarya	sparse
NCU08132 (gh13-10)	alpha amalyse; glycosyl transferase	Asco-Basidio-Planta	dense, hyperbranching
NCU00459 (cws-2)	RNA pol II promoter domain, Mitochondrial protein from FMP27	Fungal	dense, hyperbranching
NCU01393 (gh18-10)	GH18_CTS3_chitinase domain	Asco-Zygo	Normal
CWI MAPK signaling			
NCU04866 (ada6)	Fungal zn cys binuclear cluster domain	Asco-Zygo	Sparse, stunted
NCU04663 (cws-19)	Fungal zn cys binuclear cluster domain	Asco	very sparse, curvy, stunted, thin
NCU08131 (gh13-1)	alpha-amylase	Fungal-Metazoa	very sparse, curvy, thin
NCU04475 (cws-17)	no domains	Asco-Basidio	very sparse, curvy, thin
Other Signaling			
NCU08823 (bd)	ras	Fungal-Metazoa-Oomycota-Planta	dense, hyperbranching, very stunted

NCU00935 (cws-3)	Pleckstrin homology domain	Asco-Chytrid-Metazoa-Planta	sparse, hyperbranching
Transport			
NCU01911 (cws-5)	GDP/GTP exchange factor Sec2p	Fungal	Normal
NCU01254 (cws-4)	cation efflux family	Asco	stunted, curvy hyperbranching

Table 5 (a) Cell wall integrity mutants having hyphal abnormalities.

Gene no	name	homolog in yeast (evalue)	hyphal morphology
i) Sparse			
NCU04866	ada-6	TEA1 (1.3e-11)	very sparse, curvy, thin hyphae
NCU04350	chs-7	CHS3 (7.8e-135)	sparse, stunted hyphae
NCU08131	gh13-1	IMA5 (2.6e-12)	very sparse, curvy, thin hyphae
NCU04663	cws-19	No hits	very sparse, curvy, stunted, thin hyphae
NCU04352	chs-5	CHS3 (1.9e-129)	sparse
NCU04475	probable lipase B precursor	No hits	very sparse, curvy, thin hyphae
NCU00935	cws-3	No hits	sparse, hyperbranching
NCU04145	cws-13	XDJ1 (1.9e-08)	sparse
NCU05974	gh16-7	CRH1 (5.1e-52)	sparse branched, thin hyphae
NCU08560	cws-26	No hits	sparse
NCU03013	acw-10	No hits	sparse
NCU07484	gh18-8	No hits	sparse, knotted hyphae
ii) Dense			
NCU01254	cws-4	ZRG17 (2e-13)*	dense, hyperbranching, stunted
NCU06561	rdi-1	RDI1 (2.3e-31)*	dense, hyperbranching, stunted
NCU05268	chs-6	CHS3 (5.5e-13)	hyperbranching, curvy, stunted

NCU08823	bd	RAS2 (3.1e-52)	dense, hyperbranching, very stunted
NCU06579	bud3	BUD3 (4.3e-07)*	dense, hyperbranching
NCU03115	spa	SPA2 (7.1e-25)*	dense, hyperbranching, stunted
NCU08132	gh13-10	No hits	dense, hyperbranching
NCU03611	chs-1	CHS2 (5.9e-136)	dense, hyperbranching
NCU00459	cws-2	YPR117W (3.3e-125)*	dense, hyperbranching
NCU03833	cnb-1	CNB1 (1.7e-60)*	hyperbranching, long hyphae
NCU04265	inv	SUC2 (1.4e-35)*	knotted hyphae, sparse branch

Table 5 (b) Cell wall integrity mutants having sexual developmental defects.

Gene no	name	homolog in yeast (evalue)
NCU04866	ada-6	TEA1 (1.3e-11)
NCU01254	cws-4	ZRG17 (2e-13)*
NCU08131	gh13-1	IMA5 (2.6e-12)
NCU04663	cws-19	No hits
NCU04475	probable lipase B precursor	No hits
NCU01393	gh18-10	No hits
NCU01911	Sec2	SEC2 (2.1e-21)*
NCU00526	laccase1	FET3 (3.5e-44)
NCU03026	gh18-3	CTS2 (3.1e-36)

CHAPTER II

History of the fungal cell wall: Phylogenomics of cell wall polysaccharide enzymes in early-diverging fungal lineages.

ABSTRACT

Since composition of the cell wall differentiates pathogenic fungi from the plants and animals they infect, it is a common target for natural and synthetic anti-fungal compounds. Thus it is worthwhile to identify novel fungal cell wall biosynthesis and regulatory components in the newly sequenced early diverging fungal species. A combination of phylogenetics, comparative genomic and biochemical approaches were used to extend the understanding of the cell wall components to the early fungal species in an attempt to reconstruct the phylogenetic history of the fungal cell wall across a broad evolutionary time period. Profile HMMs constructed for genes involved cell wall biosynthesis, maintenance and remodeling were searched against a collection of fully sequenced genomes to reveal a new class of chitin synthase (CHS) and chitinase (CTS) gene family and the timing of gain of 1,3-beta glucan metabolism machinery in the fungal kingdom.

INTRODUCTION

The cell wall is a vital organelle of the fungal cell as it serves the dual purpose of maintaining the cell shape and protecting against physical damage while allowing exchanges of sugars, minerals and waste transport and organelle delivery mechanisms. The wall must also be strong enough to maintain turgor pressure for the growth of hyphae (Lew 2011) and through regulated exchanges maintain stable osmotic conditions inside the cell. The wall is not static and remodeling is required for morphogenesis and growth (Klis, Ram et al. 2007). The cell wall is comprised of a three-dimensional polysaccharide network of chitin, β-glucan, mannan and glycoproteins (Bernard and Latge 2001). For a majority of fungi, the central core of the cell wall is a branched -1,3 and -1,6 glucans that link to chitin via a β-1,4 linkage (Latge 2007). Various components such as 1,3-β glucan synthase and chitin synthase are targets of the clinically successful anti-fungals echinocandins and nikkomycin respectively (Latge 2007). Identifying and characterizing additional cell wall components would aid in the exploration for other potential drug targets.

Chitin is a non branched polymer of β-1,4-*N*-acetyl-glucosamine (GlcNAc). It is crystalline and extraordinarily strong, hence is known to be a characteristic stress-bearing polysaccharide of the fungal cell wall (Roncero 2002; Klis, Ram et al. 2007). Chitin biosynthesis is accomplished by a set of membrane isoenzymes called chitin synthases (CHS) that catalyze the transfer of GlcNAc residues from UDP-GlcNAc to the non-reducing end of the growing chitin chain. CHSs have been classified into six

(Roncero 2002) and more recently seven (Riquelme and Bartnicki-GarcÌa 2008) classes (CHS I to VII). These have further been grouped into Divisions each having a unique domain which distinguishes its members from the other divisions (Figure 1), for example the Division 1 contains the Pfam domains Chitin synth_1 (CS_1) and Chitin synth_1 N terminal (CS_1N) (Bowen, Chen-Wu et al. 1992); Division 2 proteins contain Chitin Synth_2 (CS_2) and Cytochrome-B5 (Cyt B5) domains with class V and VII having additional Myosin_head and DEK_C domains (Fujiwara, Horiuchi et al. 1997) and the division 3 (Class IV) genes contain only a CS_2 domain (Mandel, Galgiani et al. 2006). *N. crassa* has one copy of each of the seven classes but the Class I gene, CHS3 has been shown to be essential for survival, suggesting that it synthesizes the bulk of chitin (Riquelme and Bartnicki-GarcÌa 2008). Chitin deactetylase (CDA) catalyzes the elimination of the acetyl group from GlcNAc, leading to glucosamine. Fully deacetylated chitin is known as chitosan. Chitinase is a glycohydrolase_18 family enzyme that breaks down the chitin by the process of hydrolysis. The main chitinase operating on the cell wall of *S. cerevisiae* is the Chitinase (CTS2) that is involved in sporulation (Adams 2004). Mutations in CTS2 lead to abnormal spore wall biosynthesis and failure to form mature asci (Giaever, Chu et al. 2002)

Glucans are composed of repeating glucose residues that are assembled into chains through a variety of chemical linkages, mainly β-1,3 and β-1,6, although α-1,3- and α-1,4- linked glucans are also found in some species (Latgé JP 2006). In the present study I focus on β-1,3-glucan machinery as it is a good representative of the cell walls of the

fungal species at large. β-1,3-glucans are synthesized by a plasma membrane-bound protein complex synthase, FKS, that uses UDP-glucose on its intracellular side as a substrate. In yeast, the β-1,3-glucan synthase is encoded by genes FKS1 and FKS2. Disruption of either FKS1 or FKS2 yields mutants with slow growth rates and cell wall defects whereas the simultaneous deletion of FKS1 and FKS2 is lethal (Bowman and Free 2006). Three exo-β-1,3-glucanases have been described in *S. cerevisiae* cell wall- EXG1, EXG2 and SPR1 (SSG1). EXG1 is the main exo-glucanase involved in cell wall β-glucan assembly and exists as two differentially glycosylated isoenzymes- EXGIa and EXGIb. EXG2 is the exo-glucanase anchored to the plasma membrane via a glycosylphosphatidylinositol (GPI) anchor. SPR1 encodes a sporulation-specific exo-β-1,3-glucanase which is involved in development of heat-resistant ascospores (Larriba, Basco et al. 1993). In the present study I focus on profiling EXG1 among the fungal species examined.

Advancement in sequencing technology and subsequent drop in costs has made it possible to sequence a vast variety of fungal genomes (Martin, Cullen et al. 2011; Grigoriev, Nordberg et al. 2012). Data produced by these projects provide unparalleled opportunities to undertake studies on fungal comparative genomics and the evolution of individual protein families within the fungal kingdom (Hibbett, Stajich et al. 2013). Studies of the evolution of cell wall components in the early fungi can reconstruct the phylogenetic history of the fungal cell wall and test if the earliest fungi likely had the same components as extant species and the ubiquity of the genes needed for the

biosynthesis and degradation of the cell wall carbohydrates. Hence in my present report I sought to extend the understanding about the cell wall components to the early fungal species in an attempt to reconstruct the phylogenetic history of the fungal cell wall. This study uses a comparative genomics approach to elucidate important differences between the cell wall composition of the species belonging to the early diverging and the later diverged clades of fungi (Figure 1) (James, Kauff et al. 2006; Hibbett, Binder et al. 2007; Stajich, Berbee et al. 2009).

The early diverging clades included in this study are Microsporidia, Cryptomycota, Neocallimastigomycota, Chytridiomycota, Blastocladiomycota, Kickxellomycotina, Entomophthoromycota, Mucormycotina and the recently diverged Dikarya comprises of the Ascomycota and Basidiomycota. The species chosen from these clades were those that had fully sequenced genomes available and also were important to understand from a pathogenesis perspective. Microsporidia is the earliest diverging clade presently known (Capella-Gutierrez, Marcet-Houben et al. 2012) are obligate unicellular parasites of eukaryotes characterized by an absence of mitochondria, golgi apparatus and flagella. They grow as rigid thick walled spores outside the host, on germination it infects the host by means of a polar tube and then grow into a multinucleate plasmodium before producing more spores (Corradi and Keeling 2009). Cryptomycota is the most recently discovered phylum of fungal kingdom (Jones, Forn et al. 2011). Its only known member, *Rozella allomycis* is an intracellular parasite of 'water mold'- *Allomyces*. The infection begins with uniflagellate, wall-less zoospores of the parasite that swim to the

host, attach to the surface and form a penetration tube through which the parasite cytoplasm is injected into the host. Once inside the host, *R. allomycis* then grows as a wall-less form that feeds on the host cytoplasm (James and Berbee 2012). Chytridiomycetes such as *Batrachochytrium dendrobatidis* and are unicellular and flagellated and grow as a motile zoospore that swims towards host and changes into stationary zoosporangia upon penetrating and infecting it. Zygomycetes containing the sub-phyla- Kickxellomycotina, Entomophthoromycotina, Zoopagomycotina and Mucormycotina, are haploid and undergo meiosis to form sexual spores called 'zygospores'. The Dikarya subkingdom, which consists of Ascomycetes and Basidiomycetes is the most recently diverged. The defining feature of ascomycetes is the 'ascus', a sexual structure in which non-motile spores, called 'ascospores' are formed; some species however may be asexual. Popular examples of ascomycetes are *Saccharomyces cerevisiae*, model unicellular fungi that reproduce by budding and *Neurospora crassa*, filamentous fungi that grow vegetatively by extending hyphae and sexually through ascospores. Basidiomycota is the other phylum belonging to the Dikarya group, which consists of both yeast like and filamentous fungi that sexually reproduce through the club shaped 'basidiospores' (Stajich, Berbee et al. 2009). I employed a phylogenomic approach on the species chosen from these clades to study the history of the major gene families involved in cell wall polysaccharide biosynthesis and degradation.

Previous studies that attempted to examine the history of cell wall proteins using comparative approaches revealed that many of these gene families are ancient and vary in copy number among the Fungi (Coronado, Attie et al. 2006; Coronado, Mneimneh et al. 2007; Ruiz-Herrera and Ortiz-Castellanos 2009). I attempted to improve on these studies by employing fully sequenced representatives from each phylum and sensitive homology detection with profile HMMs trained on curated alignments of genes. Additionally, I have evaluated the history of these gene relationships in context of species relationships using phylogenetics to provide strong statistical support for the timing and patterns of duplication of these gene families. I hope to shed light on the evolutionary transitions of the cell wall in the fungal kingdom from the early flagellated form, which may have been similar to the modern Chytridiomycota or Blastocladiomycota, to the filamentous hyphal growth form in the Dikarya fungi. Because chitin and β-glucan in the cell wall constitute synapomorphies that distinguish Fungi from other Eukaryotes, I chose to focus on the genes involved in chitin and β-glucan metabolism and biosynthetic pathways to better understand diversification of these enzyme families.

MATERIAL AND METHODS

Genome sequencing- Selected genomes for this study were from the Microsporidia (represented by *Antonospora locustae, Enterocytozoon bieneusi* and *Encephalitozoon cuniculi*), Cryptomycota (*Rozella allomycis*), Neocallimastigomycota (*Piromyces sp.,)* Chytridiomycota (*B. dendrobatidis*), Blastocladiomycota (*Allomyces macrogynus*),

Kickxellomycota (*Coemansia reversa*), Entomophthoromycota (*Conidiobolus coronatus*), Mucormycota (*Phycomyces blakesleeanus*), Ascomycota (*Neurospora crassa* and *Saccharomyces cerevisiae*) and Basidiomycota (*Ustilago maydis* and *Coprinopsis cinerea*). Predicted protein sequences from annotated fully sequenced genomes were obtained from GenBank, SGD, and genome sequencing centers.

Cell wall gene identification- The Saccharomyces Genome Database was mined to catalogue the genes responsible for synthesis, degradation and organization of the cell wall for the model organism, *S. cerevisiae*. For chitin synthase, additional information from previously studies (Choquer, Boccara et al. 2004; Mandel, Galgiani et al. 2006; Riquelme and Bartnicki-GarcÌa 2008) was used augment the gene set and identify the known CHS families and their typical sequences. I developed a simple pipeline for performing a comparative homology search utilizing the profile Hidden Markov Models (Eddy 1998) comprised of cell wall genes defined in *S. cerevisiae*, *N. crassa and U. maydis*. Multiple sequence alignments of seed homologs was constructed with T-Coffee (Notredame, Higgins et al. 2000), and built into a profile-Hidden Markov Model with HMMER3 (Eddy 1998). HMMER3 was chosen to allow global-global comparisons of alignments families. Identified homologs from each genome for each cell wall gene family HMMs were identified with hmmsearch and a cutoff bit score more than log of the number of proteins in this database of selected fungal genomes and these typically resulted in expectation values less than 1E-20.

Phylogenetic analysis- The homologs from HMMER3 search were aligned with Muscle (Edgar 2004) and the resulting protein alignments were automatically trimmed for high quality regions alignments with trimAl using the –automated1 parameter (Capella-Gutierrez, Silla-Martinez et al. 2009). Next, maximum likelihood phylogenetic trees were constructed with RAxML (Stamatakis 2006) with protein substitution matrix chosen by ProteinModelSelection. Protein sequences for candidate genes were profiled for protein domain composition of gene products by searching the Pfam database with HMMER (Finn, Mistry et al. 2010). The homolog detection and phylogenetic analysis was performed as pipeline in executed in Perl that is available at https://github.com/dsain/genefam_pipeline.git.

RESULTS

Chitin synthase

Profile HMMs were developed for the entire Chitin synthase family and individually for the Division 1 and 2, 3 members. Searches of target genomes with profile HMMs of CHS identified potential homologs of CHS genes in all the clades included and a detailed list of homologs is presented in Table S1. The relationship of the CHS genes was resolved through a phylogenetic tree (Figure 3) and proved to be consistent with the Dikarya species tree previously reported (Riquelme and Bartnicki-GarcÌa 2008). However this work also includes early diverging lineages and further resolves the history of the CHS gene family to all the fungal phyla. All seven classes of CHS appear

as well-supported phylogenetic clades in the phylogeny (the homologs of the well curated species are labeled with the class number for reference). Consistent with the previous studies of the family (Roncero 2002; Ruiz-Herrera, Gonzalez-Prieto et al. 2002; Riquelme and Bartnicki-GarcÌa 2008), I was able to conclude from the phylogenetic tree that Classes I and II are sister to Class III and branch first in the Division 1 family. Similarly Classes V and VII are sister with Class IV as the earliest branch in the Division 2. Class VI is on its own branch apart from both the divisions supporting the notion that it is a completely different division (Division 3) of Chitin synthase.

The most drastic feature is the presence of fewer types of CHS classes in the early diverging clades. Whereas most of the Dikarya clades (with the exception of yeast forms) have 7 distinct classes of CHS, most of the early diverging fungal clades possess only about 1 to 5 classes. Another unique feature is the presence of independent gene expansions arising from species-specific duplication in the clades- neocallimastigomycetes, chytridiomycetes, blastocladiomycetes, kickxellomycetes, entomophthoromycetes and mucormycetes. Species from these clades all contain copies ranging from 10-40, whereas other clades- Microsporidia, Cryptomycota and the Dikarya contain only 1-10 copies (Table 1).

I examined these differences in more detail by the three specific divisions (Table 2). The Division 1 has been shown to contain classes I, II and III in the Dikarya (Riquelme and Bartnicki-GarcÌa 2008). I observed that the microsporidia and cryptomycetes don't

contain any homologs in this division. The neocallimastigomycetes, chytridiomycetes, blastocladiomycetes, kickxellomycetes, entomophthoromycetes and mucormycetes contain homologs in a single I-II-III ancestral class as opposed to three distinct classes in the Dikarya. The multiple gene expansion of this ancestral class in blastocladiomycetes and mucormycetes are worth noting. In Division 2, the Dikarya species typically contain classes IV, V and VII (Riquelme and Bartnicki-GarcÌa 2008). The Microsporidia contain only class IV homologs; whereas cryptomycetes, neocallimastigomycetes, chytridiomycetes and blastocladiomycetes possess homologs in class IV and a V-VII ancestral class. The clades Kickxellomycota, Entomophthoromycota and Mucormycotina contain homologs in all three classes- IV, V and VII, similar to the Dikarya. I found that the class IV has homologs in at least two sub-classes for all the clades except the microsporidia and ascomycetes (that only possess one homologs in only one subgroup); I named these as IV a and IV b. In addition, blastocladiomycetes and mucormycetes contain homologs in a potentially novel class VIII that clusters distinctly away from the other three classes in Division 2. The Division 3 was known to possess homologs from the Pezizomycota subgroup of the Ascomycota clade (Riquelme and Bartnicki-GarcÌa 2008) which is consistent with Class VII homolog clustering away from all the other classes in the tree (Figure 3).

By parsimony inference of the tree as shown in Figure 4, I was able to deduce that there were only two classes of CHS in the fungal ancestor, the class IV (containing the Chitin Synth_2 and Cytochrome-B5 domains), comprising of two subclasses IV a & IV b and

the ancestral class V-VII (containing additional Myosin head domain). However one of the subclasses IV b was lost when the microsporidia diverged and then again in ascomycetes, as I was not able to find any homologs of class IV b in these two clades. Also the class V-VII seems to have been lost when the Microsporidia diverged. Later after the divergence of kickxellomycetes, this ancestral class further split into separate classes- V and VII. The ancestral class I-II-III was gained after the divergence of neocallimastigomycetes and then further split into three separate classes- I, II and III after the divergence of Dikarya. There seems to have been an independent gain of class VI in the pezizomycete subgroup of the ascomycete clade. Another independent gain is the ancient and conserved synteny of the class V and VII genes in all pezizomycete lineages. From the CHS species tree (Figure 3) I observed the V and VII genes in *N. crassa* (NCU04352 and NCU04350 respectively) were both adjacent in the phylogeny and on the chromosome. Further inspection of all the major sequenced pezizomycotina lineages including *B. cinerea*, *C. immitis*, *F. oxysporum*, *M. grisea* and *S. nodorum* confirmed that these genes (Class V and VII) have remained adjacent (Figure 4) for at least 200-300 Mya (Taylor and Berbee 2006).

Additional scrutiny of the CHS tree revealed some lineage-specific features as well (Figure 3). Phylogenetic analysis helped clarify the correct Division II assignments in *N. crassa*. Its worth noting that NCU04350 which had been previously assigned to Class V (Riquelme and Bartnicki-GarcÌa 2008), was found to cluster in class VII in this analysis with high bootstrap support, and NCU04352 is a member of the Class V cluster

(and syntenic conserved with other Pezizomycotina) while it was not previously classified. Instead NCUXXXX/XP_323705 (this is a truncated version of NCU04352) was described as a class VII CHS (Riquelme and Bartnicki-GarcÌa 2008) however, it lacks a myosin domain that is a characteristic of all the members of the class V and VII suggesting that the proper V and VII assignments are NCU04352 and NCU04350 respectively.

Also, it is notable that UM10367, which groups in class VII, lacks a myosin domain. This lack of a myosin domain in the class VII genes seems to be a common characteristic of basidiomycetes, as same is the case with *C. neoformans* and *P. chrysosporium* (data not shown) and is very different from the ascomycetes. This implies that the myosin domain was lost in the basidiomycete lineage after splitting from ascomycetes, which may also have coincided with the loss of adjacency of the class V and VII copies in the basidiomycetes. Further examination of early diverging basidiomycete lineages may help clarify the order of these events.

Chitin deacetylase

I observed a similar pattern of gene duplication of the chitin deactelyase gene family as chitin synthase. The species belonging to the clades- neocallimastigomycetes, chytridiomycetes, blastocladiomycetes and mucormycetes contain copies ranging from 17-44 (exceptions are kickxellomycetes (0) and entomophthoromycetes (3)) and the microsporidia, cryptomycetes and the dikarya contain fewer i.e. 1-10 copies (with the

exception of *Coprinopsis cinerea* which has 17 copies). The phylogenetic tree (Figure 6) represents the evolutionary relationships between these homologs. I observed two broad clusters, both containing homologs from all the clades. The only exception is the chitin deactelyase homologs of *Piromyces,* which show a huge expansion in one cluster and missing in the other. Other clades showing these vast gene expansions are blastocladiomycetes and chytridiomycetes. *N. crassa* contains five putative CDA homologs, out of which the two that are annotated as chitin deacetylase (NCU09582 and NCU09508) at the broadinstitute.org are present in one cluster along with a putative homolog that also contains a CBM18 domain (NCU10651) and the other two putative show up in the other cluster. By parsimony I concluded that the fungal ancestor had two copies of CDA and as a result of species-specific duplications, there was expansion in the copy numbers of the two CDA classes.

Chitinases

The clades blastocladiomycetes, kickxellomycetes and entomophthoromycetes contain multiple copies i.e. 11-22 of chitinase whereas all the other clades contain fewer, about 1-8 (*N. crassa* is an exception as it possesses 12 copies) (Table 1). The Microsporidia contains only the CTS I homologs (containing the GH_19 domain), whereas all the other clades contain only CTS II homologs (containing the GH_18 domain) (Table 3). Cryptomycota is the only clade that contains homologs of both CTS I and CTS II. I observed that the homologs belonging to the CTS I and CTS II do not cluster as evolutionarily separate clusters as would have been expected (Figure 7). I noticed huge

gene expansions in the clades- kickxellomycetes and entomophthoromycetes. By manual parsimony (Figure 4), I was able to infer that the fungal ancestor had both CTS I and CTS II genes and the microsporidia clade lost the CTS II gene whereas the CTS I gene was lost after the divergence of neocallimastigomycetes.

β-1,3-glucan synthase

The tree showing the evolutionary relationship of β-1,3-glucan synthase (FKS1) homologs is depicted in Figure 8. Most noticeable feature about the glucan synthase gene family is that there are no significant expansions in any of the fungal species as seen in chitin synthase. More surprisingly, I observed Zygomycota that there are no FKS1 homologs present in the early clades- Microsporidia, Cryptomycota, Neocallimastigomycota, Chytridiomycota. However, beginning with the Blastocladiomycota branch, the "Zygomycota" lineages and the Dikarya, the presence of 1,3-beta glucan synthase is detected. This suggests that this gene was gained in the fungal kingdom at the same time the Blastocladiomycota diverged. Whereas evidence from the past literature suggests that mucormycetes do not contain any glucans (Bartnicki-Garcia 1968), in the present study I found homologs having >90% similarity to *S. cerevisiae*. I also found strong homologs present in another mucormycete *R. oryzae* (data not shown).

Exo-β-1,3 –glucanase

I find no homologs of EXG in the early clades- Microsporidia, cryptomycetes, neocallimastigomycetes, chytridiomycetes, which is expected, as these clades do not contain β-glucans. I was able to find homologs of EXG in all the later diverging clades except blastocladiomycetes (Figure 9). Again, not unlike β-1,3-glucan synthase, I noticed considerably less gene expansions as compared to chitin metabolism genes, for instance kickxellomycetes, entomophthoromycetes, mucormycetes and ascomycetes contain copies ranging from 2-6 (Table 1). However basidiomycetes- *C. cinerea* and *U. maydis* contain 16 and 8 copies of EXG1 respectively. Phylogeny analysis suggests that the EXG1 gene was acquired by the fungi kingdom after kickxellomycetes diverged.

DISCUSSION

Overall, the main cell wall gene families are remarkably conserved. Although this is not a novel finding, the patterns that emerge provide some clues about the specialization in some fungi. This information helps us to predict what the ancestral fungal cell wall might have been like and how it underwent change to attain the current stage. Worth noting are the genes missing in the early clades and the multiple expansions of gene families in the early diverging clades. I was also successfully able to reconstruct the evolution of specific gene families of chitin and glucan metabolism ranging from the most primitive fungi to the recently diverged clades. Identification of expansions/contractions of protein families along with gain/loss of specific genes in

fungi with diverse ecological roles can aid in understanding relationships between function and phylogeny.

Chitin

Chitin synthase

Chitin synthases are amongst the very promising targets for antifungal drug development because of their active roles in maintaining the integrity of the fungal cell walls. Recent fluorescent tagging studies have shown four of the seven *N. crassa* chitin synthases (CHS-1, CHS-3, CHS-5 and CHS-6) localized at the Spitzenkorper core of the growing fungal hyphae ((Riquelme, Bartnicki-Garcia et al. 2007; Riquelme and Bartnicki-GarcÌa 2008), M. Riquelme personal communication), suggesting an active role of chitin synthase in hyphal tip growth. There is a wealth of information available on chitin synthase gene family in higher fungi like *S. cerevisiae, N. crassa* and *A. nidulans,* however the present report is the first that comprehensively describes the chitin synthase gene family in early fungi using highly sensitive profile HMMs. In order to identify and compare all CHS among fungi a single HMM was used, and to identify exact copy numbers of Division 1 and Division 2 in individual species the Division-specific HMMs were used. Based on the phylogeny and manual parsimony, I report that there were 2 main classes of chitin synthase (CHS) in the fungal ancestor- Class IV and Class V-VII. This study was also able to discover two subclasses of class IV and a novel division 2 class VIII which had not been uncovered in the past phylogenetic

analysis (Riquelme and Bartnicki-GarcÌa 2008; Ruiz-Herrera and Ortiz-Castellanos 2009) owing to the non-availability of the genomes of the early diverging clades at that time.

The fact that the microsporidia, cryptomycota, chytridiomycetes and mucormycetes possessed fewer classes of CHS suggests that the cell wall of these primitive organisms was structurally simpler than what is observed in the Dikarya fungi. The early fungal lineages required fewer types of genes to carry out the biosynthesis and remodeling. This indicates that as the fungi evolved from the early unicellular to the recent multi-cellular fungal forms, they acquired additional genes for performing more complex functions. The absence of division 1 genes in microsporidia and cryptomycetes suggests that these primitive fungi did not need the chitin synthase 1 domain.

I noticed an interesting pattern of gene expansion in the chitin synthase family- the most early diverging species such as those belonging to Microsporidia and Cryptomycota and the recently divereged Dikarya possess very few copies of the chitin synthase. However the species belonging to the Neocallimastigomycota, Chytridiomycota, Blastocladiomycota, Entomophthoromycota and 'Zygomycota' possess a large number of independent gene expansions. This implies that the size of the chitin synthase gene family has been highly dynamic throughout evolution. Gene duplication is an important process that can contribute to the evolution of novel functions. However, the mechanisms that govern the fate of duplicated genes are not very well understood. Recent progress suggests that stress-related genes frequently are exposed to duplications

and losses, while growth-related genes show selection against change in copy number (Wapinski, Pfeffer et al. 2007). This makes sense in relation to chitin synthase gene family, as this gene family is an important part of the cell wall biogenesis and regulation.

Also I found that the myosin domain containing chitin synthase class V-VII is present in all the fungal clades except Microsporidia. In a recent study the myosin (myo) gene was also found to be lacking in the Microsporidia genome (Mast, Rachubinski et al. 2012). Why the Microsporidia fungi did not need myosin domain is an open question and is worthy of exploring. It is possible that myosin was one of the many components that were shed as a part of the major genome reduction that Microsporidia underwent in order to acquire a parasitic lifestyle (Keeling and Fast 2002). It is interesting to speculate the role of myosin domain in the fungal kingdom. Previous studies have shown that the myosin domain plays an important role in the hyphal growth in filamentous and dimorphic fungi (Weber, Gruber et al. 2003). So it is intuitive that genomes of yeasts like *S. cerevisiae* and *S. pombe* are devoid of myosin domain-containing chitin synthase class V and VII genes. Until now the myosin domain had been directly related to the process of hyphal growth and morphogenesis as it had been found in hyphally growing fungal species only. However this study shows that the zoosporic fungi belonging to the cryptomycetes, chytridiomycetes and blastocladiomycetes also need the myosin domain, which suggests that the myosin domain might be playing a broader role in the growth and development of fungi.

The ancient synteny observed in the class V-VII genes of the pezizomycetes suggests that they these genes positioned next to each other on the locus after the divergence of pezizomycotina subgroup of ascomycetes (Figure 3). This could be attributed to a need to co-regulate these genes. There is some evidence to prove this hypothesis in the gene expression data from a microarray study of vegetative growth culture of *N. crassa* in which various time points of the growing hyphal culture were sequenced (Kasuga and Glass 2008). In this study the *N. crassa* CHS class V (NCU04350) and class VII (NCU04352) genes, both appear to be up-regulated (fourfold) at the tip (1 hr) as compared to colony (9 hrs) growth timepoint. This suggests that there might be some co-regulation of these genes occurring in *N. crassa*. Class V/VII is duplicated in mucormycetes, basidiomycetes, and ascomycetes, but only ascomycetes have the syntentic pairs. This could be due to multiple losses of synteny in mucormycetes and basidiomycetes (which seems unlikely) or perhaps the genes came together on the chromosome due to the need for being co-regulated.

Chitin deacetylase

Studies in *S. cerevisiae* have shown that chitosan is important for the protection of the spores from lysis, as the double CDA mutant shows increased sensitivity to digestive enzymes (Christodoulidou, Bouriotis et al. 1996). Recent work in *C. neoformans* have shown that chitosan helps to maintain cell wall integrity (Baker, Specht et al. 2007) and the triple mutant of the three chitin deacetylases- CDA1, CDA2 and CDA 3 is avirulent in a mouse model (Lorina G. Baker Boomhower, unpublished); suggesting that the

predicted chitin deacetylases may prove to be excellent antifungal targets. Hence here I attempt to further our understanding of chitin deacetylase in the early fungi. The presence of two broad evolutionarily distinct groups of Chitin deacetylase homologs suggests that there were two copies of this gene in fungal ancestor. However, the CDA gene family although not as diverse in domain structure and function, shows similar species-specific gene duplication patterns as chitin synthase. These independent gene expansions suggests that a large proportion of cell wall chitin may be getting converted to chitosan, the deacetylated form of chitin in these species. I also observed that *E. bienusi* and *C. reversa* did not contain any CDA homologs, which is suggestive of a lack of chitosan in their cell walls. It would be worthwhile to examine the biochemical compositions of the cell walls of these species to gain more insight into this.

It is worth noting that some of the chitin deactetylases also contain a CBM (chitin binding domain), which is a lectin domain found in proteins from plants and fungi that is involved in the recognition and/or binding of chitin subunits. CBM 14 domain has been implicated in protection of the fungal cell wall from plant chitinases as shown by the work done in tomato pathogen, *Cladosporium fulvum* (van den Burg, Harrison et al. 2006). *N. crassa* contains one CDA homolog having this domain and Piromyces contains 17 such CDA homologs. This along with the unusual expansion in copy number (44 genes in total) suggests that CBM domain containing CDA homologs must have a special function in the gut-dwelling fungus *Piromyces*. It has been shown in a recent study (Bernard Henrissat, unpublished results) that *Piromyces* contains over 400

CBM18 genes, so it is possible that the expansion noticed in the CDA genes could be for the same reason as the CBM18 genes.

Chitinase

Growth and morphological development of fungi makes cell wall remodeling a necessity. Cell expansion and division, spore germination, hyphal branching and septum formation all depend on the activities of chitinases operating in the cell wall (Adams 2004). Chitinases also have aggressive roles as fungal pathogenicity factors during infection of other fungi (mycoparasitism), insects and nematodes (Wattanalai, Wiwat et al. 2004; Duo-Chuan 2006; Gan, Yang et al. 2007). Hence understanding the evolution of chitinases is of great importance. Chitinases of the two families (family GH 18 and family GH 19) do not share amino acid sequence similarity and have completely different 3-D structures and molecular mechanisms and past studies indicate they have arisen from a different ancestor. Whereas Family18 chitinases are found in bacteria, fungi, yeast, viruses, plant and animals, Family 19 members are almost exclusively present in plants (Xie, Jia et al. 2011). A single family 19 chitinase is identified in the bacterium- *Streptomyces griseus* (Watanabe, Kanai et al. 1999). However there have been no reports of a family 19 chitinase in fungi. The present study is the first to report the presence of family 19 in Microsporidia and Cryptomycota (also included in (James, Pelin et al. 2013)). By means of parsimony reconstruction, I was able to show that the fungal ancestor possessed two classes of chitinases- CTSI (GH-19) and CTS II (GH-18). There was a loss of CTS II in the Microsporidia clade and then a subsequent loss of

CTS I after the Neocallimastigomycota diverged that explains the presence of only CTS I in Microsporidia, of both CTS I and II in Cryptomycota and of CTS II in rest of the fungal clades. The chitinases identified in the current study show a trend similar to the other chitin metabolism genes in terms of multiple independent gene expansions, although the copy numbers are comparatively lower than CHS and CDA genes. Notable expansions are of the *C. coronatus* and *C. reversa* which leads to contemplate why did these species need such a high number of chitinase genes.

β-Glucan

β-1,3-glucan synthase & Exo-β-1,3 –glucanase

β-1,3-glucan machinery has been studied mostly in the Dikarya fungi like *S. cerevisiae, N. crassa, A. fumigatus* and *C. neoformans* but no reports are available about the early fungal species. Since β-1,3-glucan has been showed to be an important part of these cell walls, its only imperative that we find out more about its existence in the early fungal cell walls. Present study fills this gap in the present knowledge and shows clearly using phylogeny and manual parsimony reconstruction that β-1,3-glucan synthase (FKS1) was gained after the divergence of the chytridiomycetes. FKS1 is a long protein (1600+ amino acids) and it appears to be well conserved from *S. cerevisiae* to *A. macrogynus* (65% similar). This suggests that this protein was gained once after the divergence of chytridiomycetes and has been strongly conserved since then. Thus the presence of FKS1 in Blastocladiomycota, Mucormycota and Dikarya is as a shared derived

character that helps to group these fungi to the exclusion of Chytridiomycota- a phylogenetic node that is still ambiguous. Similarly the exoglucanse (EXG1) gene seems to have been acquired after the zygomycetes diverged. Since this gene is absent in the blastocladiomycetes (that do contain FKS1), there must be other genes responsible of degradation of β-1,3-glucan in the cell walls. It is worth exploring where fungi acquired these genes for β-Glucan metabolism from- bacteria, algae, lichens or plants?

Also interesting is to speculate why the early fungi did not need β-glucan in their cell wall. Since we know that β-1,3-glucans serve as a major structural component of the fungal cell wall, it is likely that the early fungi possessed alternative polysaccharides. I explore this question in the next chapter where the cell walls of early fungi are biochemically profiled. Another possibility is that their cell walls were not as elastic in the early Fungi and the need for elasticity evolved later as a consequence of changes in lifestyle or ecological niche such as the transition from aquatic to terrestrial. In *S. cerevisiae*, a GFP-tagged FKS1 was shown to localize to areas of polarized growth (Utsugi, Minemura et al. 2002). In *N. crassa*, FKS1 has been shown to be delivered through the Spitzenkorper to the growing hyphal tip (Verdin, Bartnicki-Garcia et al. 2009). This suggests that FKS1 is clearly involved in the active growth processes so then why do the primitive fungal clades- Microsporidia, Cryptomycota and Chytridiomycota not need this gene in their cell wall? One reason could be that since these fungi were pre-dominantly parasitic in nature their cell wall did not need to

synthesize many complex components to survive as they could just utilize their hosts' resources. One thing that is evident is that after the fungi gained the β-1,3-glucan it gave rise to dramatic changes in the construction and strength of cell wall in Fungi that allowed successful colonization of new niches in an unprecedented manner.

SUMMARY

Fungi and Animals shared a common ancestor that was most likely aquatic and flagellated unicellular organism, but evolved distinct cellular forms. The acquisition of a protective cell wall in Fungi was a major innovation that was likely part of the transition from aquatic to terrestrial. The cell walls of the early fungi differ in composition from the Dikarya fungi, comprising only chitin and carbohydrates, but no beta glucans. A key change was the gain of the FKS1/ β-1,3-glucan synthase. Here I was successfully able to demonstrate the timing of when FKS1 was acquired in Fungi. The early fungi also possessed fewer types of chitin synthase and higher number of gene expansions in a class. As the Dikarya clade evolved genes became more specialized and less duplicated. I was also able to discover new subgroups of class IV and a new class VIII in division II of chitin synthase. Also worth noting is the presence of a novel family of chitinases (glycosyl hydrolase 19) in the cell walls of early fungi.

The methodology presented in this study to find homologs using comparative genomics and phylogenetics is generally useful and can be rapidly and automatically applied to new genome sequences to identify the conserved cell wall process genes in additional

species. I expect this can improve the ease of studying the genetic diversity of various fungal cell wall components in future studies. The pipeline thus developed, to find homologs using comparative genomics and phylogenetics, can be applied to other species as and when they are sequenced to identify new cell wall homologues in those genomes. This can prove to be a useful tool to study the genetic diversity of various fungal cell wall components for the future studies. The results from this study can be corroborated by examining the biochemical compositions of the cell walls of the representative species from the clades. I attempt to accomplish this in the next chapter.

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Figure 1 Major phyla and subphyla of fungi examined in the present study (enclosed in grey box). The color scheme used to represent the different phyla are used throughout the study to link species to their corresponding clades.

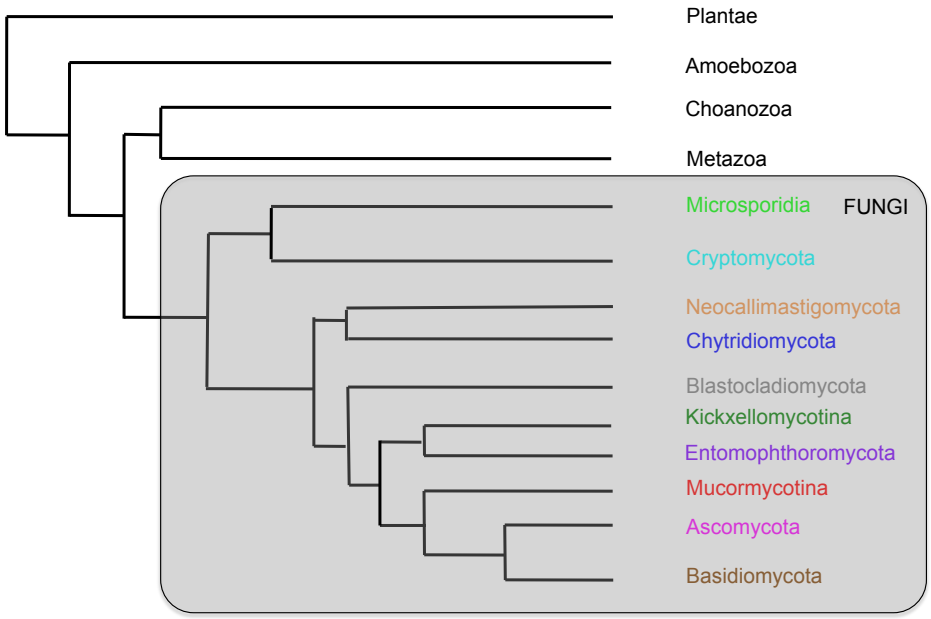

Figure 2. Domain structures for the three divisions of Chitin synthase gene.

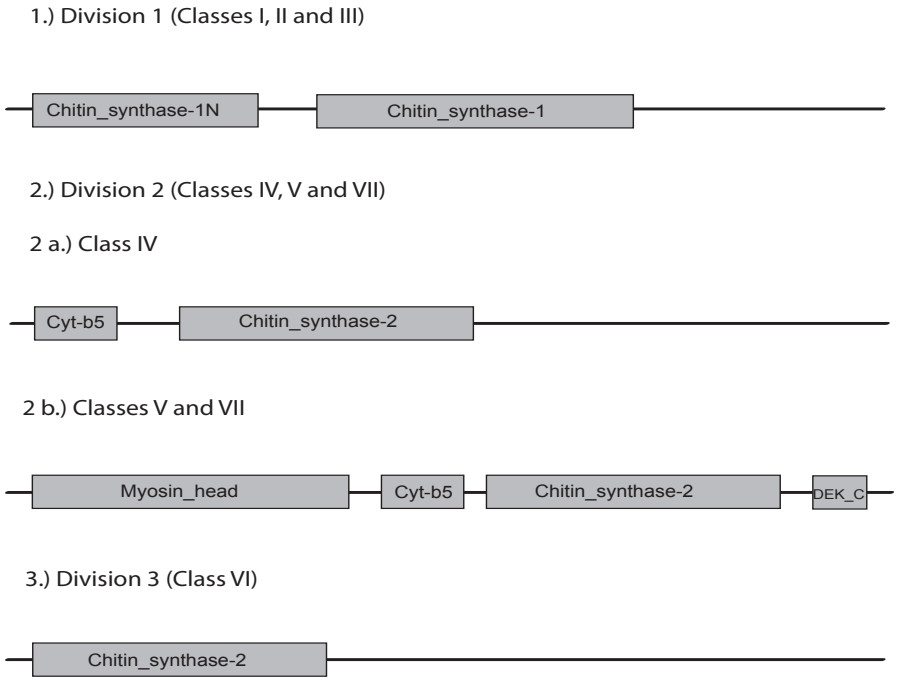

Figure 3 Phylogenetic tree of chitin synthase genes in examined species, rooted by CHS class VI built using Raxml with VTF as protein substitution model. Clades containing Division 1 (Classes I, II, II) and Division 2 (Classes VI, V, VII) and Division 3 (Class VI) are labeled. The new subclasses IV a and b and class VIII identified in this study have been marked with an asterisk. Coloring scheme of the species is consistent with the clades defined in Figure 1.

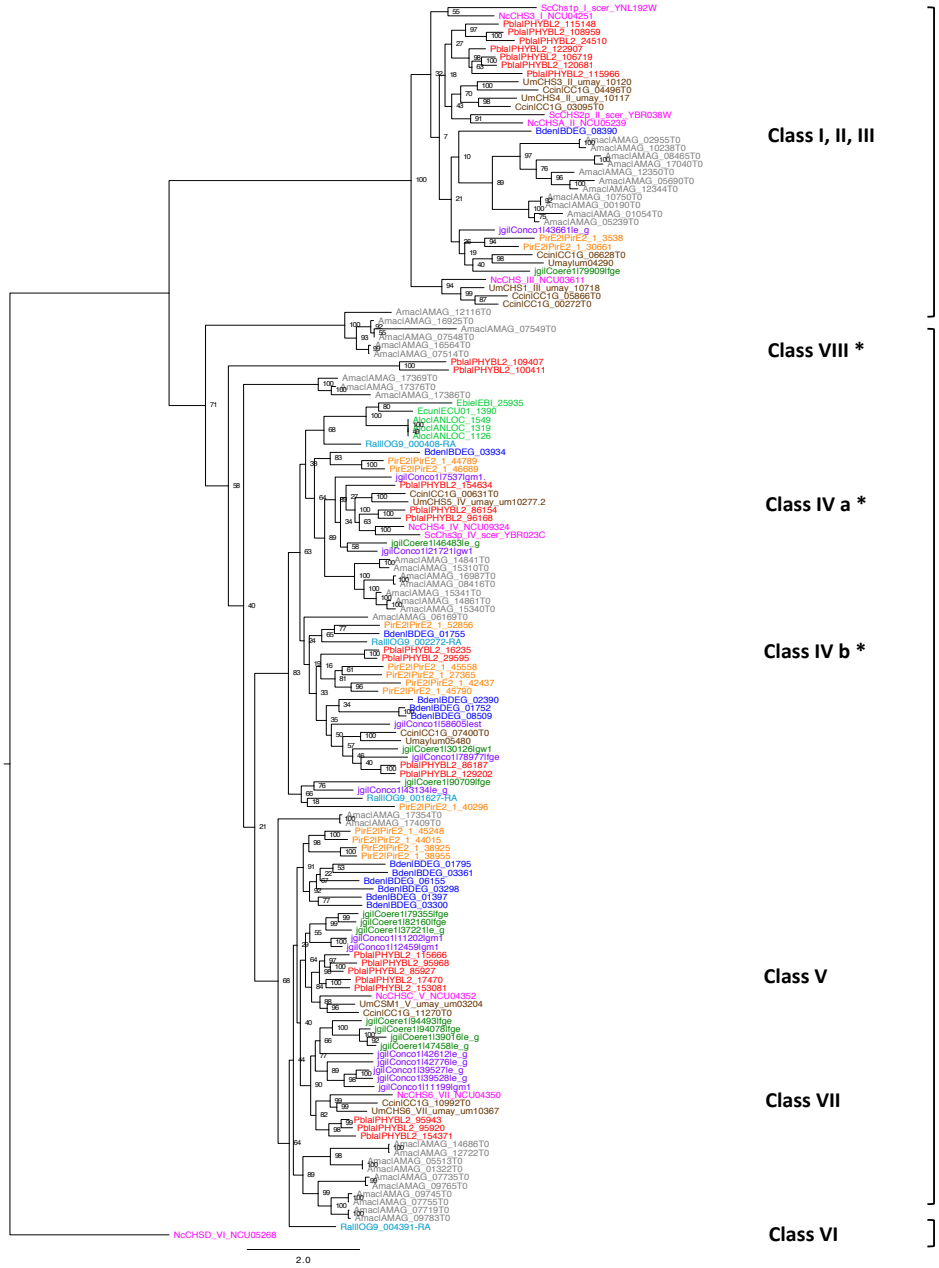

Figure 4 Reconstruction of evolution of the chitin and glucan metabolism genes using manual parsimony.

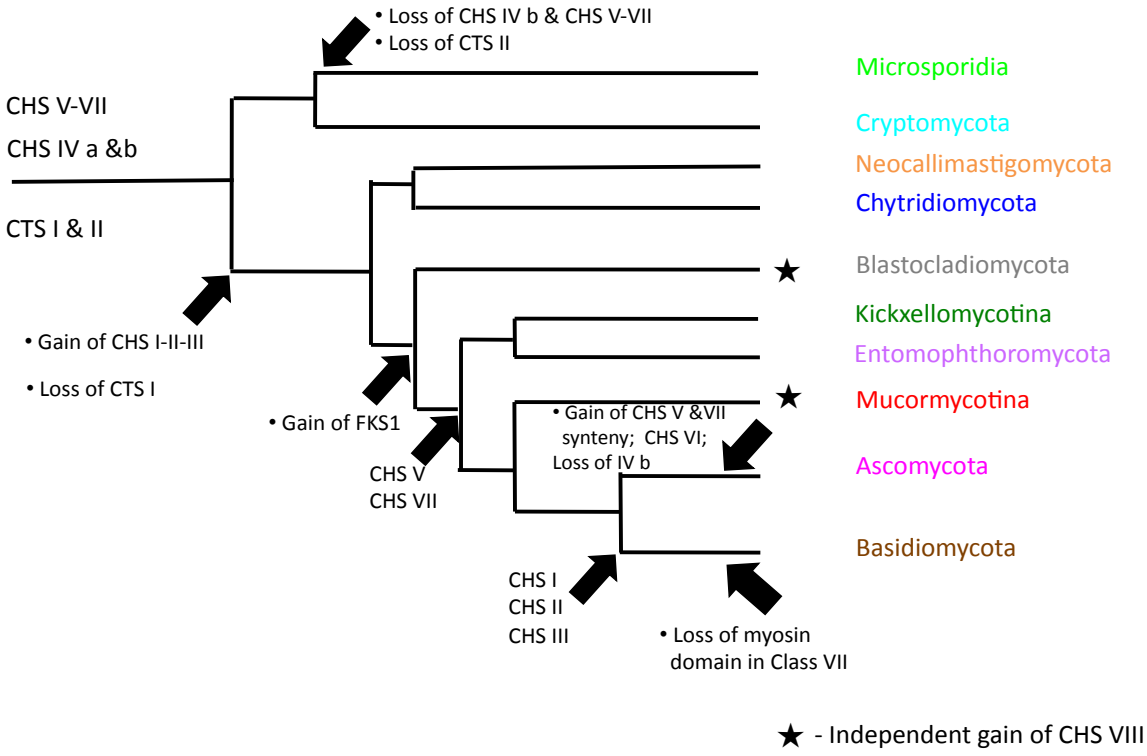

Figure 5 Phylogeny of the syntenic class V and VII class chitin synthases of *N. crassa* and other pezizomycetes- *Aspergilus nidulans, Coccidiodes immitis, Botrytis cinerea, Fusarium oxysporum, Magnaporthe grisea and Stagnospora nodorum*, rooted by the class IV chitin synthase from *A. nidulans*.

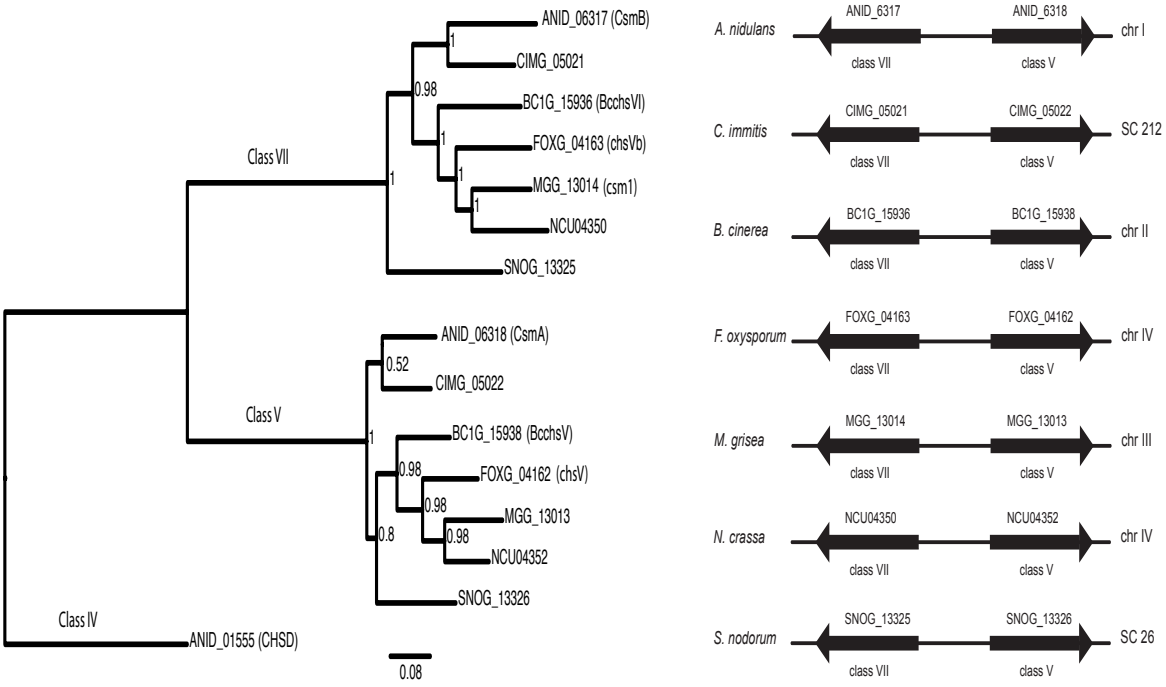

Figure 6 Midpoint rooted phylogenetic tree of chitin deacetylase genes in examined species built using Raxml with JTT as protein substitution model.

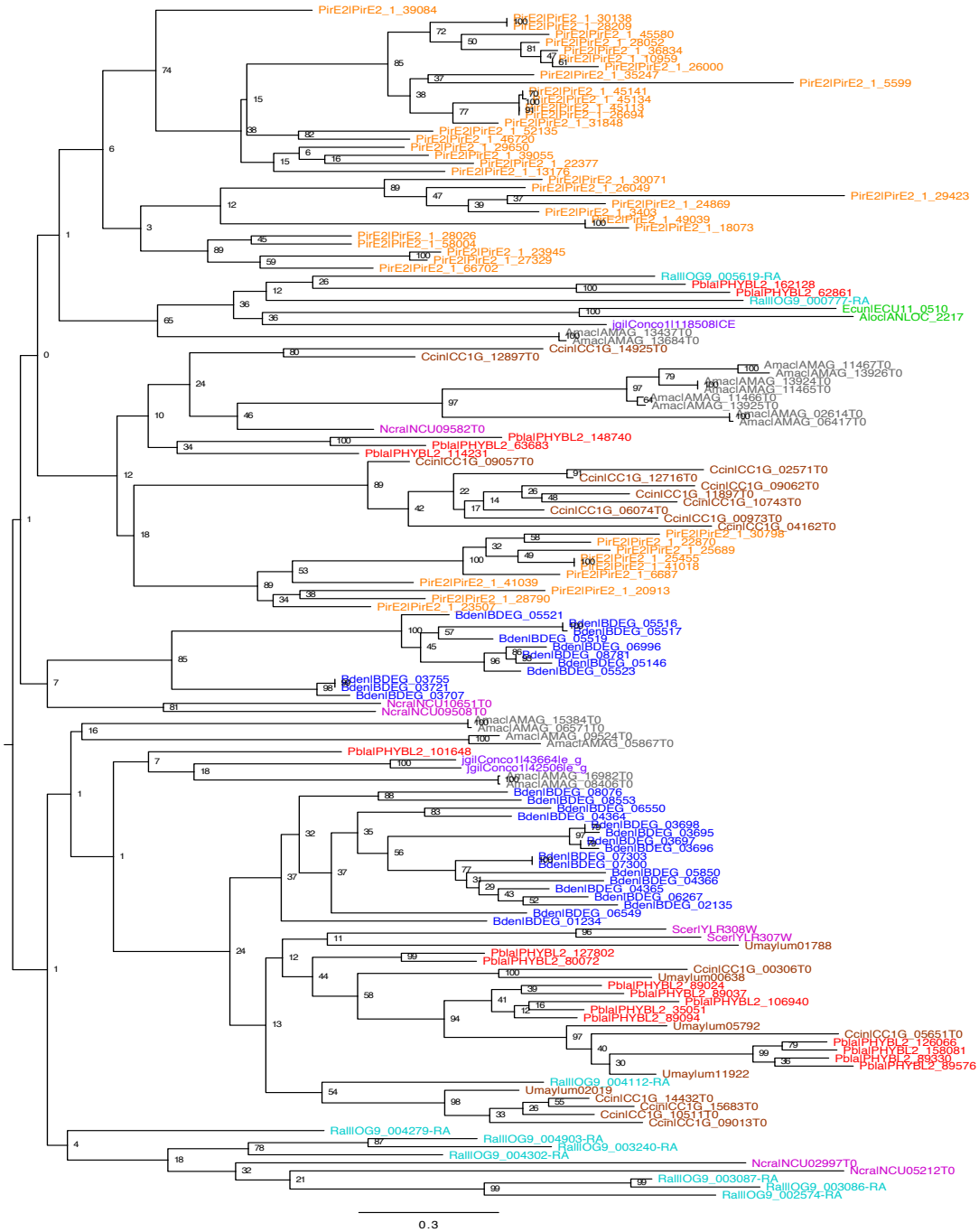

Figure 7 Midpoint rooted phylogenetic tree of chitinase genes in examined species built using Raxml with DAYHOFF as protein substitution model. The circled homologs belong to CTS class I (containing GH19 domain) and rest are CTS II (containing GH 18 domain)

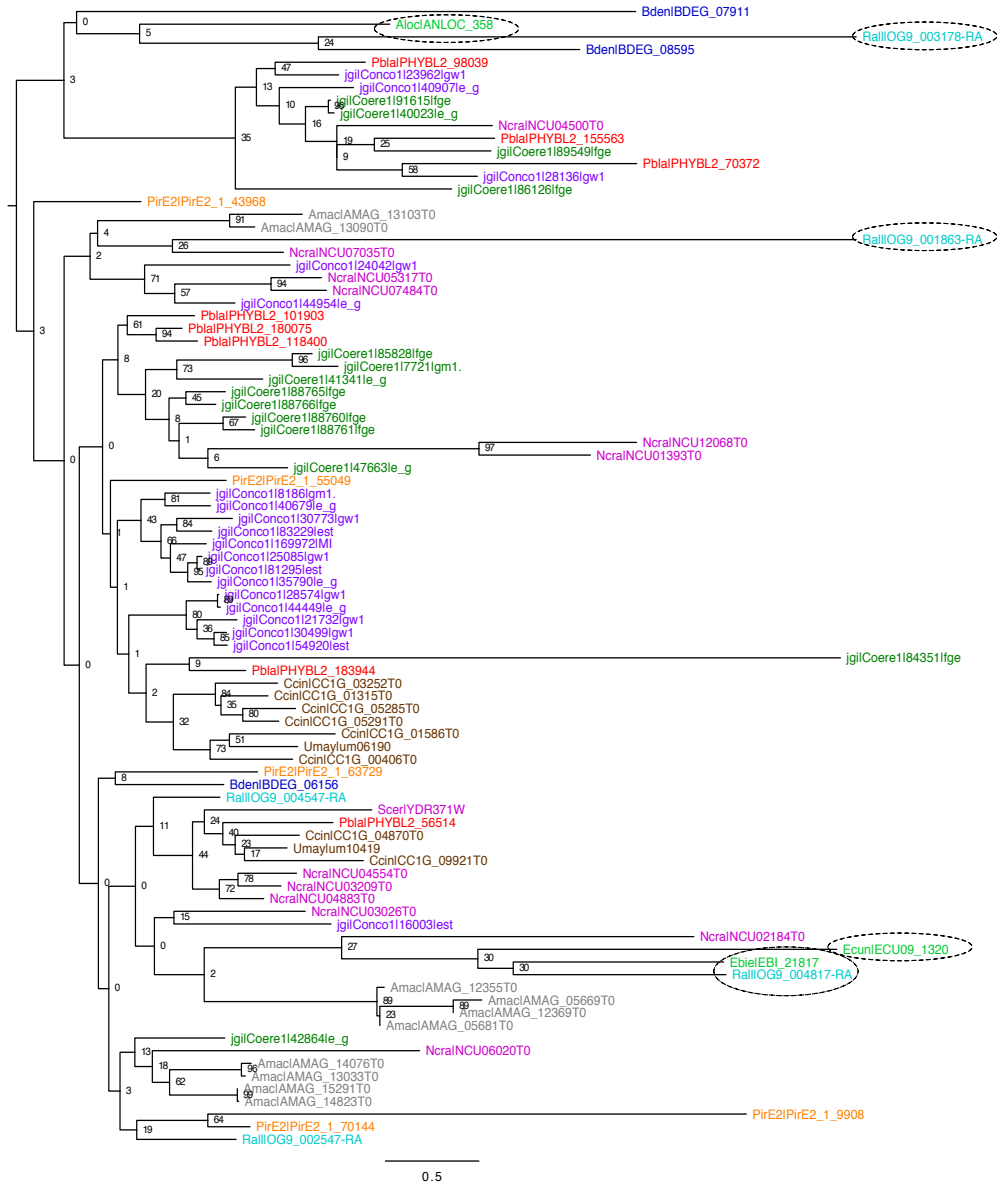

Figure 8 Midpoint rooted phylogenetic tree of β-1,3-glucan synthase genes in examined species built using Raxml with LGF as protein substitution model.

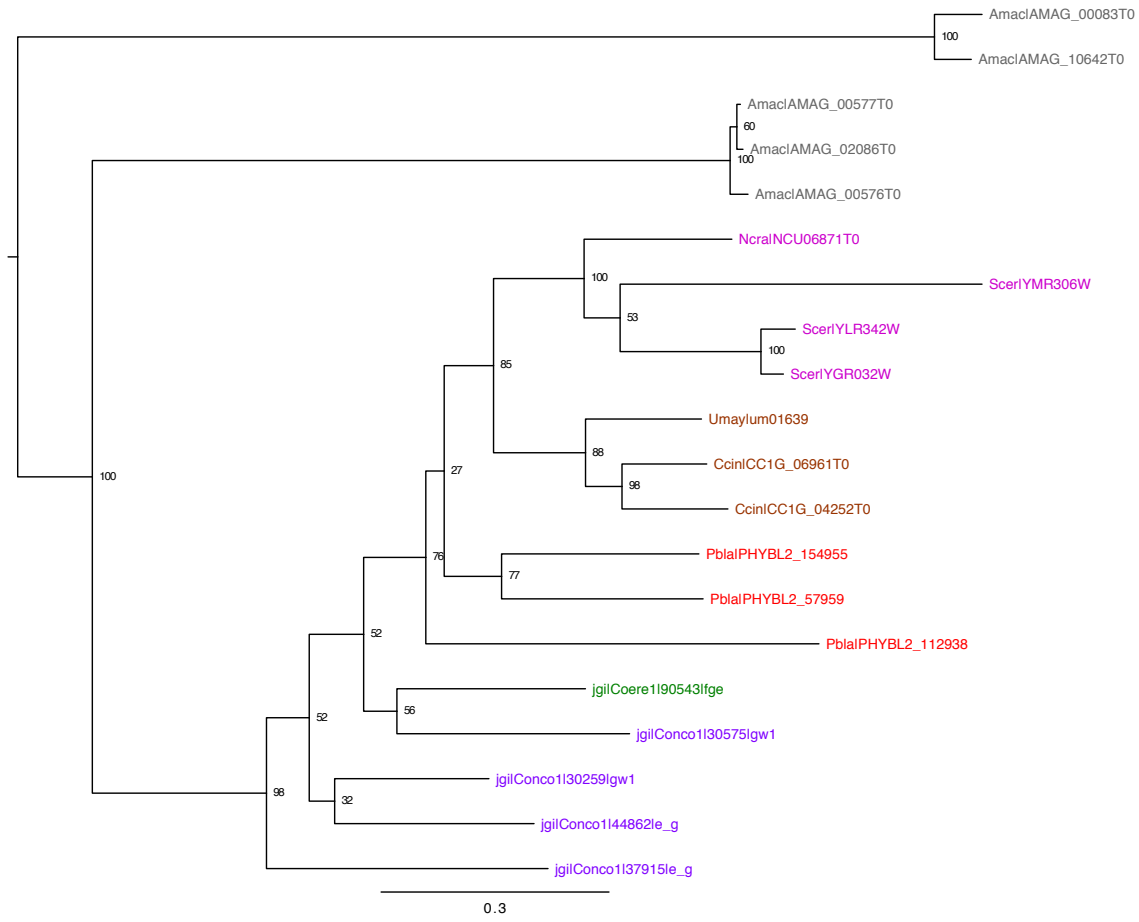

Figure 9 Midpoint rooted phylogenetic tree of exo- β-1,3-glucanase genes in examined species built using Raxml with JTT as protein substitution model.

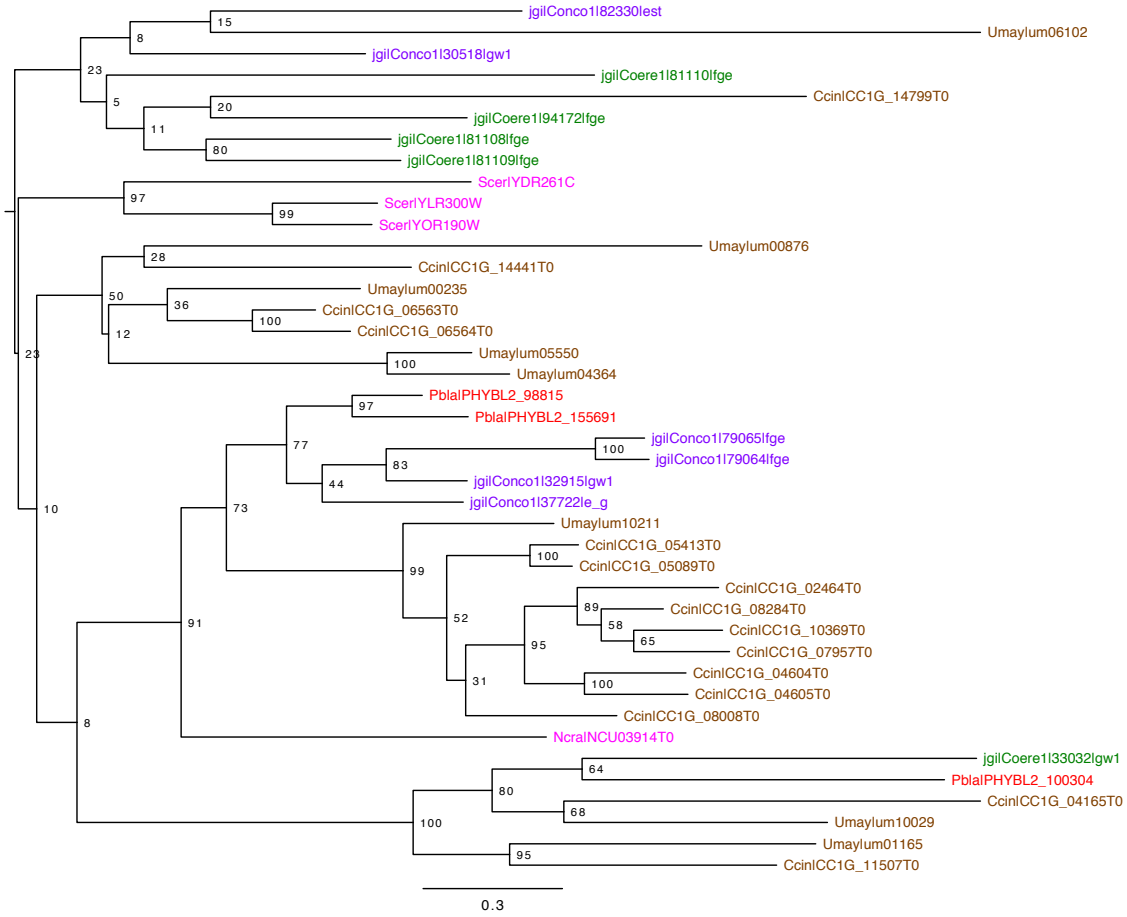

Table1 Distribution of copy number of chitin & glucan metabolism gene homologs in species examined in this study. The coloring scheme of the species is consistent with Figure 1.

Species	CHS	CDA	CTS	FKS1	EXG
Antonospora locustae	3	1	1	0	0
Enterocytozoon bieneusi	1	0	1	0	0
Encephalitozoon cuniculi	1	1	1	0	0
Rozella allomycis	4	10	6	0	0
Piromyces sp.	14	44	6	0	0
Batrachochytrium dendrobatidis	15	28	3	0	0
Allomyces macrogynus	41	18	11	5	0
Coemansia reversa	11	0	14	1	5
Conidiobolus coronatus	13	3	22	4	6
Phycomyces blakesleeanus	24	17	8	3	3
Ustilago maydis	8	6	2	1	8
Coprinopsis cinerea	9	17	8	2	19
Neurospora crassa	7	5	12	1	2
Saccharomyces cerevisiae	3	2	1	3	3

Table 2 Distribution of copy number of CHS homologs belonging to different classes in the species examined in this study. * indicates the new classes discovered in this study.

DIVISION	1	1	1	2		2	2	2	3
CLASS	I	II	III	IV* a	IV* b	V	VII	VIII*	VI
Antonospora locustae	0	0	0	3	0	0	0	0	0
Enterocytozoon bieneusi	0	0	0	1	0	0	0	0	0
Encephalitozoon cuniculi	0	0	0	1	0	0	0	0	0
Rozella allomycis	0	0	0	1	2	1 (V-VII)		0	0
Piromyces sp.	2 (Div1)			2	6	4 (V-VII)		0	0
Batrachochytrium dendrobatidis	3 (Div1)			1	4	6 (V-VII)		0	1
Allomyces macrogynus	11 (Div1)			7	1	15 (V-VII)		7	0
Coemansia reversa	1 (Div1)			1	2	3	4	0	0
Conidiobolus coronatus	1 (Div1)			2	3	2	5	0	0
Phycomyces blakesleeanus	7 (Div1)			3	4	5	3	2	0
Ustilago maydis	1	2	1	1	1	1	1	0	0
Coprinopsis cinerea	1	2	2	1	1	1	1	0	0
Neurospora crassa	1	1	1	1	0	1	1	0	1
Saccharomyces cerevisiae	1	1	0	1	0	0	0	0	0

Table 3 Distribution of copy number of CTS homologs belonging to the two classes in the species examined in this study

Species	CTS I (GH_19)	CTS II (GH_18)
Antonospora locustae	1	0
Enterocytozoon bieneusi	1	0
Encephalitozoon cuniculi	1	0
Rozella allomycis	3	2
Piromyces sp.	0	4
Batrachochytrium dendrobatidis	0	1
Allomyces macrogynus	0	11
Coemansia reversa	1	12
Conidiobolus coronatus	0	20
Phycomyces blakesleeanus	0	8
Ustilago maydis	0	2
Coprinopsis cinerea	0	8
Neurospora crassa	0	12
Saccharomyces cerevisiae	0	1

CHAPTER III

Comparative biochemical and genomic analysis of cell walls of early fungi reveals unique carbohydrate composition.

ABSTRACT

The present study explores the polysaccharide composition in cell walls of evolutionary distinct, early diverging fungi by comparing species from the early diverging phyla Chytridiomycota, Blastocladiomycota, and Mucormycota with Dikarya fungi. I used biochemical profiling by cell wall fractionation and Gas Chromatography-Mass Spectroscopy to assess the proportions of polysaccharides in cell walls. These results were examined in context of comparative genomics analyses to identify the genes responsible for the specific polysaccharide biosynthesis. The key findings of this study include distinct differences in cell walls of early diverging fungi from the rest of the fungal kingdom. The blastocladiomycete cell walls were found to be similar to ascomycetes i.e. glucan-rich and poor in chitin. Whereas chytridomycetes have a chitin-rich and glucan-poor cell wall, with mannose being the most substantial component. The mucormycetes were the most unique having the heteropolymer- 'mucoran' as the largest fraction, followed by chitin and reduced glucan composition compared to ascomycetes. My study is the first to report that cell walls in the Chytridiomycota

contain no 1,3 beta-glucan. I also demonstrate there is not a direct correlation in proportion of polysaccharides in the cell wall content with the number of copies of biosynthesis gene.

INTRODUCTION

The cell wall is essential for growth and propagation of fungi. The acquisition of hyphal growth in the early diverging lineages was enabled by changes in the localization and control of cell wall biosynthesis components (Harris, Read et al. 2005). The significance of understanding the fungal cell wall structure and composition cannot be emphasized enough, yet the amount of work done to characterize cell walls of evolutionarily distinct species has been far from adequate. The first and the only systematic study of cell wall composition in multiple fungal species presents the taxonomic classification of the fungal kingdom into seven groups based on the cell wall composition of the species (Bartnicki-Garcia 1968). At least 3 of these taxonomic groups are not valid anymore, as they included cellulose as one of their distinguishable features (Group I consisting of Cellulose/Glycogen, presently in the Amebozoa division; Group II composed of Cellulose/Glucan, oomycetes now within the Chromalveolata kingdom and Group III-Cellulose/Chitin, the (previously grouped) Protist group). Mucormycetes were included as group IV, consisting of Chitosan/Chitin. Two of the groupings have remained as the general concept of what a fungal cell wall is: group V (Chitin/Glucan, including ascomycete and basidiomycete species) and group VI (Mannan/Glucan, including the yeasts). The present study revisits this

classification by using modern biochemical characterization techniques and presents evidence that cell wall structure of the evolutionarily distinct fungal species is much more complex and varies dramatically in polysaccharide composition.

Chitin a crystalline polymer is a minor component of the fungal cell wall but is extraordinarily strong, hence is known to be a characteristic stress-bearing polysaccharide (Roncero 2002; Klis, Ram et al. 2007). The β-1,3-glucan serves as the main structural constituent acting as a scaffold for covalent linkage of other cell wall components. Other glucans including β-1,6-, α-1,3- and α-1,4- linked glucans are also found in most species, though the proportions vary (Bowman and Free 2007). Other more monosaccharides make up a small proportion of the cell wall and are found in similar distribution among examined fungi. These include Mannose, D-galactose and N-galactosamine (Ascomycetes), L-fucose (Mucormycetes and Basidiomycetes), D-glucosamine (Mucormycetes), xylose (Basidiomycetes), and D-glucuronic acid. Occasionally, the presence of small quantities of rhamnose (Hamilton and Knight 1962; Novaes-Ledieu, Jimenez-Martinez et al. 1967), ribose (Bartnicki-Garcia and Nickerson 1962; Crook and Johnston 1962; Bartnicki-Garcia 1966; Novaes-Ledieu, Jimenez-Martinez et al. 1967), and arabinose (Johnston 1965) has been reported. The role of these minor sugars is not known but understanding that is another motivation to study the fungal cell wall composition in more detail.

Much of the recent work in fungal cell wall biology has focused on genetic and biochemical work in the ascomycetes *S. cerevisiae, N. crassa* and *A. fumigatus* (Bernard and Latge 2001; Lesage and Bussey 2006; Maddi, Bowman et al. 2009). The *S. cerevisiae* cell wall consists largely of β-1,3-glucan with lesser amounts of β-1,6-glucan. The wall also contains a small amount of chitin, which is found in the bud scar formed during the separation of mother and daughter cells during budding growth. The β-1,6-glucan has been shown to crosslink the β-1,3-glucans together to create a matrix (Klis, Boorsma et al. 2006; Lesage and Bussey 2006). β-1,6-Glucan has also been shown to be associated with the glycoprotein released from the cell wall by laminarinase (β-1,3-glucanase) digestion indicating that the protein is tied into the cell via β-1,6-glucan. The cell wall proteins contain N-linked outer chain mannans with up to 200 mannose residues and smaller O-linked mannans (Kapteyn, Ram et al. 1997; Kollar, Reinhold et al. 1997). α-1,3-glucan, and galactomannans are absent from the *S. cerevisiae* cell wall. The yeast wall is organized such that the outer surface of the wall contains large amounts of the outer chain mannan and glycoprotein, with the glucans and chitin being more concentrated in the portion of the cell wall adjacent to the plasma membrane (Bowman and Free 2006). Recent studies characterizing the *N. crassa* cell wall show the presence of glucose, N-acetylglucosamine, mannose and galactose and a glucosyl linkage analysis showed the presence of large amounts of 1,3 linked glucose (Bowman and Free 2006; Maddi, Bowman et al. 2009). Studies have demonstrated the presence of β-1,3-glucan and the importance of β-1,3-glucan synthase in cell wall

biosynthesis (Taft and Selitrennikoff 1988; Tentler, Palas et al. 1997). α-1,3-glucans have been found in other fungal cell walls and the *N. crassa* genome encodes two α-1,3-glucan synthase genes (NCU02478 and NCU08132), so some of the 1,3 linked glucose could be found as an α-1,3-glucan. Linkage analyses show that cell walls of *N. crassa* are devoid of β-1,6-glucan (Fontaine, Simenel et al. 2000). The *N. crassa* cell wall glycoproteins contain N-linked galactomannan and O-linked galactomannan. The *Aspergillus fumigatus* cell wall has also been extensively studied in the past and major components are β-1,3-glucan, a mixed β-1,3-/ β-1,4-glucan, α-1,3-glucan, and chitin (Beauvais, Maubon et al. 2005; Latge 2007). β-1,6-Glucans are absent from the cell wall. The cell wall proteins are modified by N-linked galactomannans and O-linked galactomannans.

The afore mentioned studies provide an important insight into the cell wall compositions in the Ascomycota but examination of additional lineages of basidiomycetes, mucoromycetes and chytridiomycetes indicates that there is broad diversity in the cell wall structure and composition that cannot be captured in a few reference species. Now with the availability of genomic sequences of the early fungal species, it is the perfect time to undertake a biochemical-comparative genomics study to elucidate the various components of the cell walls of the different fungal species across the kingdom. Here I explore the polysaccharide composition of cell walls of evolutionary distinct, early diverging fungal species by analyzing the species-

Spizellomyces punctatus, Allomyces macrogynus, Rhizopus oryzae and *Phycomyces blakesleeanus* and correlate with the presence of cell wall biosynthesis and metabolism genes through additional annotation of the sequenced genomes. Furthermore I compare and contrast the broad differences in the cell wall compositions between the Chytridiomycetes, Mucormycotina and the Dikarya to characterize the differences between early diverging and the recently diverged fungi.

MATERIALS AND METHODS

Strains and culture methods

All analyses were performed on the mycelial or sporangia cell walls from *S. punctatus* (DAOM BR117), *A. macrogynus* (ATCC 38327), *P. blakesleeanus* (NRRL1555; mating type '-'), *R. oryzae* (RA99880) and *N. crassa* (74-OR23-1VA / FGSC 2489; mating type 'A'). *P. blakesleeanus*, *R. oryzae* and *N. crassa* strains were obtained from the Fungal Genetics Stock Center (http://fgsc.net). A. *macrogynus* and *S. punctatus* were generous gifts from Franz Lang. *R. oryzae* and *P. blakesleeanus* were grown overnight in glass culture tubes on liquid potato dextrose agar (PDA) medium at room temperature. *N. crassa*, was grown in the liquid minimal medium of Vogel (VM) (HJ 1956) under the same conditions. The mycelia were harvested from liquid on Whatman filter paper by vacuum-assisted filtration, lyophilized and stored in sealed vials. A. *macrogynus* and *S. punctatus* were grown on 1% Tryptone (10g/L tryptone and 3.2g/L glucose) agar plates at room temperature for 5 days. The sporangia were harvested by

flooding the plates with water, pouring into vials, spinning down cells in centrifuge, and removing excess water.

Biochemical characterization

Dr Hugo Melida at Royal Institute of Technology, Sweden, performed these procedures. See Appendix 2 for details.

Phylogenetic analysis of cell wall gene families

To perform phylogenetic comparison of gene families, genomes from the chytridiomycetes- *S. punctatus*, *A. macrogynus* (unpublished, Broad Institute (http://broadinstitute.org)*;* mucormycetes *R. oryzae* (version 3; (Ma, Ibrahim et al. 2009)) and *P. blakesleeanus* (JGI: http://genome.jgi.doe.gov/fungi) and the ascomycete *N. crassa* (version 10.5; (Galagan, Calvo et al. 2003)) were used. To identify homologs of cell wall polysaccharides synthesis genes, the Saccharomyces Genome Database (Cherry, Hong et al. 2012) (http://yeastgenome.org) was searched to catalogue the known chitin and glucan synthesis genes in *S. cerevisiae*. For chitin synthases, additional information from previous studies (Choquer, Boccara et al. 2004; Mandel, Galgiani et al. 2006; Riquelme and Bartnicki-GarcÌa 2008) was used to augment the gene set and identify the known CHS families and their typical sequences. I performed a comparative homology search using profile Hidden Markov Models implemented in the HMMER2 package (Eddy 1998) comprised of cell wall genes defined in *S. cerevisiae, N. crassa* and *Ustilago maydis*. Multiple sequence alignments of seed

homologs from these three species were constructed with T-Coffee (Notredame, Higgins et al. 2000) and built into a profile-Hidden Markov Model with HMMER3. HMMER3 was run with global-global comparisons of alignments families. Identified homologs from each genome for each cell wall gene family HMM were identified with hmmsearch and a cutoff bit score more than log of the number of proteins in our database of selected fungal genomes and these typically resulted in expectation values less than 1E-20.

With the aim of finding the genes responsible for polysaccharide biosynthesis and remodeling, I mapped the metabolic pathways of the monosaccharides fucose, mannose, galactose, and glucuronic acid to the genome of *R. oryzae* and *N. crassa* using Fungicyc (http://fungicyc.broadinstitute.org/). I searched the *S. punctatus, A. macrogynus* and *P. blakesleeanus* genomes with hmmsearch to identify homologs of these genes. In addition, for the fucose metabolism genes, a search on the available sequenced fungal genomes yielded homologs in the Mucormycetes *Mortierella alpina* and *M. circinelloides* and in the Basidiomycetes *Puccinia graminis sp. tritici* and *Sporobolomyces roseus*. Additionally, the sequences of fucose related glycosyltransferases genes from *Escherichia coli* (Iguchi, Thomson et al. 2009) were used to search to for any extra homologs in the Uniprot database (http://uniprot.org/). The homologs were aligned with T-Coffee and the resulting protein alignments were automatically trimmed for high quality regions alignments with trimAl using the automated1 parameter (Capella-Gutierrez, Silla-Martinez et al. 2009). Maximum

likelihood phylogenetic trees were constructed with RAxML (Stamatakis 2006) with protein substitution matrix chosen by ProteinModelSelection.

RESULTS

Analysis of cell wall polysaccharides from N. crassa

The cell wall of the ascomycete *N. crassa* by and large represents the general concept of a fungal cell wall in terms of monosaccharide composition with glucose and glucosamine as main components accounting for 73 and 9% respectively (Figure 1(a)). Almost all the glucosamine was found to be 1,4-linked and only a very little amount (≈0.1%) was found to be located at the terminal non-reducing ends of glucosamine-based polysaccharides (Figure 2), pointing to the occurrence of chitin polymers with high degrees of polymerization. A majority of the glucans in the cell wall of *N. crassa* correspond to 1,3-glucans (Figure 2). Small proportions of 1,4-linked glucose (<4%) units were detected (Figure 2). This together with the fact that 1,4,6-linked glucosyl residues were identifiable in the chromatograms (trace amounts) is indicative of the presence of residual glycogen/starch-like polymers, which apparently were not removed after several cycles of enzymatic digestions.

Mannose, galactose and *N*-acetylgalactosamine (GalN) were detected in amounts ranging from ≈9 to ≈4%, and only trace amounts glucuronic acid were identifiable (Figure 1(a)). Mannans were found to be 1,2-linked with many branches in position 6 (1,2,6-Man) (Figure 2). This together with the high proportion of terminal galactose

(both the pyranose (t-Gal*p*) and furanose (t-Gal*f*) forms detected; Figure 2) compared to the low amounts of 1,4-galactosyl units found (4 vs. 1%; Figure 2) suggests that probably mannose and galactose are making up a galactomannan with mannose as internal monosaccharide (1,2-Man), branched at some carbon 6 (1,2,6-Man) holding short galactose branches (1,4-Gal and t-Gal*p*/Gal*f*). The type of glycosidic bonds linking GalNAc could not be accurately determined due to its originally low abundance combined with the lower response factors of amino sugars in the GC/MS columns required for the separation of partially methylated alditol acetates.

Mucormycetes have a distinctive polysaccharide cell wall composition

A clearly distinct picture was found for the mucormycete cell wall monosaccharide composition. In this case glucosamine has a more substantial contribution to the total monosaccharide wall composition of *P. blaskesleeanus* and *R. oryzae* up to 16 and 34% respectively (Figure 1(a)). Again, only 1,4- and terminal-linked glucosamine residues were found via linkage analysis, indicative of chitin-chitosan polymers (Figure 2). A small proportion (≈1-3%) of the glucosamine was detected in the ASF (Figure 1(c)) pointing to the presence of glucosamine-based polysaccharides of higher solubility than chitin, although in a very small amount. On the other hand, 1,3-glucans were also detected but both in *P. blakesleeanus* and *R. oryzae* as a minor cell wall component (≈3-4%; Figures 1 and 2).

The relative low abundance of glucose polymers is apparently counterbalanced by a higher proportion of glucosamine, but the most remarkable feature of this type of cell wall is the presence, and abundance, of fucose and glucuronic acid (Figure 1). Interestingly their relative abundance seems to be paired in each of the species, being 33% and 34% for fucose-glucuronic acid in *P. blakesleeanus* and 20-23% in *R. oryzae*. Although 1,4-linked fucose was detected (≈0.8 to 1.2%), fucose-based backbones were found mostly as 1,3-linked (≈5.5 to 8%) (Figure 2). The 1,3-fucan chains are highly branched at position 2 as indicated by relative similar proportions of 1,3- and 1,2,3-linked fucosyl residues (≈5 to 10%) detected (Figure 2). Regarding glucuronic acid, it was also essentially found in the ASF (Figures 1(b) and 1(c)), forming polymers via 1,4-linkages without any branching (Figure 2). Mannose (≈3 and 7%) and galactose (≈9 and 12%) were found in both *P. blakesleeanus* and *R. oryzae*. In both cases mannose was mostly detected as 1,2-linked, while terminal-mannose represented almost half of the linked counterpart (Figure 2). Galactose was again mostly detected as a terminal monosaccharide (≈7 to 12%; although in this case only in the pyranose form) suggesting that is decorating polysaccharides with backbones based in other monosaccharides (Figure 2).

Allomyces macrogynus cell wall composition is similar to N. crassa

The cell wall of the blastocladiomycete- *A. macrogynus* was found to be similar to *N. crassa* in terms of monosaccharide composition with glucose and glucosamine as main components accounting for 86% and 7% respectively (Figure 1(a)). Almost all the

glucosamine was found to be 1,4-linked (6.12%) and only a very small amount (≈0.1%) was found to be located at the terminal non-reducing ends of glucosamine-based polysaccharides (Figure 2), pointing to the occurrence of chitin polymers with high degrees of polymerization. A majority of the glucans in the cell wall of *A. macrogynus* corresponds to 1,3-glucans (63%); small proportions of 1,4-linked glucose (<5%) units were also detected (Figure 2). This together with the fact that 1,4,6-linked glucosyl residues were identifiable in the chromatograms (trace amounts) is indicative of the presence of residual glycogen/starch-like polymers like *N. crassa*.

Mannose (4.5%), galactose (3.3%) and were identifiable (Figure 1(a)). Mannans were found to be 1,2-linked (1.26%) with many branches in position 6 (1,2,6-Man) (Figure 2). This together with the high proportion of terminal galactose (both the pyranose (t-Gal*p*) and furanose (t-Gal*f*) forms detected; Figure 2) compared to the low amounts of 1,4-galactosyl units found (4 vs. 1%; Figure 2) suggests that probably mannose and galactose are making up a galactomannan with mannose as internal monosaccharide (1,2-Man), branched at some carbon 6 (1,2,6-Man) (1.26) holding short galactose branches (1,4-Gal and t-Gal*p*/Gal*f*) (1.27 and 0.72, 1.30). *N*-acetylgalactosamine and glucuronic acid were absent from the *A. macrogynus* cell wall.

Spizellomyces punctatus possesses a mannose-rich cell wall

Similar to mucormyctes, glucosamine is present in the total monosaccharide wall composition of *S. punctatus* as 31% (Figure 1(a)). Again, only 1,4- (30.84%) and

terminal-linked (0.5%) glucosamine residues were found via linkage analysis, indicative of chitin-chitosan polymers (Figure 2). On the other hand, no 1,3 beta-glucan was found in the cell walls. 1,4-glucans were detected as a minor cell wall component (4.63%; Figures 1 and 2). This together with the fact that small amounts of 1,4,6-linked glucosyl residues were identified (0.91%) indicates the presence of residual glycogen/starch-like polymers.

The low abundance of glucose polymers is evidently compensated by a higher proportion of mannose (58%) (Figure 1(a)). Mannose is present mostly as 1,3 linked (32%) and 1,4 linked (23%), in small amounts as terminal mannose (≈3%) and in trace amounts as 1,4,6- linked (0.56) and 1,3,6- linked (0.37%). Galactose is present in small amounts (≈4%), mostly as terminal pyranose form (≈3%) and no glucoronic acid or galactosamine is present.

Glucosamine with low degrees of N-acetylation in mucormycete cell walls

Glucosamine degree of *N*-acetylation was determined by FTIR spectroscopy in the 5 species compared (Table 1). Additionally, commercial chitins with varying degrees of *N*-acetylation were included in the analyses. The *N*-acetylation degree of commercial intact crab shell chitin and partially alkali-de-acetylated chitin were 84.9% and 73.5% respectively (Table 1). In the case of *S. punctatus* cell walls chitin degree of *N*-acetylation is even higher 90.7%, whereas the *A. macrogynus* cell walls have a low *N*-acetylation degree of 51.8%. Mucormycete chitin acetylation was closer to the partially

de-acetylated one, ranging between 70.9% in *R. oryzae* and 75.3% in *P. blakesleeanus* (Table 1). This value was higher for *N. crassa*'s chitin (78.9%), however still far from the crab shell chitin.

Phylogenomic profiling of the chytridiomycete and mucormycete cell walls

Chitin synthase (CHS) genes were identified by sequence homology (Table 2 (gene numbers in Table S1)) in *S. punctatus* (17copies), *A. macrogynus* (41 copies), *R. oryzae* (29 copies) and *P. blakesleeanus* (24 copies), and phylogenetically resolved into the major classes using *N. crassa* CHS genes as reference as shown in Figure 3. In contrast to Dikarya, the mucormycetes only have four classes of chitin synthases instead of the seven, specifically the Division 1 ancestral class and classes IV, V and VII of Division 2. Also there is another possibly new class in Division 2 comprising of only mucormycetes homologs. Both *R. oryzae* and *P. blakesleeanus* have multiple copies of members of each of these classes, which indicates a multi-duplication event in the Mucormycotina lineage. Similarly, mutiple copies of chitin deactelyase (CDA) genes, were found in almost all the species except *N. crassa* and *S. punctatus* that possess only 2 and 5 copies respectively; 28 in *A. macrogynus*, 34 in *R. oryzae* and 16 in *P. blakesleeanus* (Table 2). Examination of the gene tree (Figure 4) shows multiple copies with relatively short branches of *S. punctatus*, *A. macrogynus, R. oryzae* and *P. blakesleeanus* genes, and the species-specific clades indicating expansions within the Chytridiomycets and Mucormycotina. The chitin metabolism genes appear to have

expanded in copy number in these diverging lineages as compared to Dikarya fungi (Table 2).

The glucan metabolism genes show the same pattern as chitin. *N. crassa* contains one functional copy of the putative beta-1,3-glucan synthase (FKS1), however *R. oryzae* posses 4 copies and *P. blakesleeanus* possesses 3 homologs of this gene, *A. macrogynus* has 5 copies and *S. punctatus* has None (Table 2 and Figure 5). In addition to the glycosyltransferases involved in the biosynthesis/remodeling of chitin and glucans, for the rest of cell wall components, I investigated the presence of the pathways responsible for the production of the nucleotide-sugars required by the polysaccharide synthesizing enzymes (GDP-L-fucose, GDP-D-mannose and UDP-D-glucuronate). Copy numbers of each of the enzymes involved in the biosynthesis of these nucleotide-sugars in *S. punctatus*, *A. macrogynus*, *R. oryzae*, *P. blakesleeanus and N. crassa* are shown in Table 2 (accession numbers in Supplementary Table S1). Expression of all the genes were confirmed in the hyphal cells from analysis of RNA sequencing of the mycelia from vegetative growing colony cultures of *N. crassa and R. oryzae* (data not shown).

GDP-L-Fucose can be biosynthesized through a pathway where GDP-D-mannose is first converted to GDP-4-keto-6-deoxy-D-mannose by GDP-D-mannose 4,6-dehydratase and then to GDP-L-fucose by GDP-L-fucose synthase. The genes corresponding to the GDP-mannose 4,6 dehydratase enzyme in *R. oryzae* are RO3G_05644, RO3G_12053 and RO3G_15908 and in *P. blakesleeanus* are

pbla_t_17728 and pbla_t_56971 (JGI transcript accessions 17728 and 56971 respectively). A GDP-L-fucose synthase homolog was identified in *R. oryzae* as RO3G_07382 and in *P. blakesleeanus* as pbla_t_30353**.** No homologs of these genes were identified in *A. macrogynus* and *N. crassa* consistent with the lack of detection of fucose-based polysaccharides in their cell walls (Figure 1). I was able to find a homolog of both GDP-mannose 4,6 dehydratase and GDP-fucose synthase in *S. punctatus* but the biochemical analysis shows that there is no fucose present in the cell wall of this chytrid. The phylogenetic tree (Figure 6) shows the evolutionary relationships between the fucose metabolism genes in other sequenced mucormycetes species- *M. alpina*, *M. circinelloides*; basidiomycetes- *P. graminis sp. tritici*, *S. roseus* and the bacteria- *E. coli*. I did not find any significantly similar homologs of these genes in any other species, notably None were found in any ascomycetes. No additional homologs were found in any other fungal species by searching the *E. coli* fucose biosynthetic related genes against the Uniprot database.

Several genes responsible for the biosynthesis of GDP-D-mannose (glucose-6-phosphate isomerase, mannose-6-phosphate isomerase, phosphomannomutase and mannose-1-phosphate guanylyltransferase) were identified in *A. macrogynus, S. punctatus, R. oryzae*, *P. blakesleeanus* and *N. crassa,* consistent with the biochemical observations of the sugars in these species (Table 2). Even though there is a very high amount of mannose in the *S. punctatus* cell wall, the number of genes is not very different from the other species containing small amounts of mannose.

UDP-D-glucuronate is synthesized from UDP-D-glucose by the enzyme UDP-glucose 6-dehydrogenase. A search for the genes in the glucuronic acid biosynthetic pathway identified 2 copies of UDP-glucose 6-dehydrogenase in *R. oryzae*, one in *P. blakesleeanus* and None in *A. macrogynus*. Similar to fucose, I was able to find homologs of glucuronic acid in *S. punctatus* but the biochemical analysis shows glucuronic acid lacking from its cell wall. The biochemical analysis show glucuronic acid in trace amounts in *N. crassa* (Figure 1) and a putative UDP-glucose 6-dehydrogenase could be identified in the genome which could be responsible for this production. UDP-D-galactose is synthesized from UDP-mannose by the enzyme GDP-mannose 3,5-epimerase, alongwith a concomitant production of UDP-glucose. I was able to find one copy each of this homolog in all the species except *A. macrogynus* which has 2 copies.

DISCUSSION

The cell wall is one of the unique features distinguishing fungi from the plant and animal hosts it infects, making it an excellent target for development of anti-fungal drugs. Therefore, studies of pathogenic (as well as model) fungi can provide additional avenues for combating the diseases they cause, as there is a limited spectrum of effective antifungals for these early diverging lineages. Nowadays in the era of the "omics" technologies, cell walls have become understudied and the hazard of assuming that cell walls are homogeneous along the fungal kingdom threatens the community.

As a representative member of "standard" fungal cell wall I selected *N. crassa* for this comparative study. Past studies like (Mahadevan and Tatum 1965) made a proper cell wall fractionation and by means of chemical and enzymatic digestion elucidated the main components of the cell wall but were not able to do an accurate quantification. (Bartnicki-Garcia 1968), (Tatum 1963) demonstrated the presence of glucan and chitin in the *Neurospora crassa* cell wall, and that the wall contained glucose, glucosamine, mannose, galactose and galactosamine. The recent studies (Taft and Selitrennikoff 1988; Tentler, Palas et al. 1997; Bowman and Free 2006; Maddi, Bowman et al. 2009) show presence of glucose, N-acetylglucosamine, mannose and galactose and a glucosyl linkage analysis showed the presence of large amounts of 1,3 linked glucose. The results from this study, are in line with these results and prediction, and are the most complete and accurate report of *N. crassa* cell wall composition available till date.

The cell walls of chytridiomycetes- *Spizellomyces* and *Allomyces* have rarely been studied till date and this study is the first and most detailed report. Whereas there has been no formal account of any biochemical study of the *Spizellomyces* cell wall, (Bartnicki-Garcia 1968) study reports *Allomyces* cell walls contained high amounts of chitin (around 60%) and glucan (exact amounts and linkages were not found). Pioneering reports in the mucormycetes *Mucor rouxii* (Bartnicki-Garcia and Nickerson 1962), *M. mucedo* (Datema, van den Ende et al. 1977; Datema, Wessels et al. 1977) and *P. blakesleeanus* (Van Laere, Carlier et al. 1977) pointed to the existence of a cell wall with a composition far from the common standards defined by studies in ascomycetes.

Fucose and uronic acids are exotic components potentially present in the cell wall of mucormycetes. Since a comprehensive treatise linking the biochemical architecture of mucormycete cell walls to its genomic repertoire has been lacking, the objective of the present study was to revisit the polysaccharide composition of cell walls of Mucormycotina fungi. Towards that end, this study biochemicaly analyzes the species and correlates with the presence of genes responsible for cell wall biosynthesis and metabolism through additional annotation of the sequenced genomes. Furthermore, it compares and contrasts the broad differences in the cell wall compositions between the chytridiomycota and mucormycotina and the well-studied ascomycete *N. crassa*.

Chitin governs the architecture and physical properties of fungal cell walls by forming a scaffold for other abundant polysaccharides, such as β-1,3 and 1,6 glucans. Although its high structural importance, quantitatively it usually represents no more than 10% of the whole fungal cell wall. This is the case for *N. crassa* (9%), for which similar values were reported earlier in other wild type strains (Mahadevan and Tatum 1965). *A. macrogynus* has similar levels of chitin as *N. crassa*. However the proportion of this polymer is elevated in *S. punctatus* and the mucormycetes- *R. oryzae* and *P. blakesleeanus*, especially in the first two where represents around a third of the total cell wall. These values are similar to the 35.8% of hexosamines measured in the only report for *R. oryzae*'s cell wall (Tominaga and Tsujisaka 1981), but smaller in the case of *P. blakesleeanus* than the 45.6% previously reported (Van Laere, Carlier et al. 1977). Chytridiomycetes and mucormycetes both possess four classes of chitin synthases (one

division 1 (I, II, III) ancestral class and one of each class IV, V and VII) as compared to the seven in *N. crassa*, which suggests that there was diversification of division 1 group into three classes and gain of class VI after the divergence of Dikarya fungi (possibly due to their different functional requirements) but the class IV, V and VII were necessary for even the early fungi. The fact that all the five classes show gene expansions unique to only mucormycetes, suggests that this expansion must be important for early fungi, which could be in line with the higher chitin content found in these species.

Similar observations were found for the CDA genes. Both CHS and CDA show gene expansions, which seem to be recent (based on the tree branch lengths), indicating that this expansion was important for the Chytridiomycota and Mucormycotina clade. Higher chitin contents are well supported by the observed numbers of CHS genes in chytridiomycetes and mucormycetes, however the impressive number of CDA genes is not backed by extremely low glucosamine *N*-acetylation degrees. Since the initial Bartnicki's classification of mucormycetes as class IV defined by the presence of chitosan and chitin in their cell wall (Bartnicki-Garcia 1968), this concept has remained as the defining parameter of the cell wall of the species in the clade (i.e. "unlike dikaryotic fungi, the cell wall of *R. oryzae* and other mucorales contains a high percentage of chitin and chitosan" (Ma, Ibrahim et al. 2009). My estimations of the degrees of *N*-acetylation indicate that effectively glucosamine based polymers in this group of species contain in average less acetyl groups than the ascomycetes and

especially the commercial chitin, but the differences are not that large. Actually, small proportions of these polymers resulted alkali-solubilized in the fractionation experiments, opposite to the expected behavior of chitosan-like polymers. Altogether these observations indicate that chitin is in average less acetylated in mucormycetes, but not enough to be considered as a non-acetylated polymer. This suggestion is in agreement with the studies in *M. mucedo* were no homopolymers of de-acetylated glucosamine were found (Datema, Wessels et al. 1977). The chytridiomycete *S. punctatus* on the other hand shows acetylation levels even higher than the commercial chitin; whereas the blastocladiomycete *A. macrogynus* shows very low levels. This study is the first to report the chitin acetylation levels of these fungi.

Glucan has been shown to be the major structural polysaccharide of the yeast cell wall, constituting approximately 50–60% of the wall by dry weight (Bowman and Free 2006). The presence of glucan in *N. crassa* has been verified by several studies (Riquelme, Yarden et al. 2011) but the precise amount of glucans has not been reported. This study shows that glucan forms a large fraction (73%) of the *N. crassa* cell wall with majority of it occurring as 1,3- beta glucans. The chytrid- *A. macrogynus* shows a similar trend with glucan accounting for 87% of the cell wall, more than half of which is 1,3 beta linked. Whereas, the other chytrid- *S. punctatus* shows trace amounts of glucan, majority of which corresponds to 1,4- alpha glucan (glycogen). The fact that there is no beta-1,3- glucan in *S. punctatus* cell wall corroborates the finding (in Chapter 2) that homologs of beta-1,3- glucan synthase are absent in its genome. The

mucormycetes- *R. oryzae* and *P. blakesleeanus* have very small amount (> 5%) of glucans in their cell walls (mostly 1,3 beta linked). Again (as in the case of Chitin genes), there was no direct co-relation of the amount of the monosaccharide and copy number of the synthetic genes. For instance- *N. crassa* and *A. macrogynus* both have a glucan as their major cell wall component, yet *N. crassa* posseses only one 1,3- beta glucan synthase and *A. macrogynus* has 5 copies of the same gene. Likewise even though the biochemical analysis shows that mucormycetes have considerable less glucan in their cell walls as compared to *N. crassa,* they still possess more copies of beta-1,3 synthase than *N. crassa*. This further suggests that gene copy number can only loosely relate to actual amounts of a biosynthetic compound that will be detected. Additional layers of regulation are clearly important influences of total amounts. Also its worth noting that, based on studies in *M. rouxii*, glucose was suggested to be only a component of spore but not hyphal walls (Bartnicki-Garcia and Reyes 1964). In this study, although as a minority,1,3-glucans were found in the hyphal walls of both mucormycete species.

Fucose is one of the few L-sugar components of polysaccharides. In plants is mainly found as side decorations of xyloglucan (Scheller and Ulvskov 2010), but the only known polymers based in fucose are the fucoidans. The fucoidans (also known as sulfated fucans) are polysaccharides containing mainly fucose and varying but lesser amounts of all or any of sugars like mannose, galactose, xylose and uronic acids. The fucoidans are mainly found in brown algae (like *Fucus vesiculosus* and *Ascophyllum*

nodosum) but there have been reports of their presence in marine invertebrates like sea cucumber and sea urchins. The structure and composition of fucoidans from different sources vary according to the species they are found in (Berteau and Mulloy 2003). Based on the high fucose content in the cell wall of both *R. oryzae* and *P. blakesleeanus* together with the high proportion of glucuronic acid and the presence of mannose and galactose it is tempting the establishment of links with fucoidans. However, one of the hallmarks of fucoidans is that they contain substantial percentages of sulfate ester groups (Berteau and Mulloy 2003), which does not occur in the fungal counterpart (data not shown; experiments performed as indicated in the methodology section). Indeed the liaison between these monosaccharides as members of the same heteropolymeric entities was already suggested for *M. rouxii* and *M. mucedo* (Bartnicki-Garcia 1968; Datema, van den Ende et al. 1977).

The phylogenomic approach employed in this study well supports the biochemical finding of fucose (GDP-mannose 4,6-dehydratase and GDP-L-fucose synthase found in *R. oryzae* and *P. blakesleeanus*). Apart than mucormycetes, fucose has been also reported in some pucciniomycetes (Basidiomycota), particularly in the germ tubes of *P. graminis* (Kim, Rohringer et al. 1982) and some species of *Sporobolomyces* (Takashima, Hamamoto et al. 2000). I was able to find the fucose synthesis gene homologs in *P. graminis sp. Tritici* and *S. roseus* which supports the idea that fucose is restricted to just zygomycetes and basidiomycetes (pucciniomycetes). This could be the result of a gain in Zygomycota-Dikarya ancestor and loss in Ascomycota, as well as a

horizontal transfer from plants. These possibilities should be tested in future approaches. I did not find fucose residues in any other species that were biochemically analyzed, however my homology search found putative homologs of GDP-mannose 4,6-dehydratase and GDP-L-fucose synthase in *S. punctatus* having very high identity to the mucor homologs. These could be non-functional genes or genes that have functionally diverged to synthesize some other polysacchride.

Mannose occurs as a significant component of the cell walls in conjunction with galactose as galactomannan in yeast (Lesage and Bussey 2006) and *N. crassa* (Bowman and Free 2006; Maddi and Free 2010) which is consistent with what I found in the present study. Mucormyctes- *R. oryzae* and *P. blakesleeanus* have been shown to have mannose in small quantities (Crook and Johnston 1962; Bartnicki-Garcia 1968), and again my results are in agreement. A previous report (Skucas 1967) claimed *A. macrogynus* had no mannose in its cell wall, however my analysis shows that it does contain mannose, possibly in conjunction with galactose forming galactomannan similar to ascomycetes as described above. An important finding of my study was that *S. punctatus* cell walls are composed primarily of mannose (more than half of the cell wall by weight), which makes it very unique from all the other fungal species.

Fungi are nowadays described as exhibiting similar cell wall architectures, regardless the phylogenetic position of the different lineages. The salient finding of this study is the existence of clearly distinguishable cell walls within the fungal kingdom. The combination of biochemical and phylogenomic approaches has correlated the unique

cell wall composition of chytridiomycetes and mucormycetes with a repertoire of genes required for its biosynthesis. The *Allomyces* cell walls were found to be similar to ascomyctes i.e. glucan-rich and poor in chitin. Whereas *Spizellomyces* has a chitin-rich and glucan-poor cell wall, with mannose being the most substantial component. It is worth highlighting the rare low glucan contents found in mucormycetes- *Rhizopus* and *Phycomyces*, which are replaced by high chitin levels (scaffolding role) together with heteropolymers of higher solubility (cementing role) composed mainly of fucose and glucuronic acid which show high structural similarities with the brown algae polysaccharides fucoidans. Further characterization of the "mucoran" component could allow for instance its use as diagnostic tool, of especially necessity in case of human pathogens.

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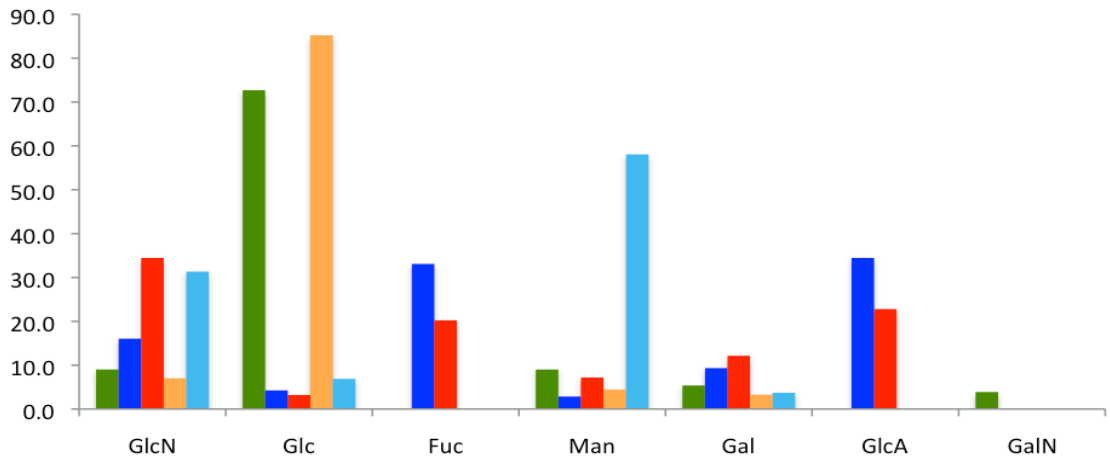
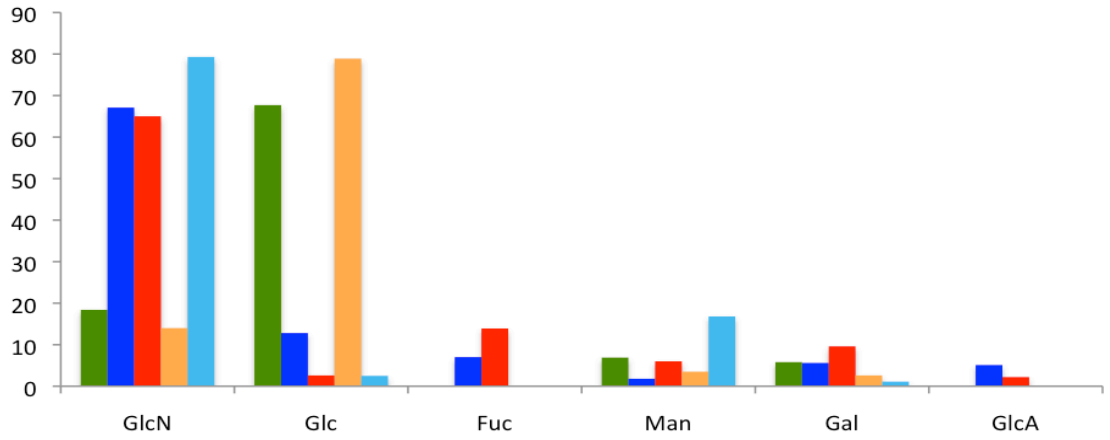
Figure 1 Monosaccharide composition (mol%) of the cell wall samples from *Neurospora crassa, Rhizopus oryzae* and *Phycomyces blakesleeanus* in a) Total cell walls; b) Alkali Insoluble Fraction; c) Alkali Soluble Fraction. GlcN, *N*-acetylglucosamine; Glc, glucose; Fuc, fucose; Man, mannose; Gal, galactose; GlcA, glucuronic acid; GalN: *N*-acetylgalactosamine. Green: *N. crassa*, Blue: *P. blakesleeanus*, Red: *R. oryzae*, Orange: *A. macrogynus* and Cyan: *S. punctatus*.

(a)



(b)



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(c)

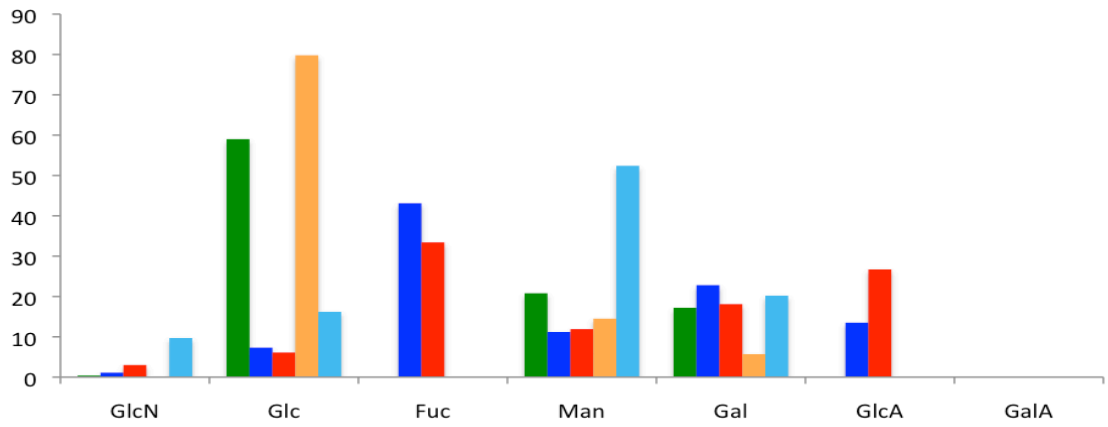

Figure 2 Glycosidic linkage analysis (mol%) of the cell wall samples from the 5 selected fungal species identified by EI-MS. GlcN, *N*-acetylglucosamine; Glc, glucose; Fuc, fucose; Man, mannose; Gal, galactose; GlcA, glucuronic acid; GalN: *N*-acetylgalactosamine. Green: *N. crassa*, Blue: *P. blakesleeanus*, Red: *R. oryzae*, Orange: *A. macrogynus* and Cyan: *S. punctatus*.

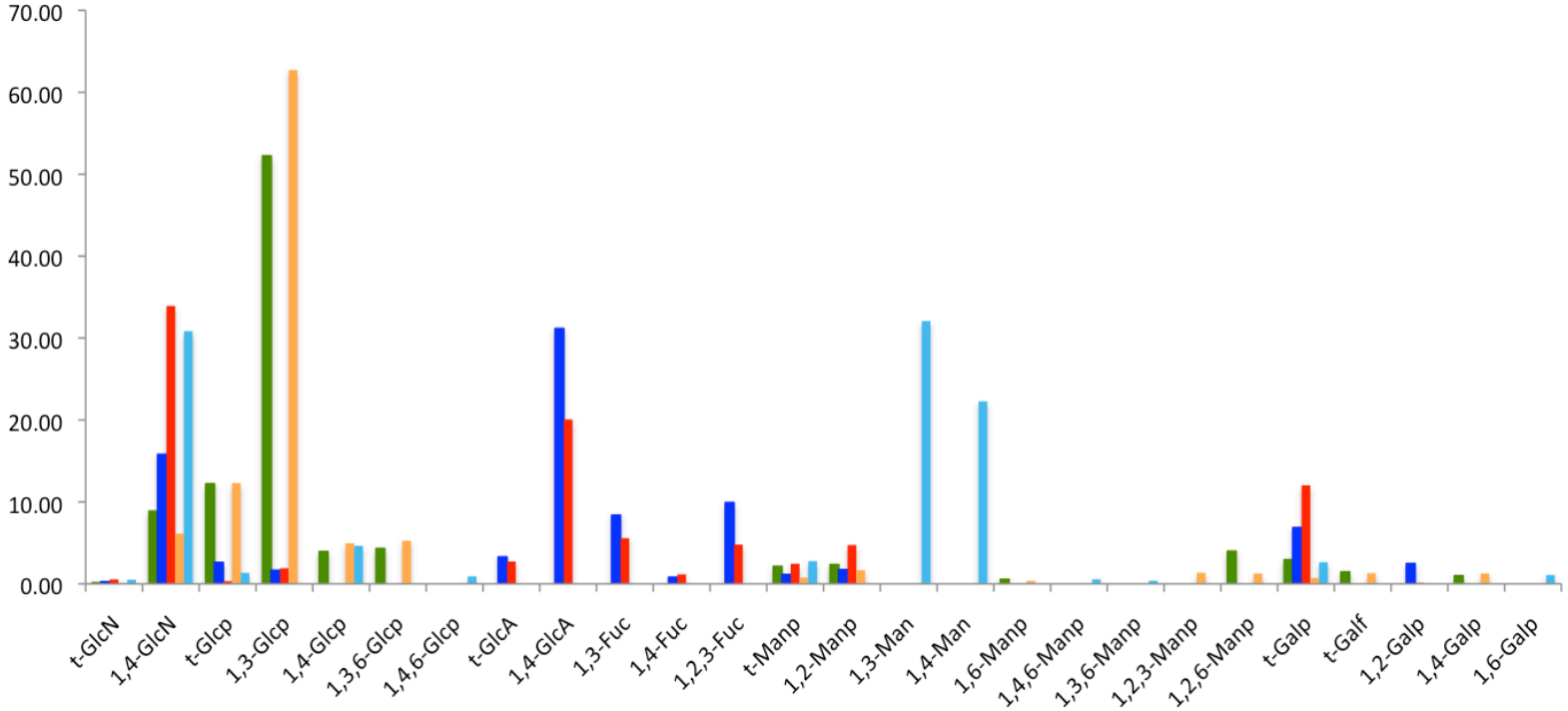

Figure 3 Phylogenetic tree of chitin synthase genes in Chytridiomycetes- *S. punctatus* and *A. macrogynus*, Mucormycetes- *R. oryzae*, *P. blakesleeanus* and Ascomycete- *N. crassa* rooted by CHS class VI. Clades containing Division 1 (Classes I, II, II) and Division 2 (Classes VI, V, VII) and Division 3 (Class VI) are labeled. All the gene accession numbers are searchable on JGI (for *S. punctatus*, *A. macrogynus*, *R. oryzae* and *P. blakesleeanus*) and Broad (for *N. crassa*).

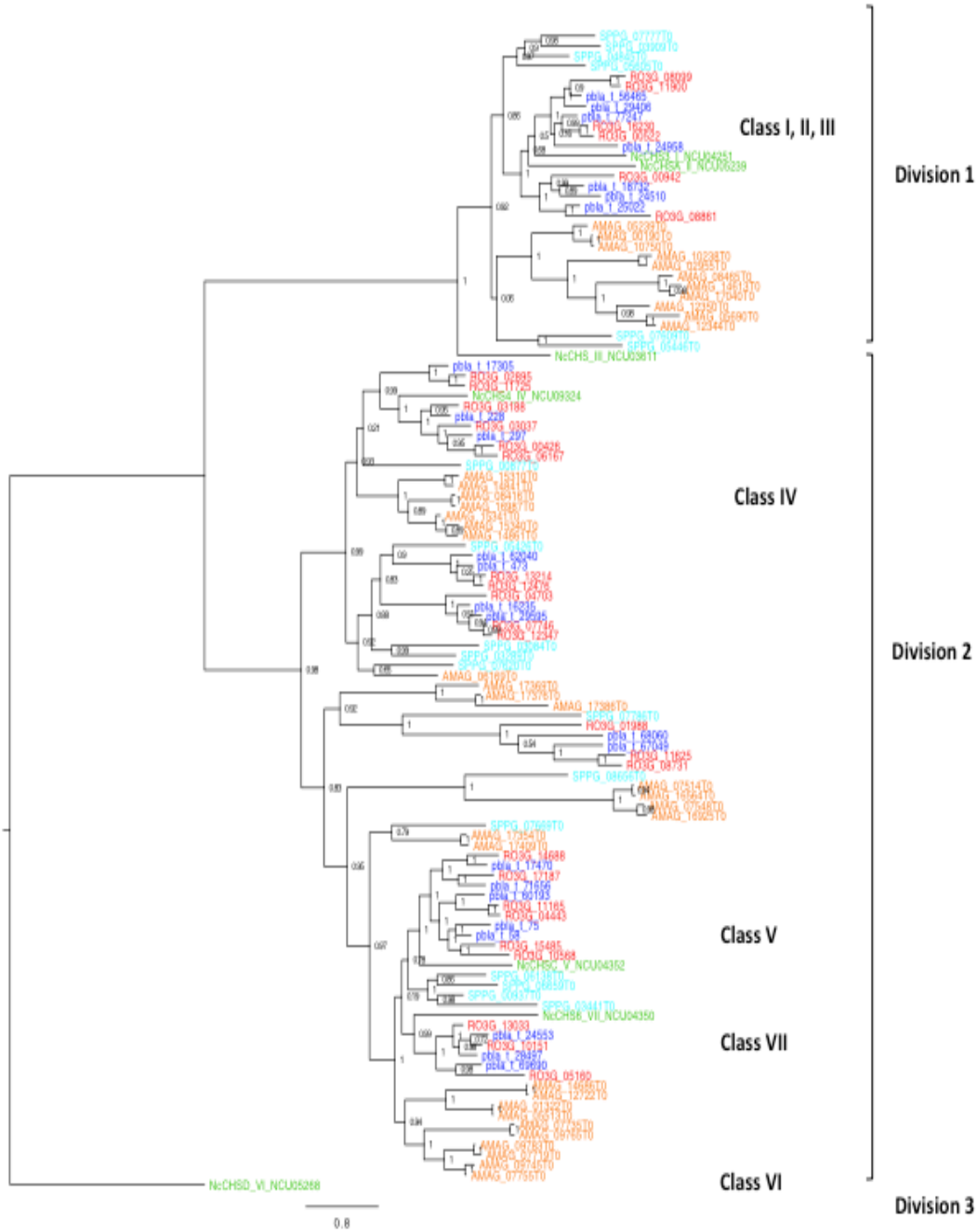

Figure 4 Midpoint rooted phylogenetic tree of chitin deactelyase genes in Chytridiomycetes- *S. punctatus* and *A. macrogynus*, Mucormycetes- *R. oryzae*, *P. blakesleeanus* and Ascomycete- *N. crassa*. All the gene accession numbers are searchable, on JGI (for *S. punctatus*, *A. macrogynus*, *R. oryzae* and *P. blakesleeanus*) and Broad (for *N. crassa*).

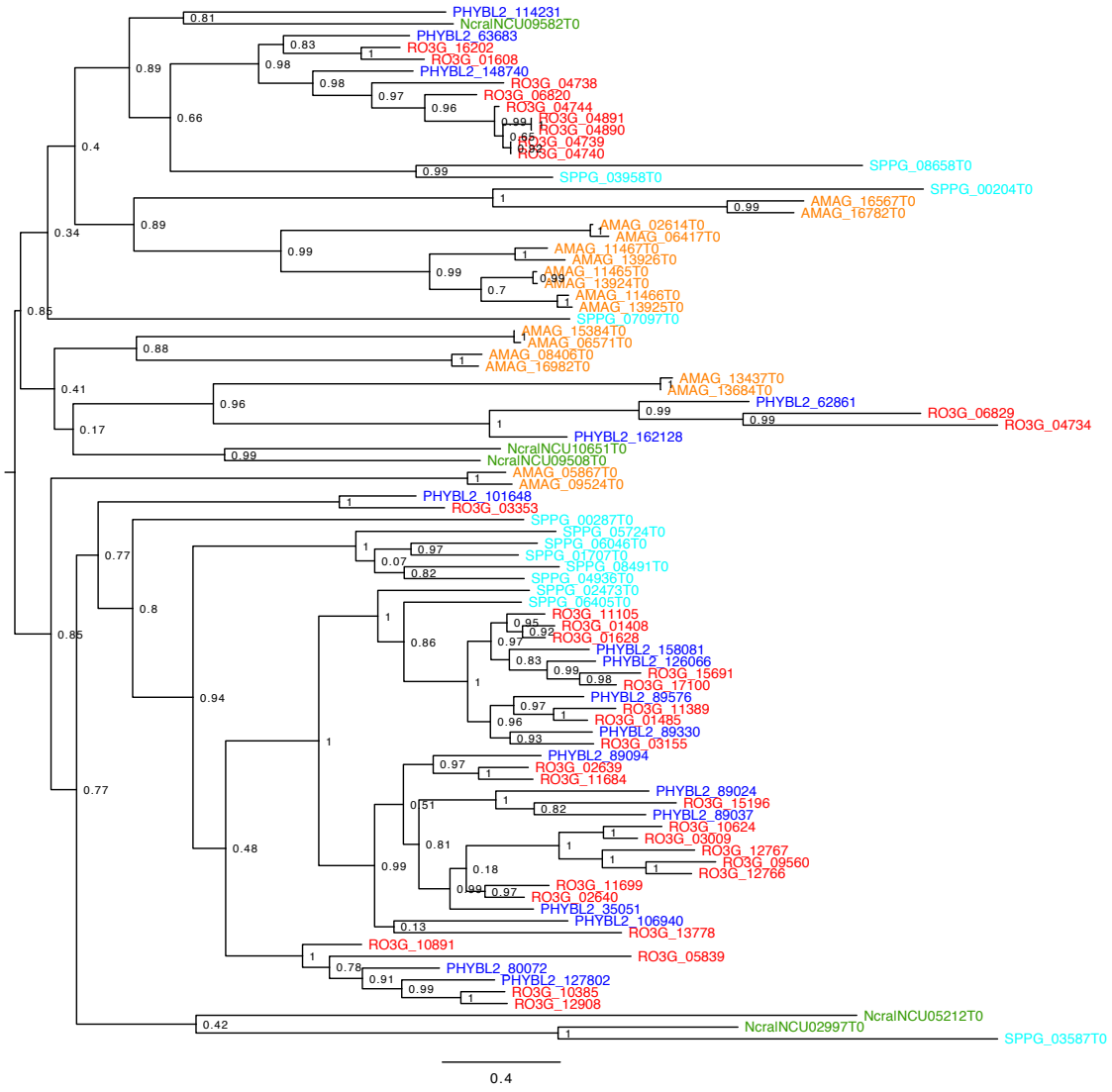

Figure 5 Midpoint rooted phylogenetic tree of β-1,3-glucan synthase genes in *N. crassa*, *S. punctatus*, *A. macrogynus*, *R. oryzae* and *P. blakesleeanus*. All the gene accession numbers are searchable, on Broad (for *N. crassa*) and JGI (for *S. punctatus*, *A. macrogynus*, *R. oryzae* and *P. blakesleeanus*).

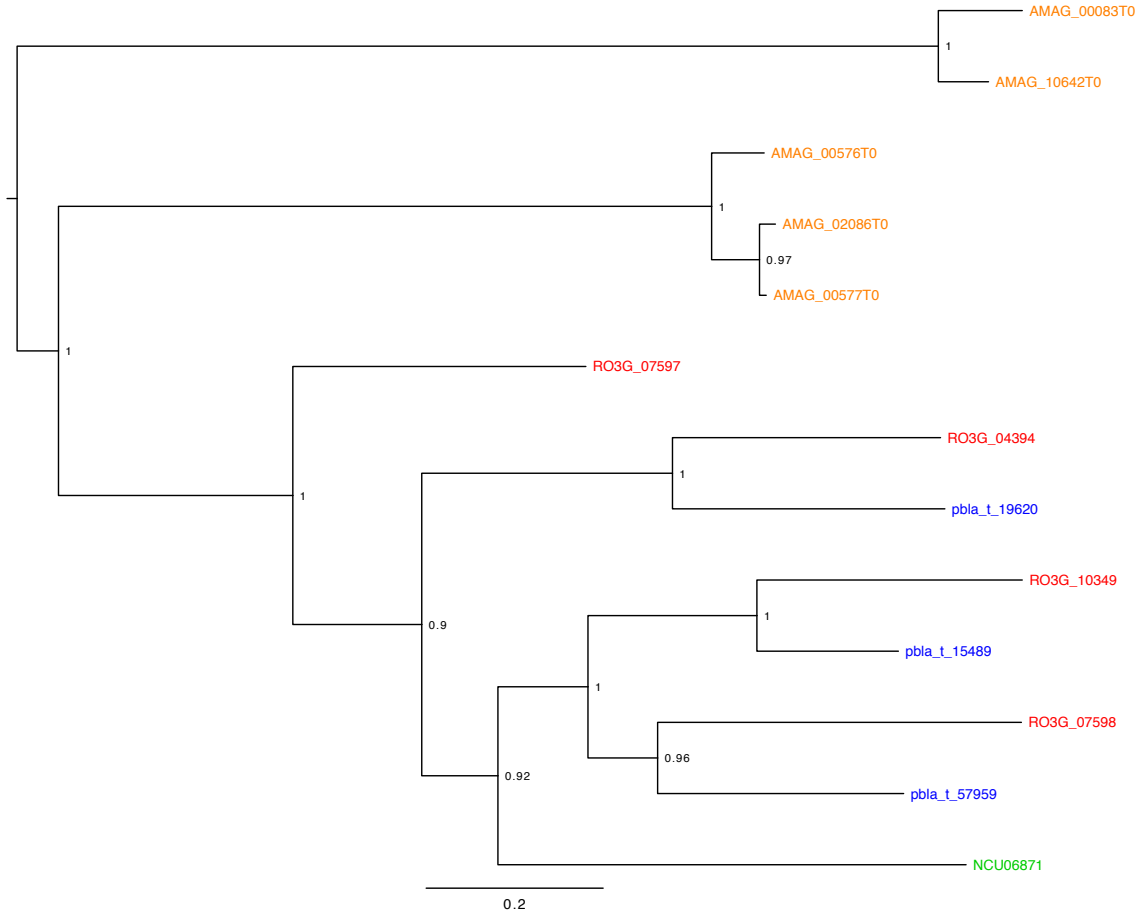

Figure 6 a) Phylogenetic tree of GDP-mannose 4,6 dehydratase genes in Mucormycetes- *R. oryzae, P. blakesleeanus, M. alpina* (Ma), *M. circinelloides* ; Basidiomycete- *P. graminis sp. Tritici* (Pg), *S. roseus* (Sr) and Bacterium- *E. coli* (Ec), rooted by Ec. All the gene accession numbers (except *M. alpina* and *S. roseus*) are searchable on JGI (for *R. oryzae, P. blakesleeanus* and *M. circinelloides*), Broad (for *N. crassa, P. graminis sp. Tritici*,) and Genbank (*E. coli*).

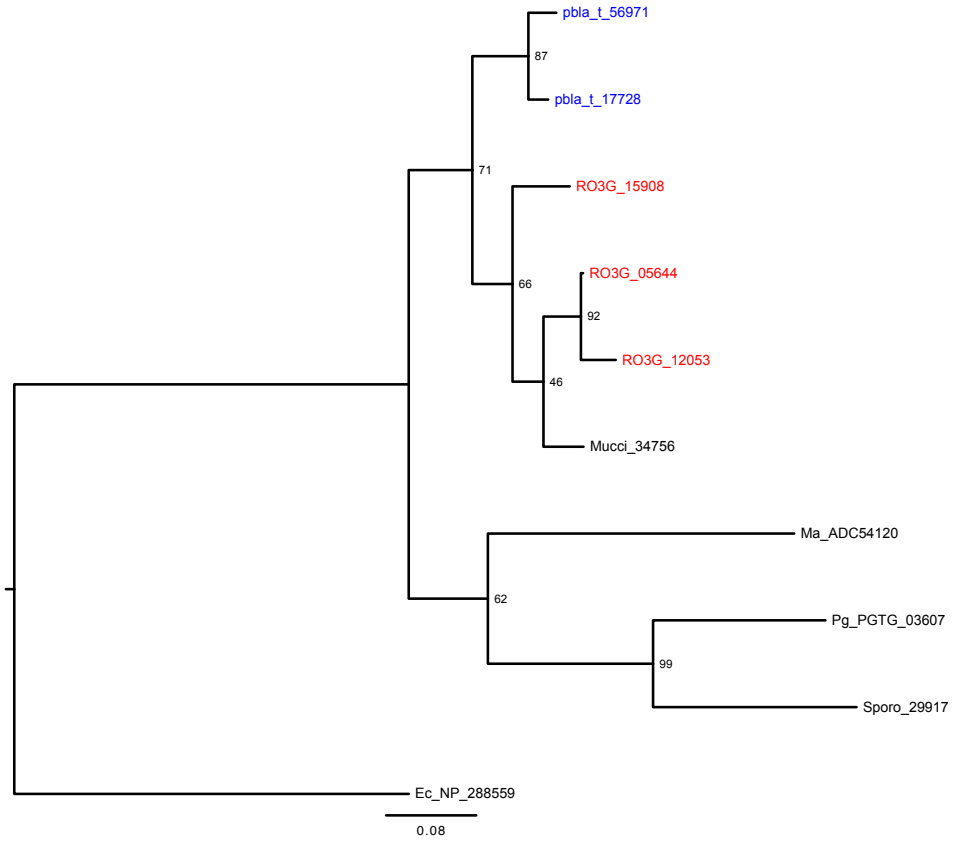

Figure 6 b) Phylogenetic tree of GDP-L-fucose synthase genes Mucormycetes- *R. oryzae, P. blakesleeanus, M. alpina* (Ma), *M. circinelloides* ; Basidiomycete- *P. graminis sp. Tritici* (Pg), *S. roseus* (Sr) and Bacterium- *E. coli* (Ec), rooted by Ec. All the gene accession numbers (except *M. alpina* and *S. roseus*) are searchable on JGI (for *R. oryzae, P. blakesleeanus* and *M. circinelloides*), Broad (for *N. crassa, P. graminis sp. Tritici*,) and Genbank (*E. coli*).

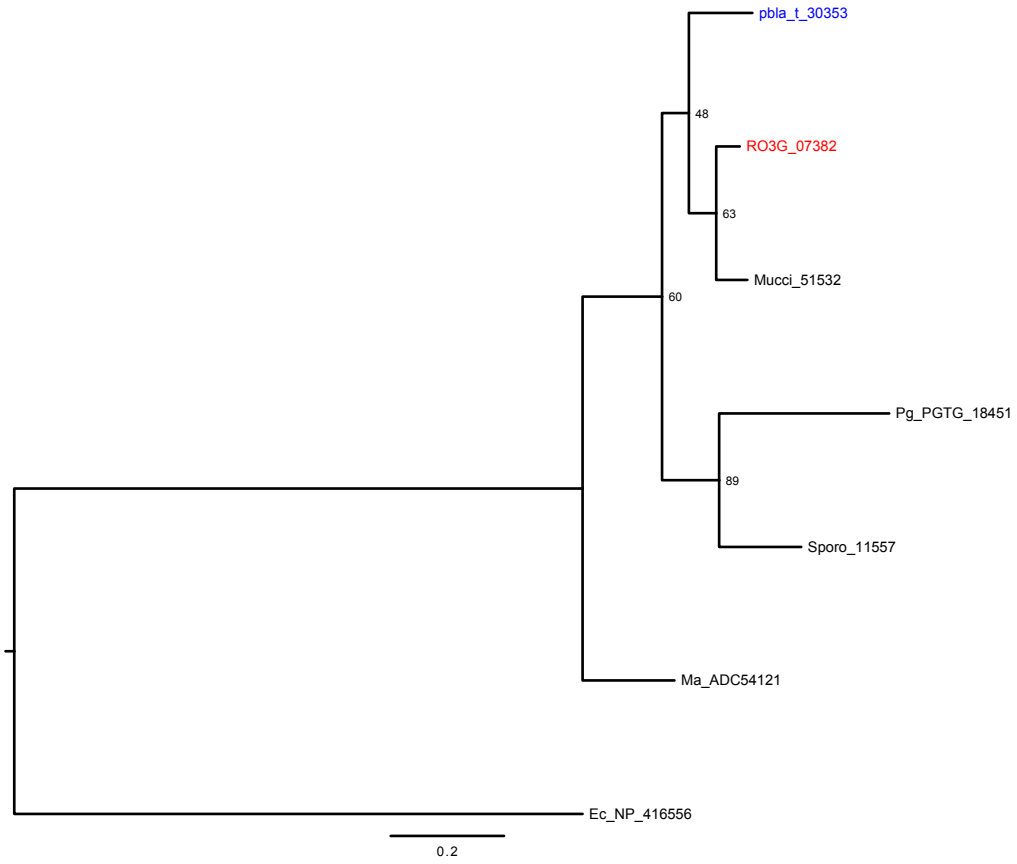

Figure 7 Comparative cell wall compositions of the species analyzed in this study.

The species are represented in their phylogenetic order.

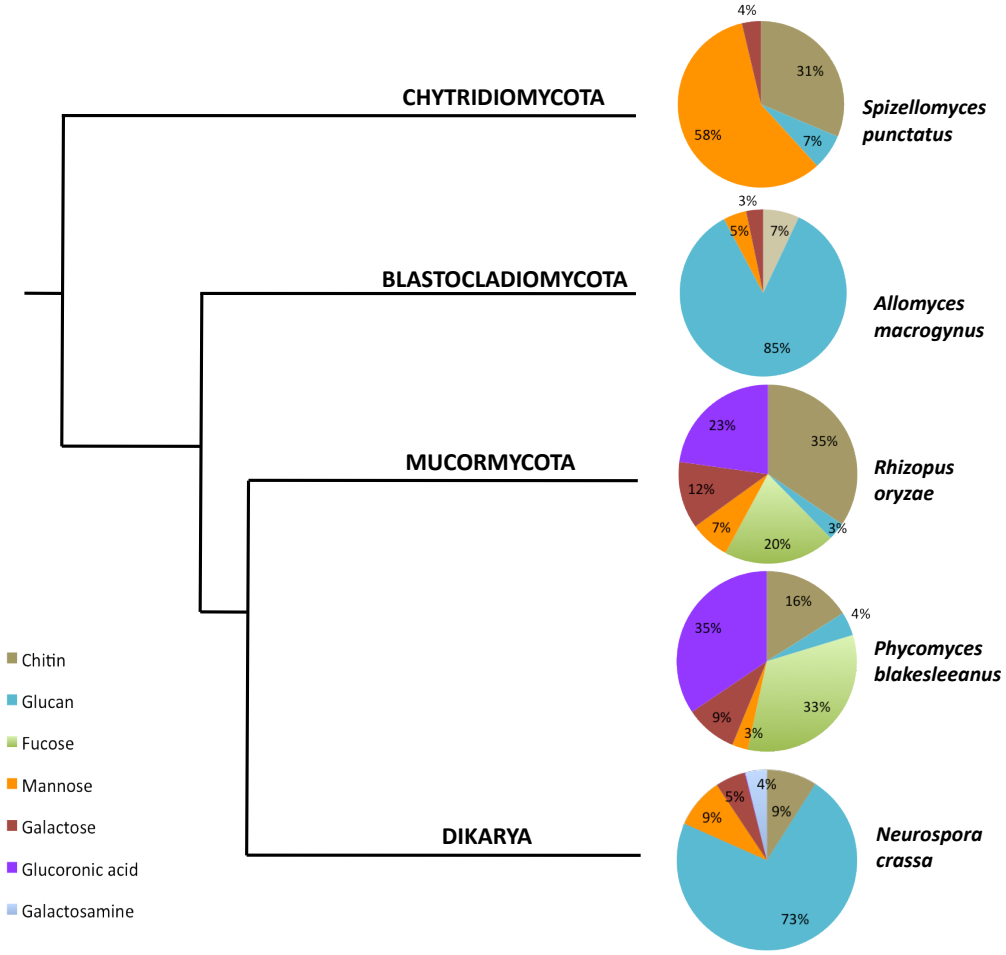

Table 1 *N*-Acetyl content (%) of chitin samples determined by FTIR spectroscopy

Source	% *N*-acetylation
Commercial crab shell chitin	84.9 ± 1.8
Partially de-acetylated chitin	73.5 ± 3.5
Neurospora crassa	78.9 ± 5.8
Phycomyces blaskesleeanus	75.3 ± 1.3
Rhizopus oryzae	70.9 ± 0.9
Allomyces macrogynus	51.8±1.4
Spizellomyces punctatus	90.7±3.4

Table 2 Distribution of copy numbers of the genes required for synthesis of the cell wall sugar monosaccharides.

Sugar	Genes	*S. punctatus*	*A. macrogynus*	*R. oryzae*	*P. blakesleeanus*	*N. crassa*
Chitin	i) Chitin synthase	17	41	29	24	7
	ii) Chitin deactelyase	5	28	34	16	2
Glucan	i) 1-3, beta glucan synthase	0	5	4	3	1
	ii) exoglucanase	0	0	2	2	1
Fucose	i) GDP-mannose 4,6-dehydratase	1	0	3	2	0
	ii) GDP-L-fucose synthase	1	0	1	1	0
Mannose	i) Glucose-6-phosphate isomerase	1	2	1	1	1
	ii) Mannose-6-phosphate isomerase	1	2	1	1	2
	iii) Phosphomannomutase	1	2	1	1	1
	iv) Mannose-1-phosphate guanylyltransferase	1	2	4	1	1
Glucoronic acid	UDP-glucose 6-dehydrogenase	2	0	2	1	1
Galactose	GDP-mannose 3,5-epimerase	1	2	1	1	1

CONCLUSION

The fungal cell wall is a structure that is essential for the fungus as well as unique from its plant/mammalian hosts and hence presents an attractive target for new antifungals. Knowledge at the molecular level of the processes and activities responsible for the biosynthesis and regulation of the cell wall components is a critical step in the design of new antifungal drugs that specifically inhibit fungal cell wall formation. Keeping this big picture in mind, I attempted to: (1) identify the various components involved in cell wall organization and morphogenesis in the model filamentous fungus- *N. crassa* (2) reconstruct the evolutionary history of the major cell wall polysaccharides in the species across the fungal kingdom and (3) understand the composition of the cell wall of lesser studied primitive fungi and correlating it to its genomic repertoire. In terms of broader impact this research makes an attempt to advance the understanding of the fungal cell wall that would eventually lead to new and improved strategies for combating the disease causing fungi.

Chapter 1

The early genomics screens in the model yeast *S. cerevisiae* (de Groot, Ruiz et al. 2001; Page, Gerard-Vincent et al. 2003) led to the identification of some of the first fungal cell wall genes. The knowledge gleaned from random mutagenesis screens (Seiler and Plamann 2003) and cell wall stress-based screens (Ram and Klis 2006) has contributed to our current understanding of the genes involved in the various processes leading to

cell wall synthesis and hyphal growth in the model filamentous fungus *N. crassa*. In addition, expression studies like (Kasuga and Glass 2008) have been instrumental in elucidating the growth and development pathways during colony establishment in *N. crassa*. Chapter 1 presents a method that attempts to enrich for cell wall genes by examining the expression of genes involved in hyphal growth. This combines data from transcriptomics studies and cell wall stress tests for the robust prediction of candidate genes involved in cell wall growth and morphogenesis. Hence, this study represents a model approach to finding genes involved in a process.

More than half of the candidate gene-set corresponded to cell wall integrity mutants. In addition, this gene-set was also enriched in functional categories such as cell wall biogenesis, polysaccharide metabolism, and directional cell growth. Whereas a similar study in *N. crassa* that tested GPI-anchored genes for having a cell wall function, found a very small proportion (~15%) of mutants to have cell wall defects (Maddi, Dettman et al. 2012). I was able to functionally annotate 32 previously un-annotated genes as cws (**c**ell **w**all **s**ensitive) on the basis of their sensitivity to cell wall stress. I chose to perform an overlap of the various defects in order to select the cell wall mutants that were defective in multiple pathways and found 14 such candidate genes. These were classified on the basis of the shared characteristics with known cell wall genes into pathways like the growth and morphogenesis, cell wall integrity signaling, Rho signaling and transport. This approach enabled us to identify genes involved the various

processes that come together to build, degrade and regulate the cell wall in the filamentous fungi.

This study produced a collection of cell wall growth and development candidate genes most of which possess homologs in other Pezizomycotina fungi improving their utility as potential targets for future drug targeting. In this direction a first step could be to screen the cws genes identified in this study against known anti-fungals (echinocandins, azoles, nikkomycin etc) to identify hypersensitive mutants. Also this study provides a valuable addition to the repertoire of genes known to be involved in hyphal morphogensis and cell wall maintenance that can be followed up using molecular and genetic techniques. For instance, the cell wall integrity mutants from this study (NCU04866 (*ada-6*), NCU04663 (*cws-19*), NCU08131 (*gh13-1*) and NCU04475 (*cws-17*)) that phenocopy the MAPK mutants involved in the CWI pathway (Park, Pan et al. 2008), could be subjected to genetic epistasis studies to determine if these genes are indeed in the same pathways as the MAPK genes. If this is found to be true then yeast two hybrid and/or differential phosphorylation experiments can be carried out to test if the genes are interactors/targets of kinases/phosphatases belonging to that pathway.

Chapter 2

Most of the previous efforts have been directed at studying the cell walls of only a few model ascomycetes like *S. cerevisiae, C. albicans, N. crassa* and *Aspergillus* sp (Odds

1985; Bernard and Latge 2001; Klis, de Groot et al. 2001; Riquelme, Yarden et al. 2011). However there are many other fungi belonging to distinct phylogenetic clades which cause world-wide crop losses as well as lethal diseases in animals and humans. The cell walls of these fungi are different from the generally accepted model (mostly based on ascomycetes). Hence there is a need to profile the cell walls of evolutionarily distinct fungi to understand the differences between their composition and structur. Chapter 2 and 3 attempt to reconstruct the genomic and compositional history of the cell walls of the fungi belonging to major phylogenetic clades using comparative genomics and biochemical approaches.

With the improvement in sequencing technology and ensuing drop in costs, it has become easier to sequence genomes and hence utilize the power of comparative genomics to study the cell walls of species belonging to other evolutionarily distinct clades like Microsporidia, Cryptomycota, Chytridiomycota and Mucormycota. Chapter 2 seeks to reconstruct the evolutionary history of these fungal cell walls and examine if the earlier diverging fungi had the same cell wall components as the recently diverged species and observe the diversification of the genes needed for the biosynthesis and degradation of the cell wall carbohydrates. One of the key findings of this study was that the early fungal species possessed multiple independent expansions of chitin metabolism genes but fewer classes than the Dikarya. This study also reveals the presence of a new class of chitin synthase and chitinase gene family. I was also able to establish the gain of 1,3-beta glucan synthase gene as a synapomorphy to successfully

group Chytridiomycota separately from Blastocladiomycota, Mucromycetes and Dikarya.

The methodology thus presented to find homologs using comparative genomics and phylogenetics is generally useful and can be rapidly and automatically applied to new genome sequences to identify the conserved cell wall process genes in additional species. I expect this can improve the ease of studying the genetic diversity of various fungal cell wall components in future studies. The pipeline thus developed, to find homologs using comparative genomics and phylogenetics, can be applied to other species as and when they are sequenced to identify new cell wall homologues in those genomes. This can prove to be a useful tool to study the genetic diversity of various fungal cell wall components for the future studies.

Chapter 3

The only comparative study of fungal cell walls of major evolutionary clades till date has been (Bartnicki-Garcia 1968) that classifies most of the fungal clades into 7 groups based on cell wall composition. Since then there have been biochemical characterization of fungal species belonging to mostly the Dikarya ((Free 2013) presents a brief review). This study is the first systematic comparative study of the polysaccharide compositions and linkages of the fungal cell wall from the major evolutionary clades i.e. Chytridiomycota, Blastocladiomycota, Mucormycotina and Dikarya. I used data from biochemical profiling by cell wall fractionation and Gas chromatography/ Mass Spectroscopy to assess the levels of each cell wall polysaccharide. I used comparative

genomics to examine how the presence of genes responsible for cell wall biosynthesis and metabolism correlated with the cell wall polysaccharide composition.

The key finding of this study was the existence of clearly distinguishable cell walls within the fungal kingdom. The blastocladiomycete cell walls were found to be similar to ascomycetes i.e. glucan-rich and poor in chitin. Whereas chytridomycetes have a chitin-rich and glucan-poor cell wall, with mannose being the most substantial component. The mucormycetes were the most unique having the heteropolymer-'mucoran' as the largest fraction, followed by chitin and a small amount glucans. This study, to the best of my knowledge, is the first to report that chytridomycetes' cell walls contain no 1,3 beta-glucan, which is in agreement with the finding in Chapter 2 that there are no homologs of beta-1,3- glucan synthase in the chytridomycete genome. I was also able to identify the genes responsible for fucose and glucoronic acid (two important components of 'mucoran' sugar fraction) in the mucormycete species presenting evidence of the genomic architecture of mucoran sugar metabolism in these species. A salient finding of this work was the finding that copy number of a biosynthetic gene doesn't always correlate with the amount of the compound synthesized, for example I was able to find multiple copies of chitin synthases in blastocladiomycete *A. macrogynus* in Chapter 2 but in the present study I observed that it contains very less amount of chitin than other species like *R. oryzae* which possesses comparable number of chitin synthases.

This study elucidating the structure and composition of these distinct clades should lead to an informed decision with regards to selection of the appropriate antifungal against a particular pathogenic fungal species. For instance since now we know that chytridiomycetes cell walls are devoid of 1,3 beta glucan, it is intuitive to think that the chytridiomycete species would be more or less resistant to drugs that target the 1,3 beta glucan synthase gene (like echinocandins). We find that this indeed is the case, (Fisher, Bosch et al. 2009) study shows that the 'frog-killing' chytridiomycete *B. dendrobatidis* showed increased resistance to caspofungin (MIC_{50}= 4-16 μL/mg as opposed to MIC_{50} =0.03 μL/mg against *Candida* sp. (Pfaller, Boyken et al. 2008)). Similarly, (Pfaller, Marco et al. 1998) study shows that *Rhizopus* sp. showed greater resistance to echinocandins (MIC_{50}= 2-8 μL/mg) as compared to other antifungals like itraconazole and amphotericin B (MIC_{50}= 1 μL/mg for both). We can attribute this to the low amount of glucans in mucormycetes. A simple next step could be to test the species profiled in this study against the various anti-fungals available to test their sensitivity. For example I hypothesize that *A. macrogynus* would be more sensitive to echinocandins as a large fraction of its cell wall is composed of 1,3-beta glucans. On the other hand *B. dendrobatidis* may be more sensitive to nikkomycin/polyoxin (bind to chitin) and pradimicin/benanomicin (bind to mannan) as it's cell wall contains major amounts of chitin and mannan. Hence findings from my work should lead to new and improved strategies for combating a multitude of fungi having varied cell wall organization.

Conclusion

Although many cell wall components have been characterized to various extents, our understanding of cell wall biosynthesis is still far from complete. The mechanism by which cell wall polysaccharides are extruded and the significance of the variations in cell wall constituents in different fungi are just examples of questions yet to be answered. Although regulatory networks that affect cell wall synthesis and maintenance have been identified, the comprehensive signaling machinery involved and the associations between different pathways have yet to be elucidated. The significance of the cell wall as a target for antifungals has long been appreciated, yet the number of successful drugs developed is very small. Important progress has been made in the analysis of the cell wall. However, our still-inadequate levels of understanding concerning the fundamental features of the cell wall, as well as our limited current capabilities in harnessing our knowledge for relevant applications in medicine, agriculture, and industry, provide us with many challenges for the future. The research presented in this dissertation has attempted to fill in a few of these gaps and the hope is that someday mankind will successfully be able to overpower the menace of fungal disease.

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Appendix 1

RNA isolation for the N. crassa RNA sequencing

Tissue of growing *N. crassa* hyphae was sampled for timepoints from three, five, and twenty hours of growth from following published methods (Kasuga and Glass 2008) with minor modifications. All sample ages were defined as number of hours of growth of the tissue based on the time when sampling was performed. Briefly, wild-type laboratory strain FGSC2489 was grown in 100x15mm sterile Petri dishes on Vogel's minimal medium (VM) (Vogel 1956) overlaid with cellophane at 25°C under constant light. After an even mycelia mat had formed in the dish (~24h), 5mm strips of cellophane were cut out of the petri dish and arranged in a straight line down the middle of a 245mm Square BioAssay Dish with VM covered with cellophane (Corning: cat. # 431111). After inoculation, the large plate was incubated at 25°C under constant light conditions. The periphery of the colony was outlined with a marker directly on the plate every 5h (with the exception of the final 3h tip growth). Measurements were taken for 30 h. Growth rate was estimated to be ~0.2 cm/h. Tissue was harvested by excising the cellophane along the marked lines from the appropriate time points using a sterile razor blade. Samples were immediately frozen in liquid nitrogen and stored at -80°C.

RNA extraction was performed using the RNeasy Plant Mini Kit (QIAGEN) using the RLC buffer for tissue lysis; all other steps were performed according to manufacturer's protocol. *N. crassa* library preparation was performed in our laboratory using the

Illumina mRNA-Seq Prep Kit (Illumina, cat number RS-100-0801) according to manufacturer's direction. mRNA-Seq library preparation was performed with 5μg of total RNA as starting material. Sequencing of the library was performed with an Illumina HiSeq2000 to generate 36 bp single-end reads at the UCR IIGB Genomics Core Facility.

Appendix 2

Biochemical characterization of fungal cell walls

Preparation of cell walls: Cell wall polysaccharides were extracted as previously described (Melida, Sandoval-Sierra et al. 2013). Briefly, the mycelia from the different species grown in the respective growth media were washed extensively and immediately frozen in liquid nitrogen. The resulting fine powders after cell disruption were subjected to ethanol extractions and the proteins in the resulting alcohol-insoluble residues (AIR) were removed by heating the samples at 80ºC for 10 min (repeated 3 times) in a 50 mM Tris-HCl buffer (pH 7.8) containing 2% (w/v) sodium dodecyl sulfate (SDS), 40 mM 2-mercaptoethanol and 10 mM EDTA. Glycogen/starch-like polymers were removed from the residue by treatments with α-amylase from porcine pancreas (Sigma type VI-A) (Melida, Garcia-Angulo et al. 2009). The residues recovered after final washes in 70% ethanol and acetone, corresponded to the purified cell walls, which were dried under vacuum and stored with desiccant. The latter were

fractionated into alkali-soluble (ASF) and alkali-insoluble (AIF) fractions using hot methanol-KOH solutions (Melida, Sandoval-Sierra et al. 2013).

Monosaccharide analysis: Prior to acid hydrolysis, carboxyl groups in cell wall polysaccharides were reduced using sodium borodeuteride ($NaBD_4$) after their activation with carbodiimide (Kim and Carpita 1992). This chemical modification allowed the chromatographic detection of uronic acids as their 6,6-dideuterio neutral sugar derivatives. The dried cell walls/fractions (5 mg) were hydrolyzed with 72% sulfuric acid at room temperature for 3 h followed by 3 h incubation at 100ºC after diluting the sulfuric acid to 1 M. *Myo*-inositol was used as an internal standard. The resulting monosaccharides were converted to alditol acetates (Blakeney, Harris et al. 1983), separated and analyzed by gas chromatography (GC) on a SP-2380 capillary column (30 m x 0.25 mm i.d.; Supelco) using a HP-6890 GC system and a HP-5973 electron-impact mass spectrometer (EI-MS) as a detector (Agilent Technologies). The temperature program increased from 180ºC to 230ºC at a rate of 1.5ºC min^{-1}.

Glycosidic linkage analysis: Polysaccharide networks in the dry carboxyl reduced cell wall samples (0.1 mg) were first swollen in 200 μL dry dimethylsulfoxide (DMSO). Ten μL of DMSO containing 0.3 mg L^{-1} sulphur dioxide and 5 μL of diethylamine were added and the samples were subsequently sonicated for 20 min and stirred under argon at room temperature for 3 h. Methylation reactions were performed using the $NaOH/CH_3I$ method (Ciucanu and Kerek 1984), by repeating 5 times the methylation step on each sample, thereby avoiding any risk of undermethylation. Partially

methylated polysaccharides were hydrolysed in the presence of 2 M TFA at 121ºC for 3 h and further derivatised to permethylated alditol acetates (Albersheim 1967). The latter were separated and analyzed by GC/EI-MS on a CP-Sil 5 CB capillary column (30 m x 0.25 mm i.d.; Agilent Technologies) with a temperature program increasing from 160ºC to 210ºC at a rate of 1ºC min^{-1}. The mass spectra of the fragments obtained from the permethylated alditol acetates (EI-MS) were compared with those of reference derivatives and by comparison to available data (http://ccrc.uga.edu/specdb/ms/pmaa/pframe.html).

Fourier transform infrared (FTIR) spectroscopy: Discs for FTIR spectroscopy were prepared in a Graseby-Specac press from mixtures of cell walls with KBr (1:100, w/w). Spectra (800-4000 cm^{-1}) were obtained on a Perkin-Elmer Spectrum 2000 instrument at a resolution of 1 cm^{-1}. All spectra were normalized and baseline-corrected with Spectrum v 5.3.1. For the calculation of the degree of *N*-acetylation of glucosamine based polysaccharides (chitin-chitosan) the ratio A_{1655} cm^{-1} (amide I band) / A_{3450} cm^{-1} (hydroxyl band) was obtained (Domszy and Roberts 1985). Standards with different degrees of *N*-acetylation (measured by NMR; N. Butchosa and Q. Zhou unpublished results) were used during the calibration.

Polysaccharide sulfate determinations: The sulfate content of polysaccharides was measured in total cell walls as well as in their AIF and ASF fractions using the gelatin/$BaCl_2$ method (Dodgson and Price 1962) after hydrolysis in 60% formic acid for 8 h at 100ºC.