Deciphering the uniqueness of Mucoromycotina cell walls by combining biochemical and phylogenomic approaches

by

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Running title: *The unique features of Mucoromycotina cell walls*

Summary

Most fungi from the Mucoromycotina lineage occur in ecosystems as saprobes, although some species are phytopathogens or may induce human mycosis. Mucoromycotina represent early diverging models that are most valuable for understanding fungal evolution. Here we reveal the uniqueness of the cell wall structure of the Mucoromycotina *Rhizopus oryzae* and *Phycomyces blakesleeanus* compared to the better characterized cell wall of the ascomycete *Neurospora crassa*. We have analyzed the corresponding polysaccharide biosynthetic and modifying pathways, and highlight their evolutionary features and higher complexity in terms of gene copy numbers compared to species from other lineages. This work uncovers the presence in Mucoromycotina of abundant fucose-based polysaccharides similar to algal fucoidans. These unexpected polymers are associated with unusually low amounts of glucans and a higher proportion of chitin compared to *N. crassa*. The specific structural features are supported by the identification of genes potentially involved in the corresponding metabolic pathways. Phylogenomic analyses of genes encoding carbohydrate synthases, polysaccharide modifying enzymes and enzymes involved in nucleotide-sugar formation provide evidence for duplication events during evolution of cell wall metabolism in fungi. Altogether, the data highlight the specificity of Mucoromycotina cell walls and pave the way for a finer understanding of their metabolism.

Keywords: Cell wall; Fucose; Mucoran; Mucoromycotina; *Neurospora*; *Phycomyces*; *Rhizopus*.

Introduction

Mucoromycotina is an early diverging lineage of the kingdom Fungi dominated by saprobe species that grow on organic material such as decaying fruits or dung. As such, they can have a negative impact on the storage of food crops such as cereals, fruits and vegetables (Ray and Ravi, 2005). In addition, some species are parasites to plants or animals (Hoffmann *et al*., 2013). Typical examples are *Rhizopus oryzae* and *Mucor circinelloides*, which are responsible for an increasing number of infections in immunocompromised humans (Roden *et al*., 2005; Skiada *et al*., 2011; Ibrahim *et al*., 2012). Other species are beneficial to mankind and exploited in industry, for instance for the production of food additives or pharmaceuticals (Shaidi *et al*., 1999). The vegetative phase of Mucoromycotina consists of fast growing mycelium and anamorph structures. Sporangiospores are produced in multi-spored sporangia characterized by the inclusion of columella (Hoffmann *et al*., 2013). Members of the clade are haploid and their sexual phase comprises the fusion of two gametangia, which leads to the formation of a resting spore designated as zygospore (Carlile *et al*., 2001). Dimorphic species include *Mucor circinelloides* and *Mycotypha* spp, which grow as hyphae under normoxic conditions and as yeasts in anaerobic conditions with 30% CO_2 (Orlowski, 1991).

The early diverging phylogenetic placement of the Mucoromycotina provides excellent models to study the early history of the fungal kingdom (Stajich *et al.*, 2009). *R. oryzae* (properly identified as *Rhizopus delemar*) and *Phycomyces blakesleeanus* are members of the clade that have served as model systems owing to the availability of their genomes (Ma *et al*., 2009; http://genome.jgi.doe.gov/Phybl2/Phybl2.info.html). However, the genetic tools available for these species are limited. This has hindered the functional characterization of Mucoromycotina genes involved in vital morphogenetic processes, such as cell wall formation. There is evidence that acquisition of hyphal growth in the early diverging lineages

was enabled by changes in the localization and control of cell wall biosynthesis components (Harris *et al*., 2005). In spite of its essential role in cell development, integrity and protection (Bowman and Free, 2006; Latgé, 2007), the cell wall remains understudied in most fungi (Latgé and Calderone, 2005).

Chitin is a linear polymer of β-1,4-linked *N*-acetylglucosamine (GlcNAc) residues that occurs in the majority of fungal walls, except from a limited number species. It is typically crystalline and extraordinarily strong, and contributes to the stress-bearing property of the cell wall (Roncero, 2002; Klis *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 ends of the growing chitin chains (Imai *et al*., 2003). CHS have been classified into six (Bowen, 1992) and more recently seven (Riquelme and Bartnicki-García, 2008) classes (CHS I to VII). Some of these proteins contain *N*-terminal myosin motor domains (Fujiwara *et al*., 1997) involved in their secretion to the plasma membrane (Schuster *et al*., 2012). Chitin is susceptible to post-synthetic modifications catalyzed by chitin deacetylases (CDA), which lead to the partial or full deacetylation of chitin to form chitosan (Zhao *et al*., 2010). Other enzymes acting on chitin and chitosan are hydrolytic proteins, *i.e.* chitinases and chitosanases, respectively (Eijsink *et al*., 2010).

Glucans represent another family of fungal wall polymers widely distributed across the fungal kingdom. They contain essentially β-1,3 and β-1,6 linkages, although α-1,3- and α-1,4-linked glucans also occur in some species (Latgé and Calderone, 2005). In *Saccharomyces cerevisiae*, β-1,3-glucans are considered to be synthesized by membrane-bound glycosyltransferases (GT) designated as FKS1 and FKS2 that belong to GT family 48 (Orlean, 2012). Genome surveys suggest that *FKS* genes are conserved across the fungal kingdom (Latgé and Calderone, 2005) and, based on the work in yeast, β-1,3-glucans are generally considered to be synthesized by FKS1 orthologues in other species. However, direct

100 experimental evidence of the biochemical activity of FKS proteins is lacking in most
101 instances. The same applies to most other putative cell wall biosynthetic enzymes.
102 As a first step toward understanding cell wall formation in Mucoromycotina, we have
103 analyzed the fine cell wall polysaccharide compositions of the mycelia from *R. oryzae* and *P.*
104 *blakesleeanus* and compared them with that of the well-studied ascomycete *N. crassa*. As
105 opposed to the latter species, studies of the cell wall structure and metabolism in
106 Mucoromycotina are limited (Bartnicki-García and Nickerson, 1962; Datema *et al*., 1977a;
107 Datema *et al*., 1977b Van Laere *et al*., 1977; Tominaga and Tsujisaka, 1981). Our analyses
108 revealed unexpected structural differences between the Mucoromycotina and *N. crassa*. Of
109 particular significance is the occurrence of high proportions of branched algal-types of fucans
110 in *R. oryzae* and *P. blakesleeanus*. These structural differences are described in detail and
111 correlated with the presence of genes potentially involved in the formation of the
112 corresponding distinguishing cell wall polysaccharides. Additional annotation of the
113 sequenced genomes was also performed and phylogenetic analyses of the key putative cell
114 wall genes identified were made to shed light on duplication events during evolution.
115
116 **Results**
117
118 *Analysis of cell wall polysaccharides from* N. crassa
119 Sugar analysis of the cell wall polysaccharides of the ascomycete *N. crassa* revealed that they
120 contain glucose (73%) and GlcNAc (9%) as the two most abundant monomers (Fig. 1).
121 Linkage analysis showed that the GlcNAc fraction was dominated by 1,4-linked residues
122 (≈99.9%) together with minute amounts (≈0.1%) of terminal non-reducing sugars (Fig. 2),
123 pointing to the occurrence of chitin chains of high degrees of polymerization. This was further
124 supported by the fact that only trace amounts of GlcNAc were detected in the alkali-soluble

125 fraction (ASF), thereby indicating that virtually all GlcNAc detected arises from alkali-
126 insoluble chitin (Fig. 3B and C).

127 Linkage analysis showed that almost all of the glucosyl residues in *N. crassa* cell walls
128 arises from 1,3-glucans (Fig. 2). The use of alkali to fractionate the cell wall revealed that the
129 alkali-insoluble fraction (AIF) represented 55.5% of the total cell wall (Fig. 3A). More than
130 50% of the cell wall 1,3-glucans were insoluble in strong bases and partitioned in the AIF
131 (Fig. 3B and C). This behavior is typical of long chains of unbranched 1,3-glucans. The
132 occurrence of 1,6-branching points in glucans typically increase their solubility. Thus, it can
133 be postulated that the 1,3,6-linked glucosyl residues indicative of such 1,6-branching points
134 most likely arose from alkali-soluble 1,3-glucans (Fig. 2 and data not shown). The proportion
135 of such branching points did not exceeded 5%, as judged by the amount of 1,3,6-linked
136 glucosyl residues detected (Fig. 2). A small proportion of 1,4-linked glucosyl units (<4%)
137 were also detected together with trace amounts of 1,4,6-linked glucosyl residues (Fig. 2),
138 indicating that residual glycogen/starch-like polymers remained in the *N. crassa* cell wall
139 preparation even after the multiple cycles of amylase treatments performed.

140 Mannose (Man), galactose (Gal) and *N*-acetylgalactosamine (GalNAc) residues
141 represented 4 to 9% of the total sugars in the cell wall polysaccharides of *N. crassa*, whereas
142 trace amounts only of glucuronic acid (GlcA) were detectable (Fig. 1). Mannans were found
143 to be 1,2-linked with many branches in position 6 as judged by the relatively high content in
144 1,2,6-mannosyl residues (Fig. 2). A significantly higher proportion of terminal galactose
145 residues occurring in both the pyranose (t-Gal*p*) and furanose (t-Gal*f*) forms (4% altogether)
146 was detected compared to ~1% of 1,4-galactosyl units (Fig. 2). Altogether these data suggest
147 the occurrence of galactomannan polymers in the wall of *N. crassa*. These polysaccharides
148 would consist of internal 1,2-mannosyl residues, some of which form 1,6 branching points
149 (1,2,6-Man) that hold short 1,4-Gal chains with either t-Gal*p* or Gal*f* at their non-reducing

ends. The type of glycosidic bonds linking the GalNAc residues could not be determined due to the low abundance of this sugar in the wall of *N. crassa* and the typical low response factors of permethylated alditol acetates of amino sugars during linkage analysis by GC/MS.

Distinguishing structural features of Mucoromycotina cell walls

Cell wall analyses of the Mucoromycotina *P. blaskesleeanus* and *R. oryzae* revealed strikingly different cell wall compositions compared to *N. crassa*. The GlcNAc content of the *P. blaskesleeanus* and *R. oryzae* walls was 2 to 2.5 fold higher than in *N. crassa*, representing 16 and 34% of the total cell wall sugars, respectively (Fig. 1). As expected, linkage analysis revealed the occurrence of the terminal- and 1,4-linked GlcNAc residues characteristic of chitin (Fig. 2). A small proportion (≈1-3%) of these residues was detected in the ASF sample (Fig. 3C) pointing to the presence of small amounts of GlcNAc-based saccharides of higher solubility than chitin. Most strikingly, and as opposed to the walls of *N. crassa* and other well characterized fungal species, the 1,3-glucan content of the *P. blakesleeanus* and *R. oryzae* mycelial walls was remarkably low and did not exceed 3-4% of the total wall glycans (Fig. 1-3). This low abundance of glucans is partly counterbalanced by the higher proportion of GlcNAc denoted above. However, the most important distinctive feature of the Mucoromycotina walls is their high content in fucose (Fuc) and GlcA (Fig. 1-3). A significantly larger proportion of these monosaccharides partitioned in the ASF (Fig. 3B and C), which represented 66.5% and 47.5% of the total cell walls of of *P. blakesleeanus* and *R. oryzae*, respectively (Fig. 3A). The cell wall of *P. blakesleeanus*, however, contained a significantly higher proportion of Fuc (33%) and GlcA (34%), compared to *R. oryzae* (20 and 23% of Fuc and GlcA, respectively) (Fig. 1). The occurrence of fucose-based glycans in such high proportions has never been reported in fungi. Linkage analysis showed that the fucose residues in the two Mucoromycotina species analyzed are essentially 1,3-linked (5.5-8% of

the total wall) although 1,4-linked fucosyl residues were also detected in smaller proportions (0.8-1.2% of the total cell walls) (Fig. 2). The 1,3-fucan chains are highly branched at position 2 in both *P. blakesleeanus* and *R. oryzae*, as indicated by the comparable relative proportions of 1,3- and 1,2,3-linked fucosyl residues in each species (5% of each fucosyl residues in the total cell walls of *R. oryzae* and 10% in *P. blakesleeanus*) (Fig. 2). In addition, the high proportion of terminal-fucose (t-Fuc) in both species (9% in *R. oryzae*; 14% in *P. blakesleeanus*) indicates that these fucans are of a short degree of polymerization. Consistent with this observation, a much higher proportion of the fucosyl residues arose from soluble fucans as judged by their significantly higher partitioning in the ASF sample during cell wall fractionation (Fig. 3B and C). GlcA was also essentially recovered in the ASF sample (Fig. 3) where it forms 1,4-linked linear chains (Fig. 2). Man represented no more than 3 and 7% of the total walls in *P. blakesleeanus* and *R. oryzae*, respectively (Fig. 1). In both species, two third of the mannose residues were 1,2-linked, the remaining corresponding to terminal Man (Fig. 2). The cell walls of *P. blakesleeanus* and *R. oryzae* contained 9 and 12% Gal, respectively (Fig. 1). The sugar essentially occurred as a terminal residue, suggesting that it decorates polysaccharides with backbones that consist of other monosaccharides (Fig. 2).

Degree of N-*acetylation of chitin*

The degree of *N*-acetylation of chitin was determined by Fourier-transform infrared (FTIR) spectroscopy in the 3 fungal species analyzed, by comparison with commercial chitins with varying degrees of *N*-acetylation (Table 1, Fig. S1 and S2). The *N*-acetylation degree of the intact and partially alkali-de-acetylated chitin from crab shells (Butchosa *et al*., 2013) were 84.9% and 73.5%, respectively (Table 1 and Fig. S1A). Chitin from the Mucoromycotina species exhibited degrees of *N*-acetylation comparable to that of the partially alkali-de-acetylated chitin from crab, with 70.9% acetylation in the case of *R. oryzae* and 75.3% for *P.*

blakesleeanus (Table 1 and Fig. S2). Chitin from *N. crassa* was significantly more acetylated (78.9%) than its Mucoromycotina counterparts.

Phylogenomic profiling of the Mucoromycotina genes directly involved in the formation or modification of cell wall polysaccharides

In the next part of our work, we undertook a phylogenomic profiling approach to shed light on cell wall metabolism in Mucoromycotina, with a primary focus on genes potentially involved in the biosynthesis of cell wall polysaccharides in *R. oryzae* and *P. blakesleeanus*. For these investigations we continued using *N. crassa* as a reference organism. The expression of all genes identified and discussed here was confirmed by RNA sequencing analyses of the mycelia from growing vegetative cultures of *N. crassa* and *R. oryzae* (Sain, Rivera, and Stajich, submitted; BioProject PRJNA233610, PRJNA188720).

Multiple copies of putative *CHS* genes were identified by sequence homology in *R. oryzae* (28 copies) and *P. blakesleeanus* (24 copies) (Table 2). The corresponding accession numbers are listed in Table S1. All *CHS* genes were phylogenetically resolved into different major classes using the *N. crassa CHS* genes as reference (Fig. 4). In contrast to Dikarya, the Mucoromycotina comprise only four classes of chitin synthases instead of seven. These are the Division 1 ancestral class I and classes IV, V and VII of Division 2 (Fig. 4). In addition, phylogenetic analysis revealed the possible existence of a new class (VIII) in Division 2, which comprises Mucoromycotina homologs only (Fig. 4). Both *R. oryzae* and *P. blakesleeanus* have multiple copies of members of each of these classes, indicating a multi-duplication event in the Mucoromycotina lineage. Multiple copies of *CDA* genes were also found in both Mucoromycotina analyzed (34 in *R. oryzae*; 16 in *P. blakesleeanus*) whereas two *CDA* copies only seemed to occur in *N. crassa* after a first round of analysis. In the latter case though, a careful examination allowed us to identify a third putative CDA homolog

(NCU10651) that contains CDA domains and an additional chitin binding domain (CBM18). Thus, the total number of *CDA* genes in *N. crassa* is most likely 3 (Table 2). Examination of the gene tree shows multiple copies with relatively short branches of *R. oryzae* and *P. blakesleeanus CDA* genes, and species-specific clades indicating expansions within the Mucoromycotina (Fig. 5). The number of genes encoding chitin and chitosan hydrolytic enzymes was comparable in all three species analyzed, with 8 to 14 genes identified that belong to glycoside hydrolase (GH) families 18 and 19 (chitinases) and 1 or 2 genes in each species from GH family 75 (chitosanase) (Table 2). Altogether the data show that, as opposed to chitinases and chitosanases, the genes related to chitin biosynthesis (*CHS*) and modification (*CDA*) have expanded in copy number in the Mucoromycotina diverging lineage compared to Dikarya fungi (Table 2).

Putative genes involved in β-1,3-glucan biosynthesis (GT48) show the same trend as those related to chitin formation and modification. *N. crassa* contains one functional copy of the putative β-1,3-glucan synthase (FKS1), whereas *R. oryzae* and *P. blakesleeanus* both possess 3 homologs of this gene (Table 2 and Fig. S3). The corresponding duplication event was revealed by phylogenetic analysis (Fig. S3). Interestingly, as opposed to *N. crassa*, the Mucoromycotina species analyzed did not contain any gene potentially involved in α-1,3-glucan biosynthesis, thereby suggesting that all 1,3-linked glucosyl residues identified experimentally (Fig. 2) arose from β-1,3-glucans. Thus, it can be inferred that these species are devoid of glucans of the α-1,3 type. The number of genes encoding putative β-1,3-glucanosyltransferases from family GH72 was more than twice as high in *N. crassa* than in both Mucoromycotina species analyzed (2) (Table 2). Enzymes belonging to this family have been shown to exhibit transglycosylase activity leading to the formation of new 1,6-branching points (Mouyna *et al.*, 1998) or 1,3-linkages (Hartland *et al.*, 1996; Mouyna *et al.*, 2000). Thus, these enzymes catalyze the formation of either branched β-1,3-glucans or β-1,3-glucans

of a longer degree of polymerization than the original substrate. The absence of 1,3,6-linked glucosyl residues in the walls of *R. oryzae* and *P. blakesleeanus* (Fig. 2) suggests that the Mucoromycotina enzymes identified here are involved in molecular mass increase of β-1,3-glucans. The Mucoromycotina species were devoid of the type of GH16 transglycosylases that form crosslinks between chitin and β-1,3-glucans, as opposed to *N. crassa* which contained 13 copies of putative genes encoding such enzymes (Table 2). Other enzymes involved in glucan metabolisms are hydrolytic proteins. Two copies of a GH5 exoglucanase were found in both Mucoromycotina species together with 3 β-1,3-glucanases (Table 2). Two of the latter enzymes are from family GH17 while the third one belongs to GH family 81 (Table S1). *N. crassa* has one GH 5 exoglucanase only but more than 3 times as many β-1,3-glucanases as *R. oryzae* and *P. blakesleeanus*, with members of GH families 17, 55 and 81 (Tables 2 and S1). In addition to 1,3-glucan hydrolytic enzymes, all species analyzed contained β-1,4-glucanases from GH families 5, 6, 12 and 45 (Table S1), with a total number of 9 genes for *N. crassa* and *R. oryzae* and 3 for *P. blakesleeanus* (Table 2). None of the genes that encode other hydrolytic enzymes in *N. crassa* were present in the Mucoromycotina species analyzed (Table 2). Altogether these data indicate the occurrence of a more comprehensive series of glucan hydrolytic activities in *N. crassa* compared to the Mucoromycotina *R. oryzae* and *P. blakesleeanus*.

As opposed to chitin and glucan synthases, the total number of putative genes involved in mannan biosynthesis was significantly lower in the Mucoromycotina species than in *N. crassa*, with no more than 3 mannosyltransferases in *R. oryzae* and 2 in *P. blakesleeanus* compared to 9 in the ascomycete (Table 2). In addition, the Mucoromycotina were devoid of genes encoding mannan transglycosylating activities that form 1,6 linkages (Table 2). A striking difference between the ascomycete and Mucoromycotina is the occurrence of a significantly higher number of mannosyltransferases involved in lipid glycosylation in *N.*

crassa, either for glycolipid biosynthesis or for the formation of dolichol-based precursors required for *N*-glycosylation of proteins (Table 2). The only type of hydrolytic enzymes acting on Man-based structures that were identified in the Mucoromycotina are one β-mannosidase and up to 9 α-1,2-mannosidases from the GH47 family (Table 2). *N. crassa* contained similar genes, but the α-1,2-mannosidases were from both GH families 47 and 92 (Tables 2 and S1). None of the other mannosidases encountered in *N. crassa*, including α-1,6-mannosidases from GH family 76, occurred in the Mucoromycotina (Table 2). Altogether, these data are consistent with the occurrence of 1,2-linked Man and the absence of 1,6-linked Man in the walls of *R. oryzae* and *P. blakesleeanus* (Fig. 2).

Compared to the families of genes presented above, a more limited number of genes could be identified that are putatively involved in Gal and GalNAc metabolism (Table 2). The Mucoromycotina species contained only one β-galactosyltransferase, but multiple α-galactosidases, whereas *N. crassa* was characterized by the absence of putative β-galactosyltransferase but the occurrence of galactanases as well as α- and β-galactosidases (Table 2). No GalNAc transferase gene was identified in the Mucoromycotina species, as opposed to *N. crassa* (Table 2).

Phylogenomic profiling of the Mucoromycotina genes involved in nucleotide-sugar biosynthesis

In addition to the analysis of genes involved directly in the biosynthesis and modification of cell wall polysaccharides, we searched the genomes of *R. oryzae*, *P. blakesleeanus* and *N. crassa* for the presence of genes involved in the biosynthesis of nucleotide-sugars that are used as precursors of wall components, namely UDP-D-Glc, UDP-D-GlcNAc, GDP-L-Fuc, GDP-D-Man and UDP-D-GlcA. The copy numbers of each of the identified genes are shown in Table 3 and the corresponding accession numbers are presented in Table S1.

Up to two copies of each of the four genes required for the biosynthesis of UDP-D-GlcNAc from glutamine and fructose were identified in all three fungal species studied (Table 3). Thus, the whole pathway for chitin biosynthesis could be reconstructed for each micro-organism. Similarly, the two genes required for UDP-D-glucose formation from glucose-6-phosphate, namely phosphoglucomutase and UDP-Glc pyrophosphorylase (UTP-glucose-1-phosphate uridylyltransferase), were identified in the ascomycete and the two Mucoromycotina. *R. oryzae* contained two copies of each gene while the two other species studied had only one copy of each gene (Table 3).

UDP-D-GlcA is synthesized from UDP-D-Glc by the enzyme UDP-Glc 6-dehydrogenase. A search for genes involved in the GlcA biosynthetic pathway identified 2 copies of UDP-Glc 6-dehydrogenase in *R. oryzae* and one in *P. blakesleeanus* (Table 3). The biochemical analysis showed the presence of traces of GlcA in the cell wall of *N. crassa* (Fig. 1). Consistent with this observation, a putative UDP-Glc 6-dehydrogenase gene, which could be responsible for the biosynthesis of UDP-D-GlcA, was identified in the genome of this species.

Several genes required for the biosynthesis of GDP-D-Man (glucose-6-phosphate isomerase, mannose-6-phosphate isomerase, phosphomannomutase and mannose-1-phosphate guanylyltransferase) were identified in *R. oryzae*, *P. blakesleeanus* and *N. crassa,* consistent with the biochemical observation of Man in the cell walls of all three species (Table 2). GDP-L-Fuc is synthesized from GDP-D-Man by the consecutive action of GDP-D-Man 4,6-dehydratase, which forms the intermediate GDP-4-keto-6-deoxy-D-Man, and GDP-L-Fuc synthase which converts the deoxy intermediate to GDP-L-Fuc. The genes corresponding to GDP-Man 4,6-dehydratase were identified in *R. oryzae* (RO3G_05644, RO3G_12053 and RO3G_15908) and *P. blakesleeanus* (pbla_t_17728 and pbla_t_56971 (JGI transcript accessions 17728 and 56971, respectively). A GDP-L-Fuc 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 *N. crassa,* consistent with the lack of detection of Fuc-based polysaccharides in the cell wall of this species (Fig. 1). The phylogenetic tree presented in Figure S4 shows the evolutionary relationship between the genes involved in Fuc metabolism in other sequenced Mucoromycotina (*Mortierella alpina*, *M. circinelloides*), Basidiomycetes (*Puccinia graminis sp. tritici*, *Sporobolomyces roseus*) and the bacterium *Escherichia coli.* No significantly similar homologs of these genes were found in any other fungal species using Fungicyc (http://fungicyc.broadinstitute.org/), hmmsearch and the Uniprot database (http://uniprot.org/); notably none were found in any ascomycete.

Discussion

Fungal cell walls contain some vital polysaccharides that are not encountered in plants and animals. Thus, the enzymes involved in their biosynthesis represent ideal targets of anti-fungal drugs for disease control. However, a better understanding of cell wall structure and biosynthesis in pathogenic fungi is needed for the rational design of new efficient inhibitors. Of particular importance are early diverging lineages that comprise severe pathogenic species, such as the Mucoromycotina, for which a limited spectrum of effective antifungals are available. Pioneering reports on the Mucoromycotina *Mucor rouxii* (Bartnicki-García and Nickerson, 1962), *Mucor mucedo* (Datema *et al*., 1977a; Datema *et al*., 1977b) and *P. blakesleeanus* (Van Laere *et al*., 1977) pointed to the existence of cell wall diversity across fungal classes. But despite these early observations, fungal cell walls have been understudied during the past decades. More recently, the sequencing of multiple fungal genomes has led to the increased use of *in silico* approaches to link gene repertoires to cell wall structures (see for instance Borkovich *et al.*, 2004; Ruiz-Herrera *et al.*, 2008; de Groot *et al.*, 2009; Balestrini *et*

350 *al.*, 2012). However, in these reports the proposed relationships between the (putative) cell
351 wall related genes and polysaccharide compositions rely essentially on experimental data that
352 are either not comprehensive, such as immunolocalization studies dependent on the
353 availability of antibodies, or that were obtained several decades ago using methods of limited
354 resolution. Thus, in most cases, an extensive treatise linking the fine biochemical architecture
355 of fungal cell walls to the genomic repertoire of the corresponding lineages is lacking. The
356 primary objective of our work was to tackle this issue in Mucoromycotina by focusing in the
357 first instance on the species *R. oryzae* and *P. blakesleeanus*. For this purpose, we have
358 revisited the cell wall polysaccharide composition of these organisms, thereby demonstrating
359 important structural differences compared to the well-studied ascomycete *N. crassa*.
360 Additional annotation of the sequenced genomes of *R. oryzae* and *P. blakesleeanus* allowed
361 the identification of multiple copies of genes involved in cell wall polysaccharide metabolism.
362 This repertoire of newly uncovered genes was correlated to our structural analyses of the
363 Mucoromycotina cell walls.

364 Chitin governs the architecture and physical properties of most fungal cell walls by
365 forming a scaffold for abundant "matrix" polysaccharides, such as β-1,3- and β-1,6-glucans.
366 Despite its high structural importance, it usually represents no more than 10% of the whole
367 fungal cell wall. This is the case for *N. crassa* in which we detected 9% of chitin, in
368 agreement with earlier reports on other wild-type strains (Mahadevan and Tatum, 1965).
369 However, the proportion of chitin was significantly higher in both *R. oryzae* and *P.*
370 *blakesleeanus*, where it represented up to 30% of the total cell walls. These values are
371 consistent with the 35.8% of hexosamines reported in the only report available on the cell
372 wall of *R. oryzae* (Tominaga and Tsujisaka, 1981), but significantly lower than the 45.6%
373 reported in 1977 for *P. blakesleeanus* (Van Laere *et al*., 1977). Mucoromycotina possess four
374 classes of CHS, *i.e.* one ancestral class (I, II or III) from Division 1 and one of each class IV,

375 V and VII from Division 2. This contrasts with the seven classes present in *N. crassa* and
376 suggests a diversification of the Division 1 group into three classes as well as a gain of class
377 VI after the divergence of Dikarya, possibly reflecting different functional requirements.
378 Classes IV, V and VII were however necessary even for early fungi. Interestingly, a distinct
379 fifth class occurring in Mucoromycotina only was identified. It was characterized by the
380 presence of a chitin synthase 2 domain together with a combination of other domains present
381 in CHS from Division 2. The fact that all five classes show gene expansions unique to the
382 Mucoromycotina suggests that these expansions are important for early fungi. In addition, the
383 higher chitin content found in Mucoromycotina compared to *N. crassa* may be a consequence
384 of *CHS* gene expansion. Since fungal life cycles typically consist of several distinct
385 developmental stages, as is the case for Mucoromycotina, it is likely that gene expansion
386 within one or several classes of a given organism reflects functional specialization at different
387 morphogenetic stages. This question may be addressed in the near future with the forthcoming
388 increasing availability of gene expression data from the different developmental stages of
389 multiple fungal species. Alternatively, in some species gene expansion may not be limited to a
390 single category of genes (*e.g.* cell wall related genes) but rather represent a more global
391 feature specific of the genome considered.

392 Similar observations as for CHS were made for the *CDA* genes. These could be
393 divided into two different groups: the first one contains the *CDA* from *N. crassa* and some
394 copies of the *R. oryzae* and *P. blakesleeanus* homologs, whereas the second group consists of
395 Mucoromycotina homologs only. Both CHS and CDA showed recent gene expansions, as
396 judged by the lengths of the tree branches. These data suggest that this expansion was
397 important for the Mucoromycotina clade. If the higher chitin content in Mucoromycotina is
398 well supported by the occurrence of multiple *CHS* genes, the impressively high number of
399 *CDA* genes is not correlated with particularly low degrees of chitin *N*-acetylation. Indeed,

even if our estimations of the degrees of *N*-acetylation of chitin indicate that the glucosamine-based polymers in Mucoromycotina contain less acetyl groups than those from ascomycetes, the chitin from Mucoromycotina still consists of up to 75% acetylated residues (Table 2). This data is in keeping with the studies in *M. mucedo* where no homopolymers of de-acetylated glucosamine were found (Datema *et al*., 1977b). It is likely that the degree of acetylation of chitin varies during the life cycle and that some CDA genes are more specifically responsible for chitin deacetylation at specific developmental stages. Expression profiling and detailed analysis of gene regulation during the different stages of the life cycle are needed to demonstrate and decipher the possible developmental specialization of the different CDA genes.

As for the *CHS* and *CDA* genes, duplications of the putative β-1,3-glucan synthase genes (*FKS1*) occurred in Mucoromycotina. However, the number of *FKS1* genes does not reflect glucan content since considerably lower proportions of 1,3-linked glucosyl residues were detected in the Mucoromycotina cell walls compared to *N. crassa.* These observations confirm that the number of gene copies only loosely relate to the actual amount of a biosynthetic compound. It is likely that not all copies are functional or that additional levels of regulation influence the total amount of final product. Studies in *M. rouxii* suggested that glucosyl residues occur in spores only, but not in hyphal walls (Bartnicki-García and Reyes, 1964), thereby implying that glucan synthase genes are developmentally regulated and not functional in hyphae. This is however contradictory with our data, which clearly showed the occurrence of small amounts of 1,3-glucans in the hyphal walls of both Mucoromycotina species analyzed.

Our phylogenomic analysis allowed the full reconstruction of the pathways involved in the biosynthesis of most of the precursors of the identified cell wall polysaccharides, namely UDP-D-Glc, UDP-D-GlcNAc, GDP-L-Fuc, GDP-D-Man and UDP-D-GlcA. In

addition, we were able to identify a number of putative transglycosylases and polysaccharide hydrolases with predicted biochemical functions that match the type of glycosidic linkages detected experimentally through cell wall carbohydrate analyses.

Fucose is one of the few cell wall monosaccharides that belong to the L series. In plants, it is mainly found as a constituent of xyloglucan side chains (Scheller and Ulvskov, 2010). The only known polymers that consist of a majority of fucosyl residues are sulfated fucans (also called fucoidans) (Berteau and Mulloy, 2003). Lower proportions of other sugars, *e.g.* Man, Gal, xylose and uronic acids are also present in fucoidans (Li *et al*., 2008). This type of polysaccharides typically occurs in brown algae, *e.g. Fucus vesiculosus* and *Ascophyllum nodosum*, although some reports have also described their presence in marine invertebrates like sea cucumber and sea urchins (Mourão and Bastos, 1987; Ribeiro *et al*., 1994). The precise sugar composition and structure of fucoidans vary considerably depending on their biological origin (Berteau and Mulloy, 2003). The high Fuc content observed in the cell walls of both *R. oryzae* and *P. blakesleeanus*, together with their unusually high proportion of GlcA and the presence of Man and Gal, strongly suggest the occurrence of fucoidan-like polymers in Mucoromycotina. The covalent association of this group of monosaccharide heteropolymeric entities has been suggested earlier for *M. rouxii* and *M. mucedo* (Datema *et al*., 1977a; Bartnicki-García and Reyes, 1968). Indeed, Datema *et al*. (1977a) defined a "glycuronan" component consisting of Fuc, Man, Gal and GlcA (molar ratio of 5:1:1:6), whereas Bartnicki-García and Reyes (1968) described the occurrence of two types of polyuronides in *M. rouxii*: (*i*) the heteropolysaccharide "mucoran" consisting of GlcA, Fuc, Man and Gal, and (*ii*) the homopolysaccharide "mucoric acid". These observations are somewhat consistent with our data, although quantitative discrepancies do exist. Our linkage analyses showed that the GlcA chains are not branched and could be forming "mucoric acid". On the other hand, the high proportion of terminal GlcA (t-GlcA)

means either that polymers of glucuronic residues are of a low degree of polymerization or that t-GlcA acts as a side decoration of a polymer whose main backbone consists of Fuc. Additional substitutions of the Fuc residues may correspond to Gal and/or GlcA residues. The protocol used for glycosidic linkage analysis provokes the β-elimination of uronic acids and the monosaccharides linked to them if no precaution is taken to protect the acidic groups by reduction with $NaBD_4$. The fact that we were able to detect permethylated derivatives only after the reduction of the GlcA residues proves the occurrence of polymers highly substituted with t-GlcA, namely "mucorans". The latter can be defined as the fungal equivalent of algal fucoidans. However, one of the characteristics of fucoidans is that they contain substantial percentages of sulfated residues (Berteau and Mulloy, 2003). Our assays of sulfated esters of sugar residues (see Experimental procedures) revealed the absence of such groups in the fungal fucoidan-like ("mucoran") polymers (data not shown).

Our phylogenomic approach supports well the biochemical detection of Fuc in Mucoromycotina, as evidenced by the identification of putative GDP-D-Man 4,6-dehydratases and GDP-L-fucose synthases in *R. oryzae* and *P. blakesleeanus*. In addition to Mucoromycotina, Fuc has been reported in some pucciniomycetes (Basidiomycota), particularly in the germ tubes of *P. graminis* (Kim *et al*., 1982) and some species of *Sporobolomyces* (Takashima *et al*., 2000). Our genome analysis identified homologs of genes involved in Fuc biosynthesis in *P. graminis sp. tritici* and *S. roseus*, which supports the concept that the occurrence of Fuc is restricted to zygomycetes and basidiomycetes. This could be the result of a gain in the Zygomycota-Dikarya ancestors and a loss in Ascomycota, as well as a horizontal transfer from host plants. These hypotheses remain, however, to be further tested.

In conclusion, the salient finding of our study is the identification of the specific structural features of the Mucoromycotina cell walls. The combination of biochemical and

phylogenomic approaches has correlated these unique cell wall compositions with a repertoire of genes required for polysaccharide biosynthesis and modification. It is worth highlighting the unusually low glucan contents found in the Mucoromycotina species. This deficiency is counterbalanced by high levels of chitin (scaffolding role) and "mucorans", *i.e.* heteropolymers of a higher solubility (cementing role) that consist essentially of Fuc and GlcA residues. Mucorans exhibit high structural similarities with fucoidans from brown algae. Further characterization of these polymers may allow the development of diagnostic detection tests for human pathogens based on specific cell wall epitopes.

Experimental procedures

Fungal strains and cultures

All carbohydrate structural analyses were performed on purified walls from mycelial cells of the following fungal strains from the Fungal Genetics Stock Center (http://www.fgsc.net): *P. blakesleeanus* (NRRL1555; mating type '-'), *R. oryzae* (RA99880) and *N. crassa* (74-OR23-1VA (FGSC 2489; mating type 'A')). All strains were grown overnight at room temperature in glass tubes containing either Vogel's liquid minimal culture medium (VM) (Vogel, 1956) for *N. crassa* or potato dextrose broth (PDB) for *R. oryzae* and *P. blakesleeanus*. The mycelia were harvested by vacuum filtration on Whatman filter paper, extensively washed with distilled water to remove the excess of culture medium, lyophilized and stored in sealed vials.

Preparation of cell walls

Cell wall polysaccharides were prepared as previously described (Mélida *et al*., 2013). Briefly, the freeze-dried cells were disrupted in liquid nitrogen using a mortar and pestle until fine powders were obtained. The latter were subjected to ethanol extractions (Mélida *et al*.,

2013) and the resulting alcohol-insoluble residues (AIR) were depleted in proteins by heating the samples three consecutive times at 80ºC for 10 min in a 50 mM Tris-HCl buffer pH 7.8 containing 2% (w/v) sodium dodecyl sulfate, 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) (Mélida *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 in a desiccator until fractionation into alkali-soluble (ASF) and alkali-insoluble (AIF) fractions using hot methanol-KOH solutions (Mélida *et al*., 2013). These extraction procedures were repeated independently and in exactly the same conditions on two separate mycelial cultures of each species. Monosaccharide and linkage compositions were determined as described below by analyzing three technical replicates of each of the two independent cell wall samples prepared from each species, thereby generating a total of six values for each species and type of analysis. The latter were averaged and data are presented as means (± S.D.) of each set of six determinations.

Monosaccharide analysis

The dried purified cell walls and the corresponding ASF and AIF preparations (5 mg) were hydrolyzed with 72% sulfuric acid at room temperature for 3 h. The acid was then diluted to 1 M and the samples were heated at 100ºC for 3 h. *Myo*-inositol was used as an internal standard. The resulting monosaccharides were converted to alditol acetates as described previously (Blakeney *et al*., 1983) 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}. Uronic acid residues were analyzed in the same chromatographic conditions as above, after conversion to

525 6,6-dideuterio neutral sugar derivatives. For this purpose, the corresponding carboxyl groups
526 in the intact cell wall carbohydrates were activated with carbodiimide and reduced in the
527 presence of sodium borodeuteride ($NaBD_4$), prior to acid hydrolysis of the polysaccharides
528 (Kim and Carpita, 1992).
529
530 *Glycosidic linkage analysis*
531 Polysaccharide networks in the dry carboxyl reduced cell wall samples (0.1 mg) were first
532 swollen in 200 µL dry dimethylsulfoxide (DMSO). Ten µL of DMSO containing 0.3 mg L^{-1}
533 sulphur dioxide and 5 µL of diethylamine were added and the samples were subsequently
534 sonicated for 20 min and stirred under argon at room temperature for 3 h. Methylation
535 reactions were performed using the $NaOH/CH_3I$ method (Ciucanu and Kerek, 1984), by
536 repeating 5 times the methylation step on each sample, thereby avoiding any risk of
537 undermethylation. Partially methylated polysaccharides were hydrolysed in the presence of 2
538 M TFA at 121ºC for 3 h and converted to permethylated alditol acetates (Albersheim *et al*.,
539 1967). The latter were separated and analyzed by GC/EI-MS on a CP-Sil 5 CB capillary
540 column (30 m x 0.25 mm i.d.; Agilent Technologies) with a temperature program increasing
541 from 160ºC to 210ºC at a rate of 1ºC min^{-1}. The mass spectra of the fragments obtained from
542 the permethylated alditol acetates (EI-MS) were compared with those of reference derivatives
543 and by comparison to available data (http://ccrc.uga.edu/specdb/ms/pmaa/pframe.html).
544
545 *Fourier-transform infrared (FTIR) spectroscopy*
546 Discs were prepared from mixtures of purified dry cell walls and KBr (1:100, w:w) using a
547 Graseby-Specac press. FTIR spectra were recorded in the 800-4000 cm^{-1} range with a
548 resolution of 1 cm^{-1}, using a Perkin-Elmer Spectrum 2000 instrument. They were normalized
549 and baseline-corrected with Spectrum v 5.3.1. The degree of *N*-acetylation of chitin and

chitosan was estimated from the A_{1655} cm^{-1} (amide I band) / A_{3450} cm^{-1} (hydroxyl band) ratio (Domszy and Roberts, 1985), by comparison with standards with known degrees of *N*-acetylation (Butchosa *et al*., 2013).

Analysis of sulfated polysaccharides

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

Phylogenetic analysis of cell wall gene families

Genomes from the mucormycetes *R. oryzae* (version 3; Ma *et al*., 2009) and *P. blakesleeanus* (JGI: http://genome.jgi.doe.gov/fungi) and the ascomycete *N. crassa* (version 10.5; Galagan *et al*., 2003) were used to perform phylogenetic comparison of gene families. Homologs of cell wall polysaccharide biosynthetic genes were identified by searching the *Saccharomyces* Genome Database (Cherry *et al*., 2012) (http://www.yeastgenome.org/cgi-bin/seqTools). The well characterized *CHS* genes from *S. cerevisiae* (Choquer *et al*., 2004; Mandel *et al*., 2006; Riquelme and Bartnicki-García, 2008) were used to augment the gene set and identify *CHS* families and their typical sequences in *R. oryzae*, *P. blakesleeanus* and *N. crassa*. A comparative homology search was performed using cell wall genes identified in *S. cerevisiae, N. crassa* and *Ustilago maydis* and profile Hidden Markov Models (HMM) implemented in the HMMER3 package (Eddy, 2009). For this purpose, multiple sequence alignments of seed homologs from these three species were constructed with T-Coffee (Notredame *et al*., 2000) and HMMER3 was run with global-global comparisons of alignments families. Homologs of each cell wall gene family were identified from each genome with hmmsearch and a cutoff bit

score higher than the log of the number of proteins in our database of selected fungal genomes. This typically resulted in expectation values lower than 1E-20.

The search for genes responsible for polysaccharide biosynthesis or remodeling was performed by mapping the metabolic pathways of fucose, mannose, galactose and glucuronic acid to the genome of *R. oryzae* and *N. crassa* using Fungicyc (http://fungicyc.broadinstitute.org/). The *P. blakesleeanus* genome was searched with hmmsearch to find homologs of these genes. In addition, a search on available mucormycetes (*M. alpina* and *M. circinelloides*) and ~~B~~asidiomycetes (*P. graminis sp. tritici* and *S. roseus*) genomes was performed to identify homologs of genes involved in fucose metabolism. The sequences of *Escherichia coli* glycosyltransferase genes involved in the biosynthesis of fucosylated glycans (Iguchi *et al*., 2009) were used to search for additional homologs in the Uniprot database (http://uniprot.org/).

The homologs were aligned with T-Coffee and the corresponding protein alignments were automatically trimmed for high quality regions alignments with trimAl using the automated1 parameter (Capella-Gutiérrez *et al*., 2009). Maximum likelihood phylogenetic trees were constructed with RAxML (Stamatakis, 2006) using protein substitution matrices selected with the ProteinModelSelection script part of RAxML.

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787

788 **Figure Legends**

789

790 **Fig. 1.** Monosaccharide composition (mol%) of the total cell walls from *Phycomyces*
791 *blakesleeanus*, *Rhizopus oryzae* and *Neurospora crassa*. Values are means ± S.D. of six
792 determinations as described in Experimental procedures. Fuc, fucose; Man, mannose; Gal,
793 galactose; Glc, glucose; GlcA, glucuronic acid; GlcNAc, *N*-acetylglucosamine; GalNAc: *N*-
794 acetylgalactosamine.

795

796 **Fig. 2.** Glycosidic linkage analysis (mol%) of the total cell walls from *Phycomyces*
797 *blakesleeanus*, *Rhizopus oryzae* and *Neurospora crassa*. Values are means ± S.D. of six
798 determinations as described in Experimental procedures. The different glycosidic linkages
799 and pyranose or furanose forms of each monosaccharide were deduced from EI-MS spectra.
800 Nomenclature used for the names of the sugar derivatives: Fuc, fucose; Man, mannose; Gal,
801 galactose; Glc, glucose; GlcA, glucuronic acid; GlcNAc, *N*-acetylglucosamine; "p" and "f" at
802 the end of a monosaccharide abbreviation indicate that the residue occurs in the pyranose or
803 furanose form, respectively; "t-" indicates a "terminal" monosaccharide, *i.e.* a
804 monosaccharide that occurs at the nonreducing end of a glycan; numbers separated by a
805 comma indicate linkage type, *e.g.* 1,2-Manp stands for 1,2-linked mannopyranose
806 (coorresponding to 1,2,5-tri-*O*-acetyl, 3,4,6-tri-*O*-methyl D-mannitol).

807

808 **Fig. 3.** Analysis of the alkali-insoluble (AIF) and alkali-soluble (ASF) fractions isolated from
809 the cell walls of *Phycomyces blakesleeanus*, *Rhizopus oryzae* and *Neurospora crassa*. A,
810 Relative proportions of the AIF and ASF fractions with respect to the total cell walls of *P.*
811 *blakesleeanus* (Pb), *R. oryzae* (Ro) and *N. crassa* (Nc). B and C, Monosaccharide
812 composition (mol%) of the AIF and ASF samples, respectively. Values are means ± S.D. of

six determinations as described in Experimental procedures. Fuc, fucose; Man, mannose; Gal, galactose; Glc, glucose; GlcA, glucuronic acid; GlcNAc, *N*-acetylglucosamine; GalNAc: *N*-acetylgalactosamine.

Fig. 4. Phylogenetic tree of chitin synthase genes in *Phycomyces blakesleeanus*, *Rhizopus oryzae* and *Neurospora crassa* built using RAxML with VTF as protein substitution model. Clades containing Division 1 (Classes I, II, II), Division 2 (Classes VI, V, VII) and Division 3 (Class VI) are labeled. Gene identifiers for *N. crassa* and *R. oryzae* are published locus identifiers. For unpublished *P. blakesleeanus* annotation, the numeric ID is the searchable transcript ID at the JGI (http://genome.jgi.doe.gov/pages/search-for-genes.jsf?organism=Phybl2).

Fig. 5. Phylogenetic tree of chitin deacetylase genes in *Phycomyces blakesleeanus*, *Rhizopus oryzae* and *Neurospora crassa* built using RAxML with WAGF as protein substitution model.

838 **Tables**

839

840 **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
Phycomyces blaskesleeanus	75.3 ± 1.3
Rhizopus oryzae	70.9 ± 0.9
Neurospora crassa	78.9 ± 5.8

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857 **Table 2.** Copy numbers of genes involved biosynthesis and modification of cell wall
858 polysaccharides in *Rhizopus oryzae*, *Phycomyces blakesleeanus* and *Neurospora crassa*.

Cell wall biosynthetic genes (CAZy family)	*R. oryzae*	*P. blakesleeanus*	*N. crassa*
Chitin metabolism:			
i) Chitin synthase (GT2)	28	24	7
ii) Chitinase (GH18-19)	14	8	10
iii) Chitin deacetylase (CE4)	34	16	3
iv) Chitosanase (GH75)	2	1	1
Glucan metabolism:			
i) β-1,3-Glucan synthase (GT48)	3	3	1
ii) α-1,3-Glucan synthase (GT5)	0	0	2
iii) β-1,3-Glucanosyltransferase (GH72)	2	2	5
iv) Chitin- β-1,6-Glucanosyltransferase / Endo-1,3- β-glucanase (GH16)	0	0	13
v) β-1,3-Glucanase (GH17, 55, 81)	3	3	10
vi) α-1,3-Glucanase (GH71)	0	0	4
vii) β-1,4-Glucanase (GH5, 6, 12, 45)	9	3	9
viii) Exoglucanase (GH5)	2	2	1
ix) Glucanase b (GH64)	0	0	2
x) Cellobiohydrolase (GH6, 7)	0	0	2
xi) β-1,6-Glucanase (GH30)	0	0	1
Fucose metabolism:			
i) α-Fucosyltransferase	4	2	0
Glucuronic acid metabolism:			
i) β-Glucuronidase (GH79)	0	0	1

Mannose metabolism:			
i) α-1,6-Mannosyltransferase (GT22, 32, 34)	0	0	5
ii) α-1,2-Mannosyltransferase (GT4)	0	0	1
iii) α-1,3-Mannosyltransferase (GT69)	0	0	1
iv) Mannosyltransferase	2	1	1
v) β-Mannosyltransferase	1	1	1
vi) GPI-Mannosyltransferase (GT22, 50, 76)	0	0	4
vii) Dolichyl-phosphate-mannose-protein mannosyltransferase	7	4	3
viii) Dolichyl-phosphate β-D-mannosyltransferase	1	2	1
ix) Dolichyl-phosphate-mannose-glycolipid α-mannosyltransferase (GT58)	1	1	1
x) Glycolipid 2-α-mannosyltransferase (GT15)	9	9	5
xi) α-1,6-mannotransglycosylase / α-1,6-mannanase (GH76)	0	0	2
xii) α-1,2-Mannosidase (GH47, 92)	9	6	8
xiii) α-Mannosidase (GH38)	0	0	1
xiv) α-1,6-Mannosidase (GH76)	0	0	7
xv) β-Mannosidase	1	1	2
Galactose metabolism:			
i) β-Galactosyltransferase	1	1	0
ii) β-1,6-Galactanase (GH30)	0	0	1

iii) β-Galactosidase (GH2, 35, 53)	0	0	6
iv) α-Galactosidase	4	6	1
GalNAc metabolism:			
i) Acetylgalactosaminyltransferase	0	0	1

878 **Table 3.** Copy numbers of genes involved in nucleotide-sugar biosynthesis in *Rhizopus*
879 *oryzae*, *Phycomyces blakesleeanus* and *Neurospora crassa*.

Nucleotide sugar pathway	*R. oryzae*	*P. blakesleeanus*	*N. crassa*
UDP-GlcNAc (chitin biosynthesis)			
i) Glutamine-fructose 6-phosphate amidotransferase	2	2	1
ii) Glucosamine 6-phosphate N-acetyltransferase	1	2	1
iii) N-acetylglucosamine-phosphate mutase	2	1	1
iv) UDP-N-acetylglucosamine pyrophosphorylase 1	2	1	1
UDP-Glc (glucan biosynthesis)			
i) Phosphoglucomutase	2	1	1
ii) UTP-glucose-1-phosphate uridylyltransferase	2	1	1
GDP-Man (mannan biosynthesis)			
i) Glucose-6-phosphate isomerase	1	1	1
ii) Mannose-6-phosphate isomerase	1	1	2
iii) Phosphomannomutase	1	1	1
iv) Mannose-1-phosphate guanylyltransferase	4	1	1
GDP-Fuc (fucan biosynthesis)			
i) GDP-Mannose 4,6-dehydratase	3	2	0
ii) GDP-L-Fucose synthase	1	1	0
UDP-GlcA (glucuronan biosynthesis)			
UDP-Glucose 6-dehydrogenase	2	1	1

880

881 **Supporting information**

882 **Fig. S1.** FTIR spectra of chitin (top) and partially de-acetylated chitin (bottom). Amide I
883 (A_{1655} cm^{-1}) and hydroxyl bands (A_{3450} cm^{-1}) are dash-highlighted.

884

885 **Fig. S2.** FTIR spectra of purified cell walls from *N. crassa* (top), *P. blakesleeanus* (middle)
886 and *R. oryzae* (bottom). Amide I (A_{1655} cm^{-1}) and hydroxyl bands (A_{3450} cm^{-1}) are dash-
887 highlighted.

888

889 **Fig. S3.** Phylogenetic tree of β-1,3-glucan synthase genes in *Neurospora crassa, Rhizopus*
890 *oryzae* and *Phycomyces blakesleeanus* built using RAxML with LGF as protein substitution
891 model.

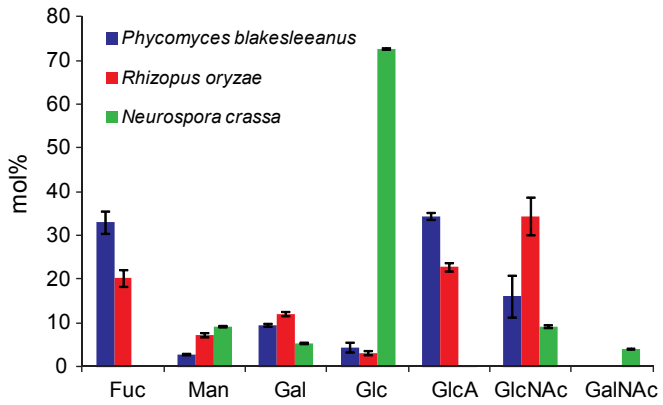
892

893 **Fig. S4.** Phylogenetic trees of GDP-L-fucose biosynthetic genes. A, GDP-mannose 4,6-
894 dehydratase genes in *R. oryzae*, *P. blakesleeanus*, *M. alpina*, *M. circinelloides*, *P. graminis*
895 *sp. tritici*, *S. roseus* and *E. coli* built using RAxML with LGF as protein substitution model.
896 All the gene identifiers are published accessions or searchable at the JGI
897 (http://genome.jgi.doe.gov/pages/search-for-genes.jsf). B, GDP-L-fucose synthase genes.

898

899 **Table S1.** Homologs of the cell wall biosynthetic genes in *R. oryzae*, *P. blakesleeanus* and *N.*
900 *crassa*.

Figure 1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mol%
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Rhizopus oryzae
Neurospora crassa
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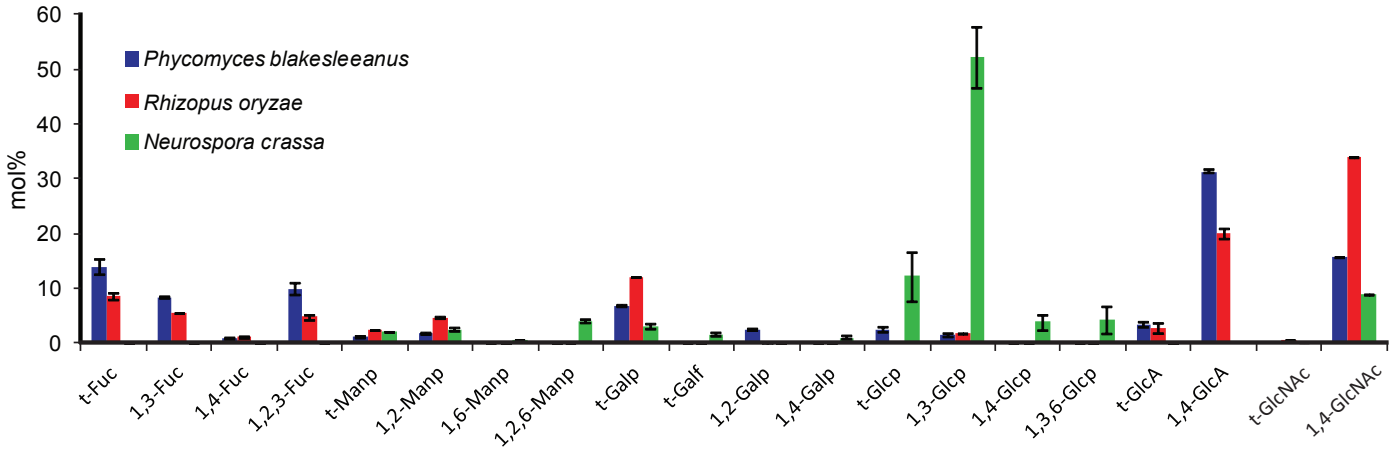
Man
Gal
Glc
GlcA
GlcNAc
GalNAc

Figure 2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Phycomyces blakesleeanus
Rhizopus oryzae
Neurospora crassa
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1,4-Fuc
1,2,3-Fuc
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1,2-Manp
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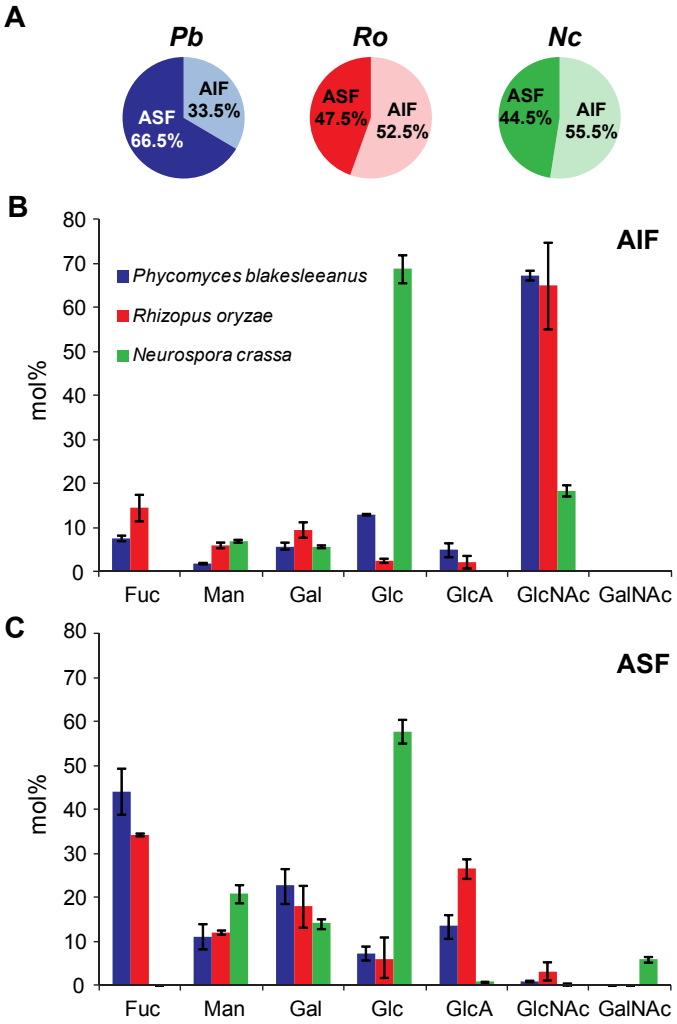
Figure 3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Phycomyces blakesleeanus
Rhizopus oryzae
Neurospora crassa
mol%
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GlcA
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ASF
mol%
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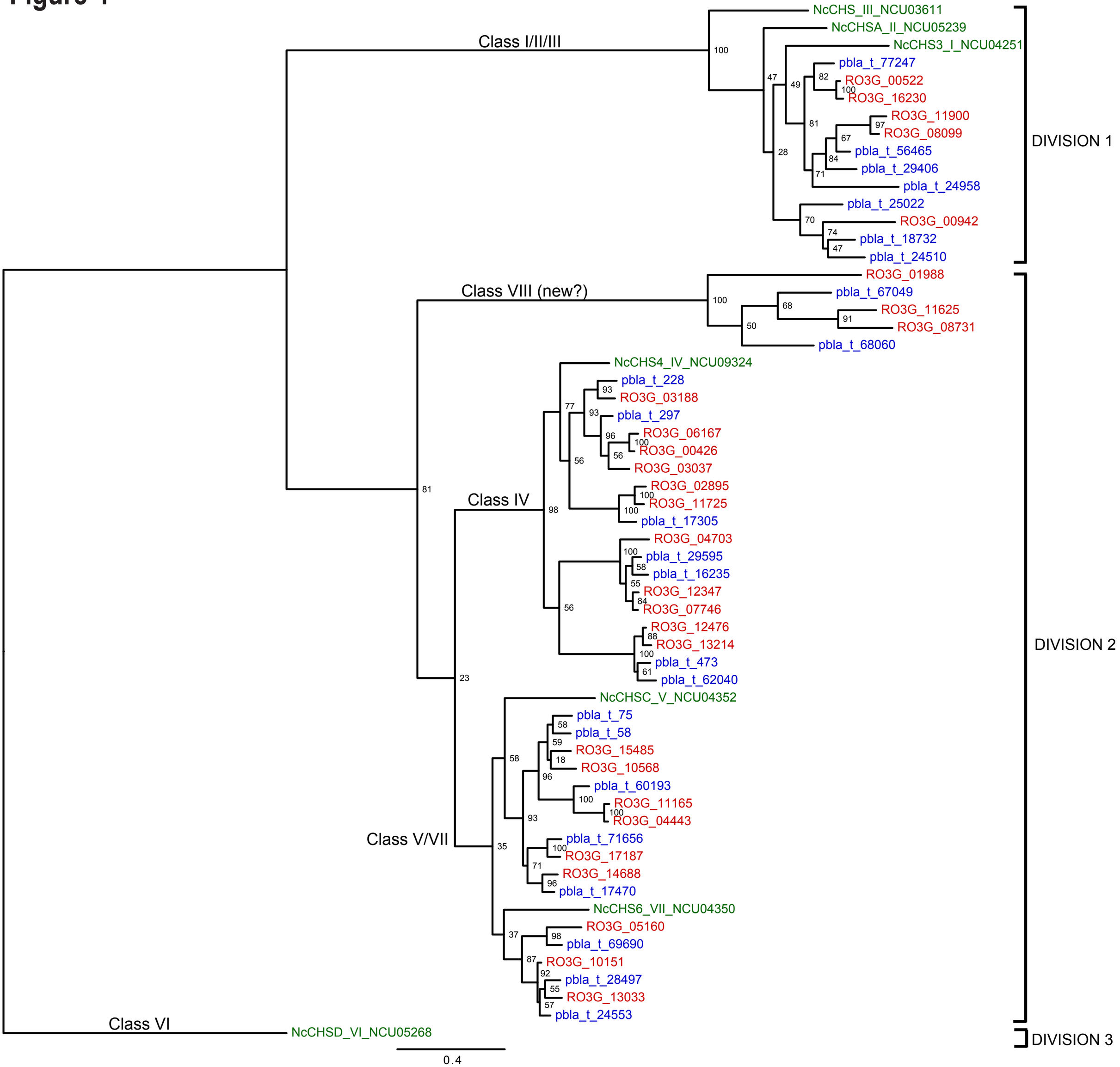
Figure 4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pbla_t_25022
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pbla_t_18732
pbla_t_24510
DIVISION 1
Class VIII (new?)
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RO3G_13214
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pbla_t_62040
Class V/VII
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pbla_t_58
RO3G_15485
RO3G_10568
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DIVISION 3
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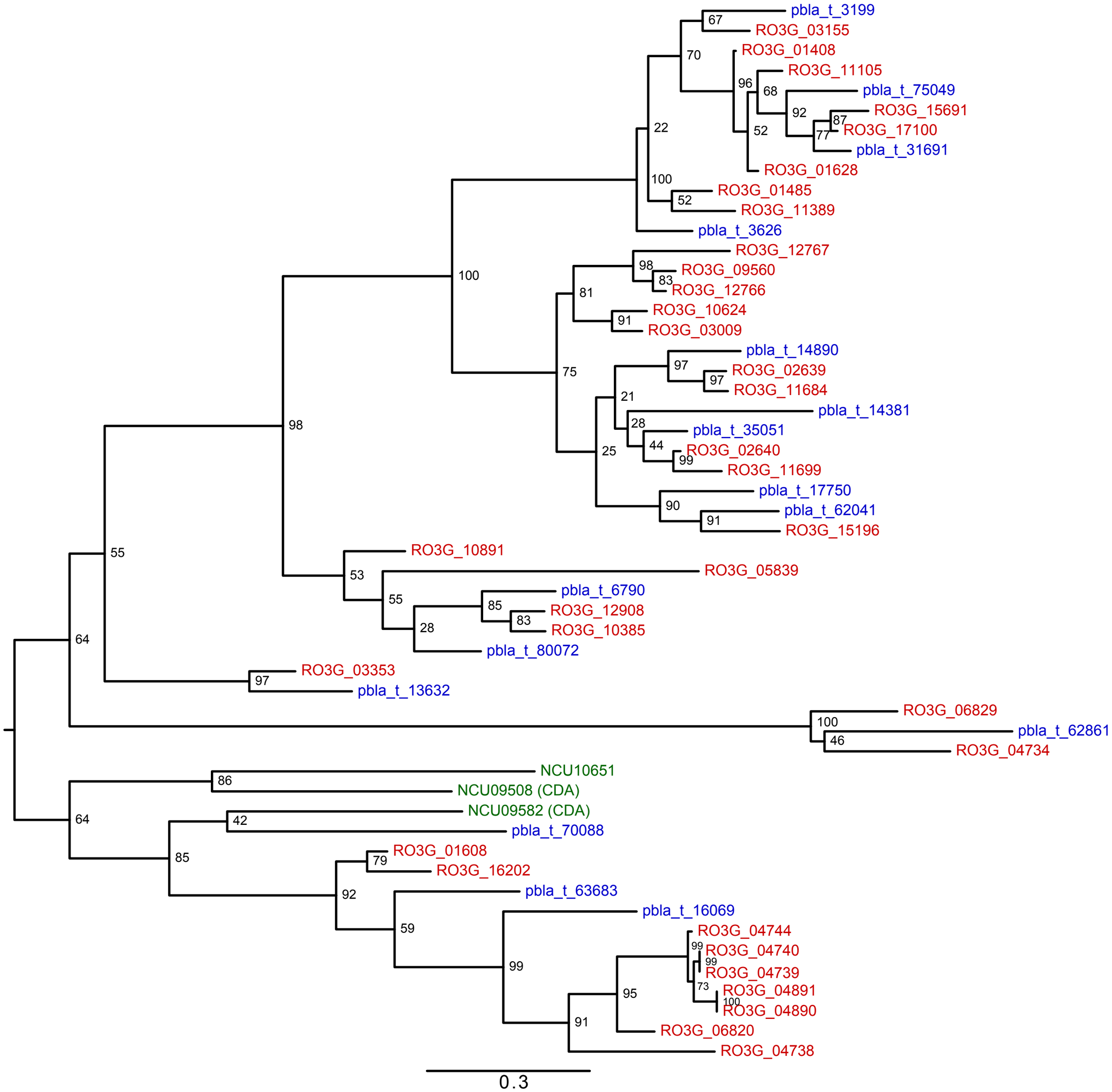
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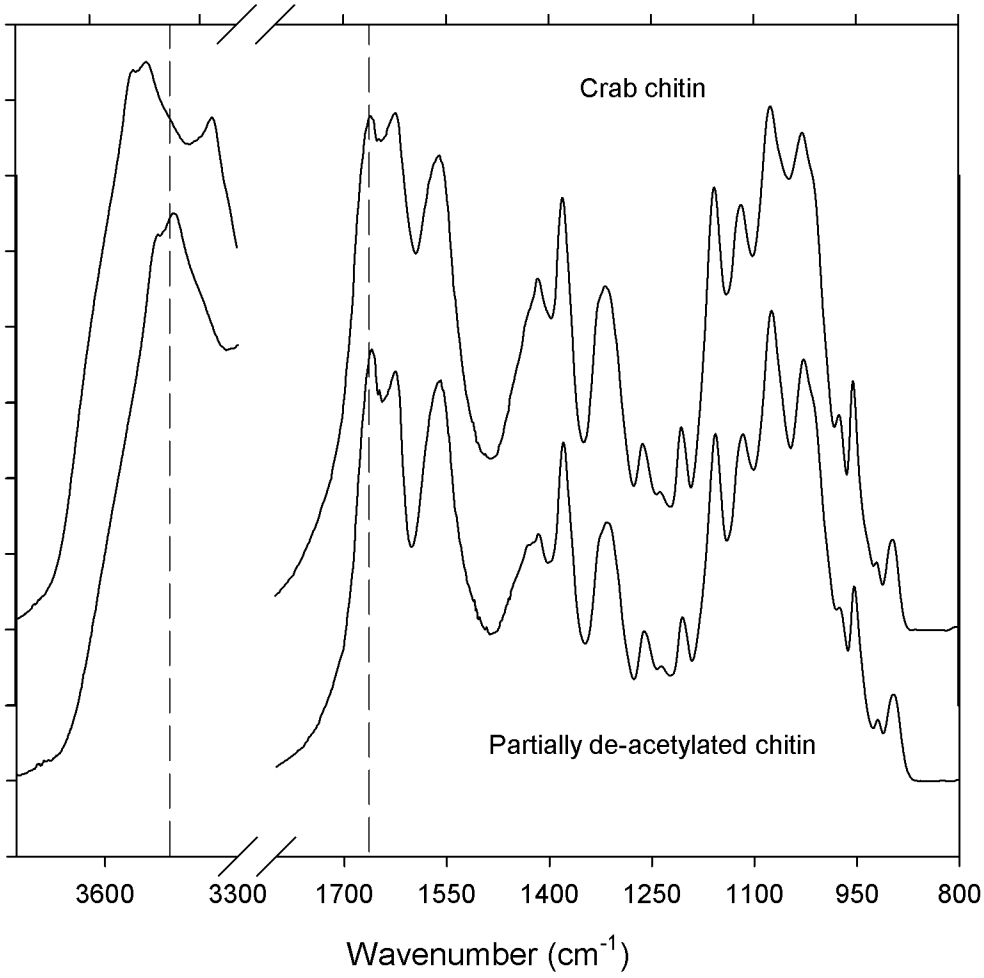
Figure S1


Crab chitin
Partially de-acetylated chitin
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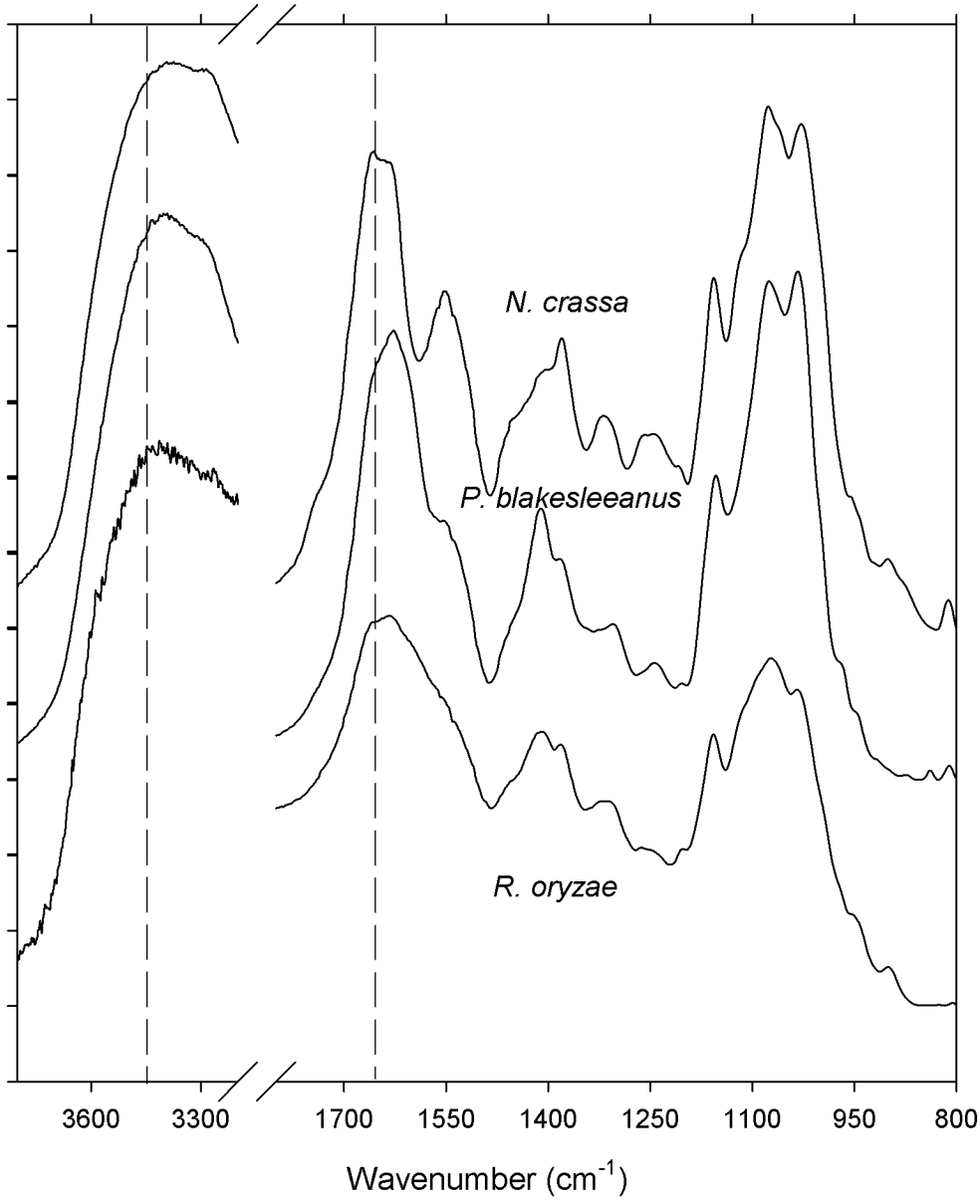
1700
1550
1400
1250
1100
950
800
Wavenumber (cm-1)

Figure S2


N. crassa
P. blakesleeanus
R. oryzae
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3300
1700
1550
1400
1250
1100
950
800
Wavenumber (cm-1)

Figure S3

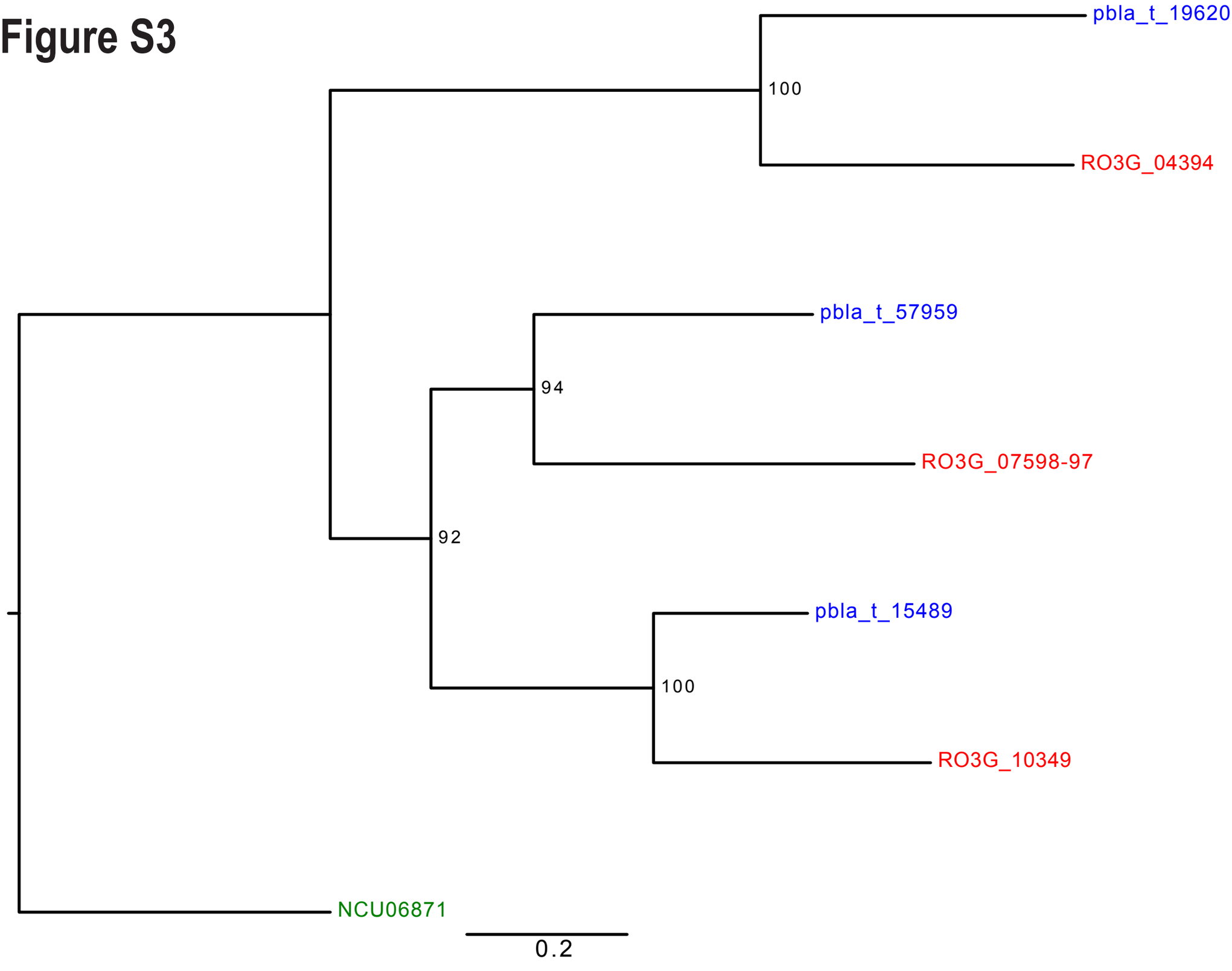
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Figure S4A

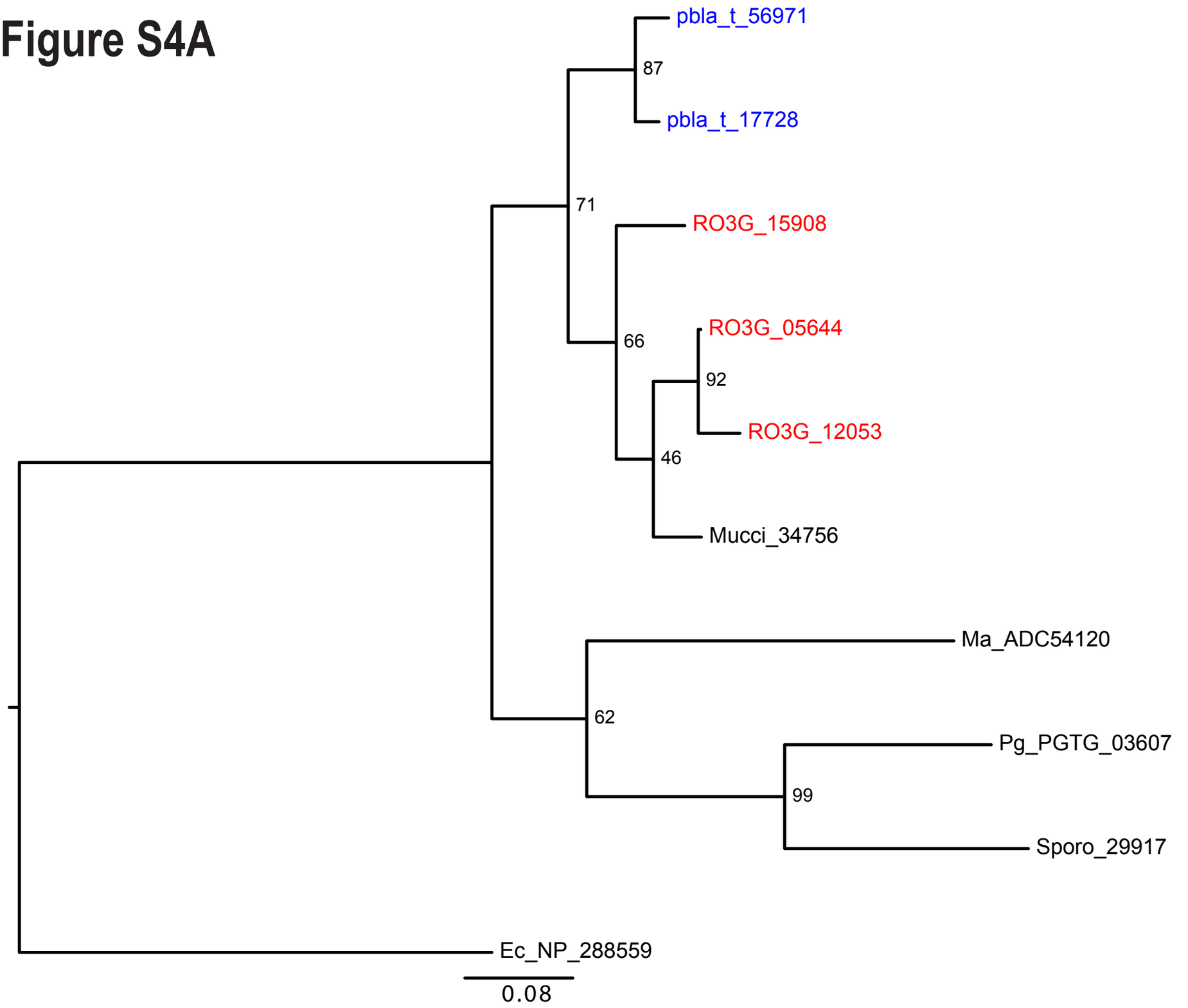
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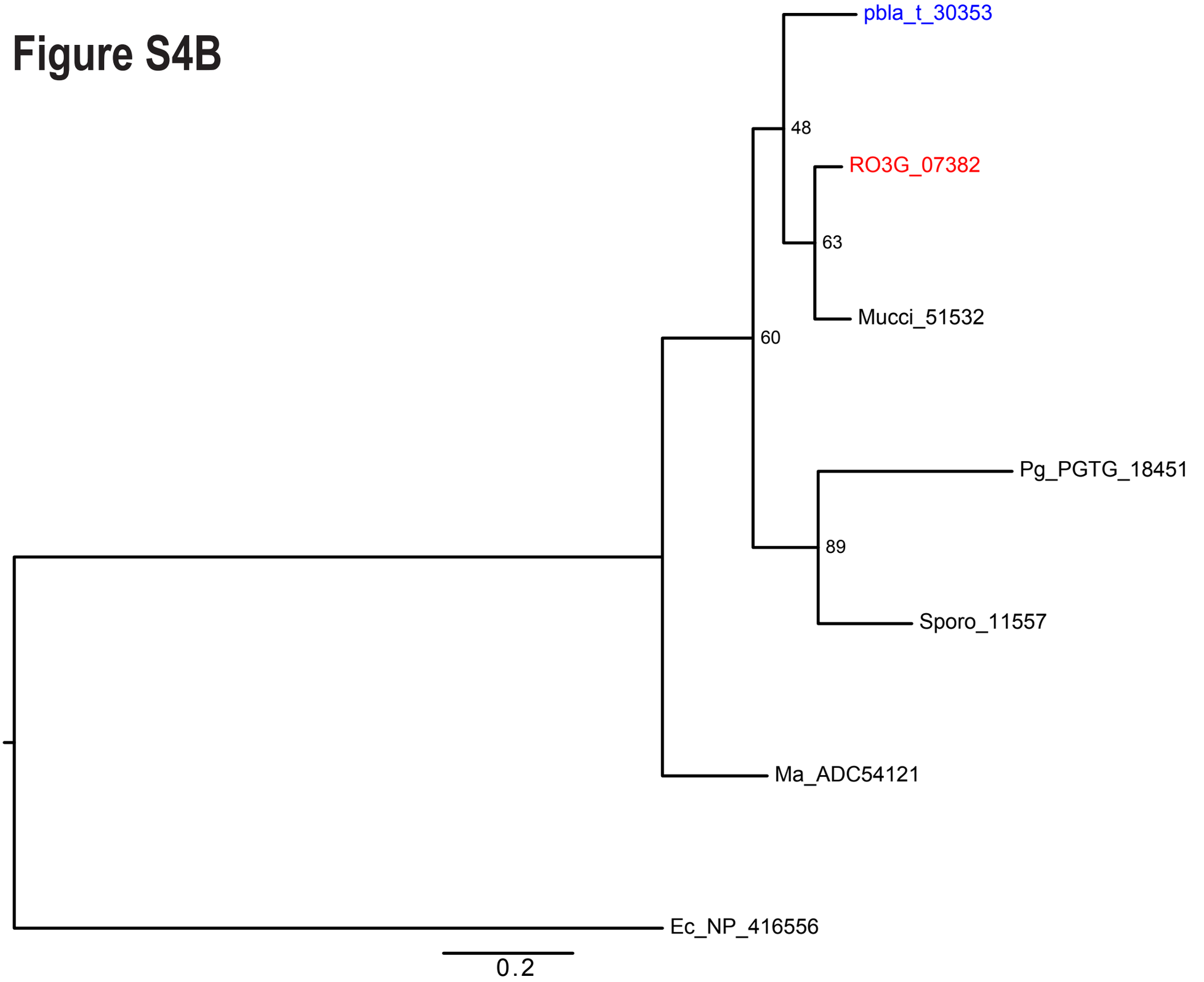
Figure S4B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0.2

Cell Wall component	(Putative) Activity (CAZy#)	*R. oryzae*	*P. blakesleeanus*	*N. crassa*
Chitin	**Chitin synthase** (GT2)	RO3G_00426 RO3G_00522 RO3G_00942 RO3G_01988 RO3G_02895 RO3G_03037 RO3G_03188 RO3G_04443 RO3G_04703 RO3G_05160 RO3G_06167 RO3G_07746 RO3G_08099 RO3G_08731 RO3G_10151 RO3G_10568 RO3G_11165 RO3G_11625 RO3G_11725 RO3G_11900 RO3G_12347 RO3G_12476 RO3G_13033 RO3G_13214 RO3G_14688 RO3G_15485 RO3G_16230 RO3G_17187	pbla_t_16235 pbla_t_17305 pbla_t_17470 pbla_t_18732 pbla_t_228 pbla_t_24510 pbla_t_24510 pbla_t_24553 pbla_t_24958 pbla_t_25022 pbla_t_28497 pbla_t_29406 pbla_t_29595 pbla_t_297 pbla_t_473 pbla_t_56465 pbla_t_58 pbla_t_60193 pbla_t_62040 pbla_t_67049 pbla_t_68060 pbla_t_69690 pbla_t_71656 pbla_t_75 pbla_t_77247	NCU05239 NCU03611 NCU04251 NCU04350 NCU09324 NCU05268 NCU04352
	Chitinase (GH18-19)	RO3G_01334 RO3G_02538 RO3G_04523 RO3G_05550 RO3G_07611 RO3G_08153 RO3G_10112 RO3G_10252 RO3G_11152 RO3G_13635 RO3G_13934 RO3G_14659 RO3G_16099 RO3G_16170	pbla_t_73140 pbla_t_79434 pbla_t_68507 pbla_t_56514 pbla_t_69395 pbla_t_20513 pbla_t_20051 pbla_t_70372	NCU06029 NCU05317 NCU04500 NCU04554 NCU04883 NCU07035 NCU07484 NCU03026 NCU03209 NCU02184
	Chitin deacetylase (CE4)	RO3G_01408 RO3G_01485 RO3G_01608 RO3G_01628 RO3G_02639 RO3G_02640 RO3G_03009 RO3G_03155 RO3G_03353 RO3G_04734 RO3G_04738 RO3G_04739 RO3G_04740 RO3G_04744 RO3G_04890 RO3G_04891 RO3G_05839 RO3G_06820 RO3G_06829 RO3G_09560 RO3G_10385 RO3G_10624 RO3G_10891 RO3G_11105 RO3G_11389 RO3G_11684 RO3G_11699 RO3G_12766 RO3G_12767 RO3G_12908 RO3G_15196 RO3G_15691 RO3G_16202 RO3G_17100	pbla_t_13632 pbla_t_14381 pbla_t_14890 pbla_t_16069 pbla_t_17750 pbla_t_31691 pbla_t_3199 pbla_t_35051 pbla_t_3626 pbla_t_62041 pbla_t_62861 pbla_t_63683 pbla_t_6790 pbla_t_70088 pbla_t_75049 pbla_t_80072	NCU09582 NCU09508 NCU10651
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	Glucosamine 6-phosphate N-acetyltransferase	RO3G_16355	pbla_t_63405 pbla_t_39828	NCU01902
	N-acetylglucosamine-phosphate mutase	RO3G_11445 RO3G_02795	pbla_t_27766	NCU07458
	UDP-N-acetylglucosamine pyrophosphorylase 1	RO3G_12286 RO3G_02460	pbla_t_29621	NCU02109
	β-1,4-mannosyl-glycoprotein 4-β-N-acetylglucosaminyltransferase (GT17)	RO3G_05157	pbla_t_32322	NCU07455
	β-1,3-glucan synthase (GT48)	RO3G_04394 RO3G_10349 RO3G_07597+RO3G_07598	pbla_t_15489 pbla_t_19620 pbla_t_57959	NCU06871
	β-1,3-glucanosyltransferase (Gel) (GH72)	RO3G_16395 RO3G_08263	pbla_t_72230 pbla_t_69942	NCU06781 NCU06850 NCU07253 NCU08909 NCU01162
	Endo-1,3-β-glucanase / Chitin β-1,6-glucanosyltransferase (Crh; Egl; Crf; Mwg) (GH16)	absent	absent	NCU00061 NCU01353

Glucan				NCU04168 NCU04431 NCU04959 NCU05789 NCU05974 NCU06504 NCU07134 NCU08072 NCU09117 NCU09672 NCU09904
	β-1,3-glucanase (GH17)	RO3G_15315 RO3G_11313	pbla_t_61012 pbla_t_73268	NCU09175 NCU09326 NCU07347 NCU06381
	β-1,3-glucanase (GH55)	absent	absent	NCU07523 NCU04850 NCU08097 NCU09791 NCU05105
	β-1,3-glucanase (GH81)	RO3G_03264	pbla_t_79096	NCU07076
	β-1,4-glucanase (GH5; GH6; GH12; GH45)	RO3G_00819 RO3G_01991 RO3G_05150 RO3G_10952 RO3G_04939 RO3G_03324 RO3G_11028 RO3G_00383 RO3G_08461	pbla_t_12110 pbla_t_68678 pbla_t_17693	NCU00762 NCU05882 NCU03254 NCU07190 NCU05121 NCU08227 NCU03996
	β-1,4-glucanase (non-classified)	only GH5s and GH45s		NCU05969 NCU07974
	Exoglucanase (GH5)	RO3G_01991 RO3G_00819	pbla_t_12110 pbla_t_68678	NCU03914
	Glucanase b (GH64)	absent	absent	NCU06505 NCU01080
	β-glucosidase (GH1; GH3)	RO3G_04557 RO3G_04265 RO3G_01985 RO3G_04558 RO3G_09386	pbla_t_75597 pbla_t_17174	NCU00130 NCU00709 NCU03641 NCU04952 NCU05577 NCU08054 NCU08755
	Lytic polysaccharide mono-oxygenase (GH61; AA9)	absent	absent	NCU01050 NCU07898 NCU02916 NCU01867 NCU03328 NCU08760
	Cellobiohydrolase (GH6; GH7)	absent	absent	NCU07340 NCU09680
	β-1,6-glucanase (GH30)	absent	absent	NCU04395
	SMI1_KNR4 (Regulation 1,3-glucan synthase)	absent	absent	NCU04189
	α-1,3-glucan synthase (GT5)	absent	absent	NCU02478 NCU08132
	α-1,3-glucanase (GH71)	absent	absent	NCU06010 NCU04691 NCU08473 NCU07355
	Phosphoglucomutase	RO3G_14405 RO3G_09121	pbla_t_80186	NCU10058
	UTP-glucose-1-phosphate uridylyltransferase	RO3G_01972 RO3G_04526	pbla_t_30945	NCU02797
Fucose	**α-Fucosyltransferase**	RO3G_02285 RO3G_06317 RO3G_08874 RO3G_11107	pbla_t_1985 pbla_t_38491	absent
	GDP-mannose 4,6-dehydratase	RO3G_05644 RO3G_15908 RO3G_12053	pbla_t_17728 pbla_t_56971	absent
	GDP-L-fucose synthase	RO3G_07382	pbla_t_30353	absent
Glucuronic acid	**β-glucuronidase** (GH79)	Absent	Absent	NCU00937
	UDP-glucose 6-dehydrogenase	RO3G_07219 RO3G_01042	pbla_t_41049	NCU08228 NCU04936
	α-1,6-mannosyltransferase (GT32)	Absent	Absent	NCU07338 NCU08232
	α-1,6-mannosyltransferase (GT22; GT34)	Absent	Absent	NCU07472 NCU03035 NCU06762
	α-1,6-mannotransglycosylase / α-1,6-mannanase (GH76)	Absent	Absent	NCU08127 NCU03770
	α-1,2-mannosyltransferase (GT4)	Absent	Absent	NCU06779
	α-1,3-mannosyltransferase (GT69)	Absent	Absent	NCU05916
	Mannosyltransferase	RO3G_11862 ??		

Mannan	**Mannosyltransferase** (GT22)	RO3G_14311	pbla_t_39723	NCU04454
	β-mannosyltransferase (GT1)	RO3G_15616	pbla_t_3272	NCU07261
	GPI mannosyltransferase (GT22; GT50; GT76)	Absent	Absent	NCU06057 NCU05960 NCU00193 NCU11399
	Dolichyl-phosphate-mannose-protein mannosyltransferase	RO3G_00012 RO3G_00855 RO3G_02309 RO3G_02310 RO3G_05284 RO3G_07144 RO3G_11796	pbla_t_16604 pbla_t_610 pbla_t_67788 pbla_t_74350	NCU01912 NCU09332 NCU01648
	Dolichyl-phosphate β-D-mannosyltransferase	RO3G_05000	pbla_t_29748 pbla_t_80017	NCU07965
	Dolichyl-phosphate-mannose-glycolipid α-mannosyltransferase (GT58)	RO3G_14582	pbla_t_36590	NCU06552
	Glycolipid 2-α-mannosyltransferase (GT15)	RO3G_02104 RO3G_04233 RO3G_04985 RO3G_04986 RO3G_09130 RO3G_09375 RO3G_14430 RO3G_14697 RO3G_15769	pbla_t_21457 pbla_t_15728 pbla_t_33463 pbla_t_18532 pbla_t_18223 pbla_t_63494 pbla_t_12196 pbla_t_12106 pbla_t_20553	NCU04037 NCU06166 NCU01388 NCU06541 NCU05680
	α-1,2-mannosidase (GH47)	RO3G_00387 RO3G_00961 RO3G_04245 RO3G_06558 RO3G_07187 RO3G_07642 RO3G_08089 RO3G_11920 RO3G_17402	pbla_t_58576 pbla_t_61958 pbla_t_75563 pbla_t_69806 pbla_t_11194 pbla_t_39476	NCU01059 NCU02091 NCU02235 NCU02778 NCU03134 NCU05836 NCU07067 NCU09028
	α-1,2-mannosidase (GH92)	Absent	Absent	NCU04798 NCU07269
	α-mannosidase (GH38)	Absent	Absent	NCU07404
	Mannan endo-1,4-β-mannosidase	RO3G_08893	pbla_t_17226	NCU08412
	β-mannosidase			NCU00890
	Mannan endo-1,6-α-mannosidase (GH76)	Absent	Absent	NCU02216 NCU04262 NCU07005 NCU09937 NCU06319 NCU00086 NCU02032
	Phosphomannomutase	RO3G_07083	pbla_t_77105	NCU02829
	Mannose-1-phosphate guanylyltransferase	RO3G_04395 RO3G_09234 RO3G_10153 RO3G_13031 RO3G_16791	pbla_t_33436	NCU11213
Galactan	**Galactosylxylosylprotein 3-beta-galactosyltransferase**	RO3G_04907	pbla_t_76055	
	Endo-β-1,6-galactanase (GH30)	Absent	Absent	NCU09702
	β-galactosidase (GH2; GH35)	Absent	Absent	NCU00810 NCU05956 NCU00642 NCU04623 NCU00985
	α-galactosidase	RO3G_06273 RO3G_08126 RO3G_02868 RO3G_13408	pbla_t_40742 pbla_t_74883 pbla_t_14560 pbla_t_76792 pbla_t_32958 pbla_t_79267	NCU02550
	Arabinogalactan endo-1,4-β-galactosidase (GH53)	Absent	Absent	NCU00972
Galacturonic acid	**Polygalacturonase** (GH28)	RO3G_02738 RO3G_04731 RO3G_06012 RO3G_06021 RO3G_07548 RO3G_08338 RO3G_11297 RO3G_15015 RO3G_15419	pbla_t_14560 pbla_t_76792 pbla_t_79267 pbla_t_74883 pbla_t_40742 pbla_t_32958 pbla_t_15372 pbla_t_57294 pbla_t_58157	NCU06961 NCU02369
GalNAc	**Acetylgalactosaminyltransferase**	Absent	Absent	NCU07487