Functional Differentiation of Glycoside Hydrolases family 18 in Filamentous Ascomycetes

Georgios Tzelepis
Faculty of Natural Resources and Agricultural Sciences
Department of Forest Mycology and Plant Pathology
Uppsala

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Cover: Colony morphology of *Neurospora crassa* Δ*gh18-10* strain, *Aspergillus nidulans* WT, *Trichoderma atroviride* Δ*Eng18B* strain and *Clonostachys rosea* WT.
(Photo: Georgios Tzelepis)

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Abstract

Glycoside hydrolases (GH) family 18 includes enzymes such as chitinases and endo-β–*N*-acetylglucosaminidases (ENGases). They are clustered into three distinct phylogenetic groups A, B and C, and further subdivided into several subgroups. Chitinases are responsible for chitin degradation and are involved in hyphal growth, nutrient acquisition, autolysis and mycoparasitic interactions. ENGases cleave the *N*,*N*'-diacetylchiotobiose moiety from high mannose *N*-linked glycans, and cytosolic ENGases together with peptide:*N*-glycanases (PNGases) are involved in the endoplasmic reticulum associated degradation process (ERAD) of misfolded glycoproteins.

This study investigated the functional role of these enzymes in different aspects of fungal biology using gene deletion and gene expression techniques. Putative GH18 ENGases were enzymatically characterized by heterologous expression in *Saccharomyces cerevisiae*, and their deglycosylation activity was investigated. The role of ENGases in the generation of free *N*-glycans was further studied using HPLC techniques. The deletion of the *gh18-10* gene, encoding a putative cytosolic ENGase, in *Neurospora crassa* resulted in a slower growth rate on carbon rich and chitin media, while an increased tolerance to abiotic stress and increased conidiation rate were also observed. In addition, the *gh18-10* deletion strain displayed lower extracellular protein secretion and reduced levels of extracellular protease activity. All of these data indicate that improper secretion might result in a more rigid cell wall, affecting the hyphal growth and the tolerance to abiotic stress. The current study proved that the Eng18A and Eng18B ENGases are active deglycosylating enzymes, while the cytosolic Eng18B ENGase in *Trichoderma atroviride* is the main factor for free *N*-glycans generation. Furthermore, this enzyme was able to degrade the RTL protein complex, which is an ERAD-specific substrate, implying a potential role in the ERAD pathway. This study also showed that chitinase genes belonging to C-II subgroup and displaying similarities to the *Kluyveromyces lactis* killer toxin were induced during interspecific interactions. Deletion of certain C-II chitinase genes in *Clonostachys rosea* and *Aspergillus nidulans* reduced their growth inhibitory activity of culture filtrates against *Botrytis cinerea* and *Rhizoctonia solani*, while reduced conidiation and altered responses to abiotic stress were also observed, indicating that these chitinases might be involved also in cell wall modification and conidiation.

Keywords: chitinases, deglycosylation, ENGases, ERAD, GH18, killer-toxins

Author's address: Georgios Tzelepis, SLU, Department of Forest Mycology and Plant Pathology,
P.O. Box 7026, SE-75007 Uppsala, Sweden
E-mail: Georgios.Tzelepis@ slu.se

Dedication

To my family

”Wonder is the beginning of wisdom”
Socrates

“The roots of education are bitter, but the fruits are sweet”
Aristotle

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I **Tzelepis, G.**, Melin, P., Funck Jensen, D., Stenlid, J. and Karlsson, M. (2012). Functional analysis of glycoside hydrolase family 18 and 20 genes in *Neurospora crassa*. *Fungal Genetics and Biology* 49: 717-730.

II **Tzelepis, G.**, Hosomi, A., Hossain, J.T., Hirayama, H., Dubey, M., Funck Jensen, D., Suzuki, T. and Karlsson, M. Endo-β-*N*-acetylglucosamidases (ENGases) in the fungus *Trichoderma atroviride*: Possible involvement of the filamentous fungi-specific cytosolic ENGase in the ERAD process (manuscript).

III **Tzelepis, G.**, Melin, P., Stenlid, J., Funck Jensen, D. and Karlsson, M. (2014). Functional analysis of the C-II subgroup killer toxin-like chitinases in the filamentous ascomycete *Aspergillus nidulans*. *Fungal Genetics and Biology* 64:58-66.

IV **Tzelepis, G.**, Dubey, M., Funck Jensen, D. and Karlsson, M. Identifying glycoside hydrolase family 18 genes in the mycoparasitic fungal species *Clonostachys rosea* (manuscript).

Additional Publications and Manuscripts

Strandberg, R., **Tzelepis, G.**, Johannesson, H. and Karlsson, M. (2013). Co-existence and expression profiles of two alternative splice variants of the pheromone receptor gene *pre-1* in *Neurospora crassa*. *Archives of Microbiology* 195:773-780.

Karlsson, M., Brandström-Durling, M., Choi, J., Lackner, G, **Tzelepis, G.**, Nygren, K., Dubey, K.M., Kosawang, C., Zapparata, A., Wang, J., Amby D. B., Jensen, B., Sarrocco, S., Lagopodi, L.A., Vannaci, G., Collinge B.D., Hoffmeister, D., Henrissat, B., Lee, Y.H. and Funck Jensen, D. (2014). Insights on the evolution of mycoparasitism from the genome of *Clonostachys rosea* (manuscript).

The contribution of Georgios Tzelepis to the papers included in this thesis was as follows:

I He analyzed the phenotypes of GH18 gene deletion strains in different conditions, validated the deletion strains, extracted RNA, synthesized cDNA, carried out RT-qPCR, analyzed the data, and wrote the paper with comments and suggestions from co-authors.

II He characterized the enzymatic function of ENGases, extracted free *N*-glycans, carried out HPLC analysis, analyzed the data in collaboration with the co-authors and wrote the paper with comments and suggestions from co-authors.

III He constructed the deletion constructs and deleted the C-II chitinase genes, evaluated the deletion strains, carried out phenotypic analysis of deletion strains, extracted RNA, synthesized cDNA, conducted RT-qPCR and wrote the paper with comments and suggestions from co-authors.

IV He analyzed the genome searching for GH18 genes, constructed the deletion cassette, deleted the C-II chitinase gene in collaboration with co-authors, validated the deletion strains, analyzed the phenotypes, extracted RNA and prepared cDNA, carried out RT-qPCR and wrote the paper with comments and suggestions from co-authors.

Abbreviations

CAZy	Carbohydrate Active Enzyme
CBM	Carbohydrate Binding Module
EDEM	ER degradation enhancing α-mannosidase-like protein
ENGase	Endo-*N*-acetyl-β-D-glucosaminidase
ER	Endoplasmic Reticulum
ERAD	ER Associated Degradation
fOs	Free oligosaccharide
GH	Glycoside hydrolases
Glc	Glucose
GlcNAc	*N*-acetyl-D-glucosamine
GN1	Oligosaccharide with single GlcNAc at its reducing terminus
GN2	Oligosaccharide with *N,N*-diacetylchitobiose at its reducing terminus
GPI	Glycosylphosphatidylinositol
HPLC	High Performance Liquid Chromatography
Man	Mannose
NAGase	*N*-acetylhexosaminidase
PA	Pyridylamination
PBS	Phosphate buffer saline
PDA	Potato dextrose agar
PDB	Potato dextrose broth
PNGase	Peptide:*N*-glycanase
SC	Synthetic cross
SDS	Sodium dodecyl sulphate
SMS	Synthetic minimal salts
VM	Vogel's medium
WT	Wild type
YE	Yeast extract

1 Introduction

1.1 Chitin and chitinases

1.1.1 Chitin as a structural component of the fungal cell wall.

The fungal cell wall is a dynamic structure that provides hyphae with the appropriate strength to cope with osmotic pressure and other environmental stress conditions. Concurrently, it must allow the communication between the inner parts of the cell with the extracellular environment. Furthermore, the cell wall is responsible for maintaining the hyphal shape and integrity, while also retaining the appropriate plasticity to allow hyphal growth (Bowman & Free, 2006).

The composition of the fungal cell wall is different from those of oomycetes and plants, consisting mostly of β-1.3 and β-1.6 glucans, chitin and glycoproteins. Chitin, derived from the Greek word *chiton* (χιτων), meaning garment, is a biopolymer that consists of β-1.4-linked *N*-acetylglucosamine (GlcNac) and it is the second most abundant biopolymer in nature after cellulose (Tharanathan & Kittur, 2003). In addition to its presence in the fungal cell wall, chitin is one of the main components of insect and crustacean exoskeletons. In filamentous fungi, the cell wall consists of 10-20% chitin (de Nobel *et al.*, 2000; Bartnicki-Garcia, 1968), while yeasts contain only 1-2% (Klis *et al.*, 2002). Despite the fact that chitin is not the main component of the fungal cell wall, its contribution to cell wall integrity is crucial. Disruption of chitin synthesis increased the osmotic instability and led to a malfunctioned cell wall (Specht *et al.*, 1996).

The chitin chain is synthesized by the involvement of chitin synthases, which are membrane enzymes responsible for catalyzing the transfer of GlcNac from uridine (UDP)-*N*-acetylglucosamine to chitin chain. The newly synthesized chitin polymers form hydrogen bonds resulting in formation of microfibrils that are inserted into the cell wall immediately adjacent to the

plasma membrane (Bowman & Free, 2006). In this process chitosomes play an important role carrying the chitin synthases to the cell surface (Bracker *et al.*, 1976). Chitosomes are small spheroidal vesicles approximately 40-70 nm in diameter and they are accumulated in the hyphal tip, where chitin is synthesized (Bartnicki-Garcia, 2006).

Since chitin is responsible for cell wall integrity, and is absent from mammalian cells, is a potential target for antifungal treatment. The natural compound nikkomycin Z, produced by *Streptomyces* species, is known as a chitin synthases inhibitor (Chaudhary *et al.*, 2013). However, compounds targeting chitin synthesis are not efficient enough against mycoses, and they are used in combination with other anti-fungal agents (Georgopapadakou & Walsh, 1996).

1.1.2 Chitin hydrolysis

The chitin biopolymer is mainly hydrolyzed by chitinases (EC 3.2.1.52) which are responsible for cleaving the β-1.4-bond releasing oligomeric, dimeric (chitobiose) and polymeric GlcNAc products. These enzymes are categorized into two different glycoside hydrolases families (GH) 18 and 19 based on amino acid similarities (Henrissat, 1991), while *N*-acetylhexosaminidases (NAGases), responsible for chitobiose degradation, belong to GH20 according to the Carbohydrate Active Enzyme database (CAZy) (Cantarel *et al.*, 2009). Between GH18 and GH19 there are limited similarities in the amino acid sequence, and they display different three-dimensional structures (Kezuka *et al.*, 2006; Perrakis *et al.*, 1994). Moreover, the catalytic mechanism is also different; GH19 chitinases produce α-anomeric products while GH18 produce β-anomeric products (Brameld & Goddard, 1998).

Depending on the cleavage patterns, chitinases are categorized into endo- and exochitinases. Endochitinases are able to cleave the chitin chain at random positions, while the exo-acting enzymes can cleave only from the polymer ends releasing chitobiose (*Figure 1*) (Horn *et al.*, 2006). The catalytic clefts are different between endo- and exo- chitinases; endochitinases have shallow clefts (Hurtado-Guerrero & van Aalten, 2007), while exochitinases have tunnel-shaped clefts (van Aalten *et al.*, 2001).

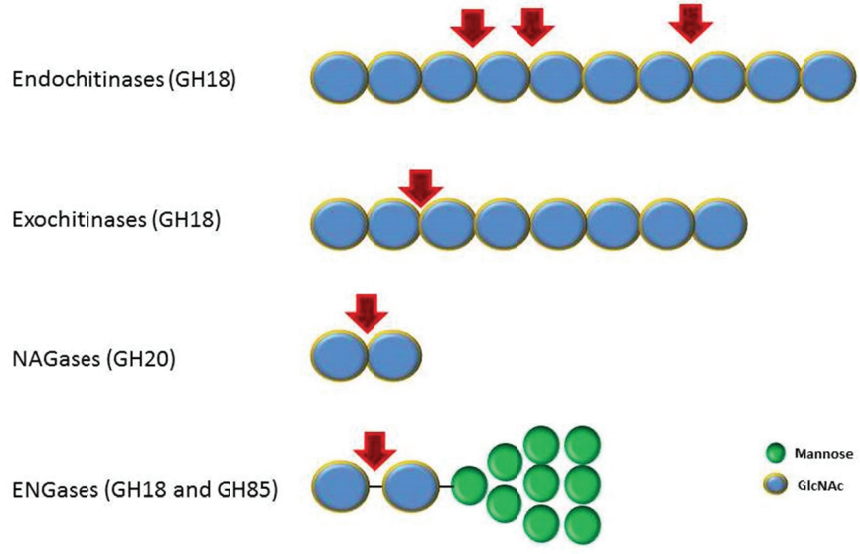

Figure 1. The cleavage patterns of GH18 and GH20 enzymes. Endochitinases cleave the chitin chain in random positions, exochitinases are able to degrade chitin only from the polymer ends, NAGases can degrade the chitobiose and ENGases are responsible for cleaving the *N,N'*-diacetylchiotobiose moiety from high mannose *N*-linked glycans.

Chitinases are found in a plethora of organisms from bacteria to primates. In bacteria they are involved in chitin degradation for nutrient acquiring purposes (Park *et al.*, 1997; Wang & Chang, 1997) and for chitin recycling (Keyhani & Roseman, 1999). Plant chitinases are induced as a part of non-specific plant responses to various biotic and abiotic stress agents (Dumas-Gaudot *et al.*, 1996). Induction of chitinases has been observed in plants as a defense response to attack by fungi (Benhamou *et al.*, 1993), while transgenic plants overexpressing these enzymes showed higher resistance to some plant pathogens and to abiotic stress conditions (Dana *et al.*, 2006). In addition, plant chitinases have also been reported to be involved in interactions with symbiotic microorganisms. For instance, induction of chitinases was observed during the nodulation process in soybeans (Staehelin *et al.*, 1994). Increased chitinase activity has been reported in ectomycorrhizal symbiosis (Albrecht *et al.*, 1994) and also in arbuscular mycorrhizal interactions (Dumas *et al.*, 1989)

Chitinases are also present in mammalian cells. It is hypothesized that they are involved in innate immune responses against parasites, digesting cell walls, egg shells and cysts produced by fungi, insects, nematodes and protozoans (Fusetti *et al.*, 2002). Furthermore, these enzymes may play a role in digestion of chitin-containing food, such as mushrooms, crustaceans etc. (Boot *et al.*, 2005).

1.1.3 Categorization of fungal chitinases

Fungal chitinases belong exclusively to GH18 according to the CAZy database (Cantarel *et al.*, 2009). Phylogenetic analyses have shown that these enzymes are categorized into three distinct phylogenetic groups A, B and C, which are further subdivided into subgroups (A-II to A-V, B-I to B-IV and C-I to C-II) (Karlsson & Stenlid, 2008; Seidl *et al.*, 2005). In addition to the catalytic domain, they usually contain different carbohydrate binding modules (CBM). Although these modules are not essential for their catalytic activity, they can enhance the enzyme binding efficacy (Suzuki *et al.*, 1999).

Chitinases in group A have a narrow catalytic cleft suggesting exochitinase activity. They contain a GH18 catalytic domain and they are present in all fungal species. The number of genes encoding group A chitinases varies between two in *Ustilago maydis* and 12 in *Fusarium oxysporum*. Chitinases in this group usually contain a signal peptide at the N-terminal, indicating that they are targeted to the secretory pathway (*Figure 2*) (Seidl, 2008). One of the most conserved and highly expressed chitinases in the fungal kingdom is the ECH42, which belongs to this group. This protein has been studied thoroughly in *Trichoderma* species, and it plays variable roles in fungal life cycle (Gruber & Seidl-Seiboth, 2012).

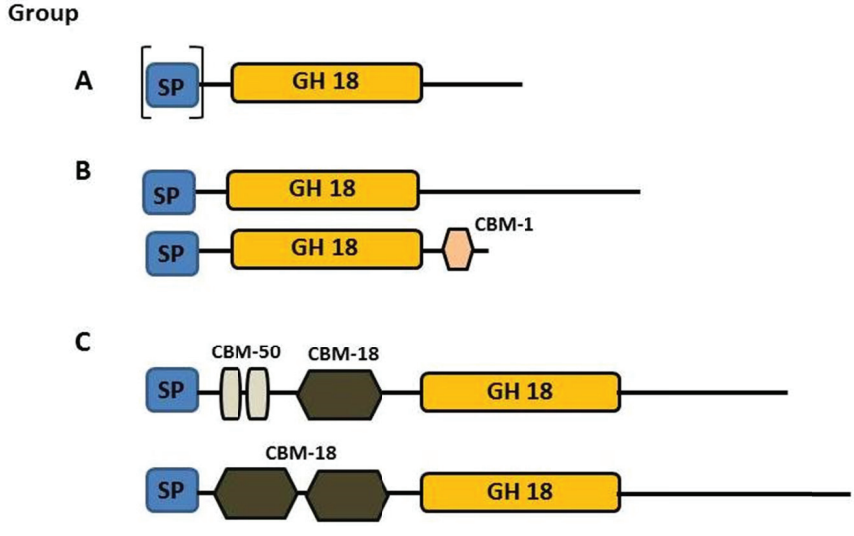

Figure 2. The modular structure of group A, B and C chitinases. Abbreviations: SP: signal peptide, GH18: glycoside hydrolases family 18, CBM-1: carbohydrate binding module family 1, cellulose-binding, CBM-18: carbohydrate binding module family 18, chitin-binding, CBM-50: carbohydrate binding module family 50, peptidoglycan binding LysM.

GH18 enzymes belonging to group B vary in size and domain structure (*Figure 2*). They have a shallow and wider substrate binding site compared to group A chitinases indicating that they are enzymes with endochitinase activity. However, members of the B-V subgroup are phylogenetically associated with group B but they encode GH18 enzymes with mannosyl glycoprotein endo-*N*-acetyl-β-D-glucosaminidase (ENGase) type activity (*Figure 1*) (Stals *et al.*, 2010). Group B includes either small or large enzymes usually contain a signal peptide. The small group B chitinases frequently contain a cellulose binding domain (CBM-1). This domain is exclusively found in fungi and can bind not only to cellulose, but also to other polysaccharides such as chitin (Boraston *et al.*, 2004). The number of chitinases containing the CBM-1 domain varies among the species. For instance, *Trichoderma* species have several group B chitinases containing this domain, while in contrast no CBM-1 domain was identified in some *Aspergillus* species (Seidl, 2008). The large group B chitinases usually contain a glycosylphosphatidylinositol structure (GPI anchor) in the C-terminal indicating that they are localized to the cell wall (Yamazaki *et al.*, 2008). It is interesting that the number of group B chitinases varies among fungal genomes. For instance, mycoparasitic *Trichoderma* species show a significantly higher number of these chitinases compared to saprophytic ones, indicating that the parasitic lifestyle exert selection for increased number of group B chitinase genes (Ihrmark *et al.*, 2010).

Chitinases belonging to group C share homology with the α/β subunit of a killer toxin produced by *Kluyveromyces lactis* yeast cells. This toxin consists of three subunits α, β and γ, where the α subunit has a chitinase function which modifies the permeabilization and thus facilitates the transfer of the γ subunit (the active toxin) into the antagonist cell (Magliani *et al.*, 1997). Similar function has been hypothesized also for group C chitinases implying an important role during fungal-fungal interactions (Gruber & Seidl-Seiboth, 2012).

Group C chitinases are predicted to be large proteins containing several CBMs (*Figure 2*). Particularly, they contain a variable number of CBM-18 (chitin binding domain) and CBM-50 (LysM, peptidoglycan binding) domains. LysM motifs are short peptide domains that have general carbohydrate binding properties (Seidl-Seiboth *et al.*, 2013). The exact function of the LysM domains in fungal proteins is not clear, but it is widely believed that they enhance the enzyme carbohydrate binding ability. For instance, in the plant pathogenic fungus *Cladosporium fulvum*, the Ecp6 protein contains three LysM motifs and has been shown to block plant immune responses triggered by chitin (de Jonge *et al.*, 2010). Moreover, an effector domain, called Hce2,

has been recently shown to be present in some C group chitinases (Stergiopoulos *et al.*, 2012). Evolution analyses of these chitinases in different *Trichoderma* species revealed variable patterns. For instance, the mycoparasitic species *T. atroviride* and *T. virens* have respectively 9 and 15 genes that encode C group chitinases, while the saprophytic *T. reesei* displays a significant decrease in the number of these genes (Gruber & Seidl-Seiboth, 2012).

1.1.4 Function and regulation of fungal chitinases

In general, fungal chitinases are suggested to be involved in different aspects of fungal biology, for instance in hyphal growth, sporulation, autolysis and mycoparasitic interactions, for nutrient acquisition etc. (Duo-Chuan, 2006). Despite the fact that chitinases have been enzymatically characterized in several fungal species, studies investigating the specific role of these enzymes in fungal biology are limited.

The role of chitinases in fungal growth and sporulation was studied in *Saccharomyces cerevisiae*. In this species, deletion of the chitinase *CTS1* gene led to cell clumping and failure of the cells to separate after division (Kuranda & Robbins, 1991). Deletion of the *CTS2* chitinase gene resulted in abnormal spore wall synthesis and failure to form mature asci, indicating a crucial role in sexual development (Giaever *et al.*, 2002). Similarly, the deletion of the *CHT3* chitinase gene in *Candida albicans* led to formation of chains of unseparated cells (Dunkler *et al.*, 2005). Finally, in *K. lactis* the nucleus-encoded *KlCts1* chitinase gene plays a role in cytokinesis, since its deletion resulted in defective cell separation (Colussi *et al.*, 2005).

In filamentous fungi, studies reported that the Tac6 chitinase in *T. atroviride*, which is a member of the C group, is involved in hyphal growth. However, a mutation was observed in the catalytic residue possibly indicating an inactive enzyme (Seidl-Seiboth *et al.*, 2013). Furthermore, chitinases in *Aspergillus niger* are involved in cell wall modification. Increased amounts of GlcNAc were observed in spore walls of the Δ*ctcB*;Δ*cfcI* strain compared to the wild type (WT), even though these deletions did not affect the colony morphology (van Munster *et al.*, 2013).

Fungal chitinases are also involved in autolysis. Transcriptional analysis showed induction of chitinase genes in several fungal species during carbon starvation conditions (Yamazaki *et al.*, 2007; Seidl *et al.*, 2005; Jaques *et al.*, 2003). In *Aspergillus fumigatus* the deletion of the *chiB1* chitinase gene demonstrated the autolytic function of this enzyme, since this mutant showed lower chitinolytic activity during the autolytic phase of batch cultures (Jaques *et al.*, 2003). In comparison, *A. nidulans* data revealed that the endochitinase

ChiB plays an active role in autolysis (Pocsi *et al.*, 2009). Additionally, chitinases have a potential role to play in nutrient acquisition, since induction of chitinase genes on media, where chitin is the sole carbon source, has been reported in several fungal species (Seidl, 2008).

The role of fungal chitinases in mycoparasitism has been studied more thoroughly. During the mycoparasitic attack, mycoparasites destroy the prey fungal cell wall and then feed on the cell contents, indicating that chitinases play an important role in this interaction, hydrolyzing the cell wall chitin. In *T. atroviride,* the *ech42* chitinase gene was highly induced during mycoparasitic interactions (Zeilinger *et al.*, 1999; Carsolio *et al.*, 1994). However, deletion of this gene does not affect the *T. atroviride* efficacy against *R. solani* or *Sclerotium rolfsii* in cotton plants (Carsolio *et al.*, 1999). Similarly, the deletion of the *ech42* gene (ortholog to *ech42*) in *C. rosea* did not affect its biocontrol ability against *Fusarium culmorum* and *Alternaria radicina* in plant bioassays (Mamarabadi *et al.*, 2008). In contrast, Woo *et al.* (1999) reported reduced antifungal activity in the *Trichoderma harzianum* Δ*ech42* strain against *Botrytis cinerea in vitro*. Recently, the role of killer-toxin like chitinases in interspecific interactions has attracted attention. As was previously mentioned, mycoparasitic *Trichoderma* species display an increased number of group C chitinase genes and all of these genes in *T. atroviride* were induced during contact with *B. cinerea* mycelia (Gruber *et al.*, 2011b).

1.2 The *N*-glycoprotein degradation process and ENGases

1.2.1 The *N*-glycosylation process and glycoprotein folding

The Endoplasmic Reticulum (ER) is the site in eukaryotic cells where the synthesis of secreted proteins occurs. *N*-glycosylation is one of the most common and crucial posttranscriptional modifications of eukaryotic proteins which takes place in the ER. During the biosynthetic process of oligosaccharides ($Glc_3Man_9GlcNAc_2$), monosaccharides are added to a lipid carrier (dolichol-pyrophosphate) by the function of the oligosaccharyl transferase (OST complex) in the ER membrane (Kornfeld & Kornfeld, 1985).

N-glycans play an important role in the physicochemical properties of glycoproteins. They affect protein solubility, heat stability and cellular distribution, and are also involved in correct folding and targeting to the secretion pathway (Varki, 1993). The role of *N*-glycans in protein folding was demonstrated in early 1990s. This process starts when a glycan has been added to the nascent protein. Firstly, the glucose residues are trimmed by the glucosidases I and II enzymes leaving the oligosaccharide with a single glucose residue (*Figure 3*). Then, the monoglucosylated protein is bound to the

calnexin/calreticulin proteins. These proteins work as chaperones promoting the protein folding and preventing the accumulation of misfolded glycoproteins (*Figure 3*). Removal of the single glucose by glucosidases II results in the glycoprotein release from these chaperones. Correctly folded glycoproteins, trimmed by α-1.2 mannosidases, are free to leave the ER and usually are transferred to Golgi apparatus for further modification and translocation, while the misfolded ones are reglucosylated by an enzyme called UDP-Glc:glycoprotein and rebinds to the chaperones (Jackson *et al.*, 1994; Ou *et al.*, 1993). Proteins that consistently fail to be folded properly are trimmed by the ER-degradation enhancing α-mannosidase proteins (EDEMs) and are retrotranslocated to cytosol for further degradation by the ERAD pathway (Hirsch *et al.*, 2003).

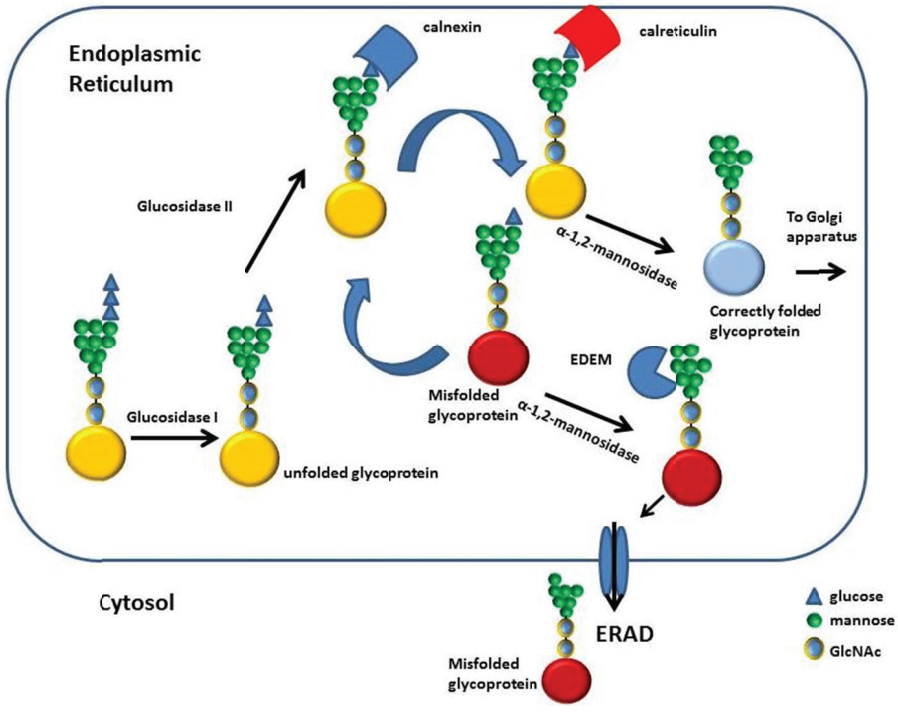

Figure 3. The calnexin/calreticulin cycle. The glucose residue is trimmed by the glucosidases I and II enzymes. Then, monoglucosylated proteins are bound to the calnexin/calreticulin chaperones responsible for correct folding. The cleavage of the glucose monomer indicates the exit from the cycle. When a protein is folded properly, trimmed by α-1.2 mannosidases and leaves the ER. If a protein is not folded properly it is reglucosylated by the UDP-Glc:glycoprotein and rebinds to the calnexin/calreticulin or is cleaved by EDEM and retrotranslocated to the cytosol for degradation by the ERAD.

1.2.2 The Endoplasmic Reticulum Associated Degradation Process

It is known that when misfolded glycoproteins are exported to the cytosol, the glycan chain must be cleaved from the protein in order to be degraded efficiently by the proteasome (Suzuki & Funakoshi, 2006). This removal is conducted by the action of the ubiquitous, cytoplasmic peptide:*N*-glycanase (PNGase), which releases free *N*-glycans to the cytosol (*Figure 4*). This enzyme cleaves the amide bond in the side chain of glycosylated–asparagine residue and generates free *N*-glycans with a chitobiose structure at the reducing terminus (GN2) (Suzuki *et al.*, 2000). Enzymatically active PNGases have been reported in a wide range of eukaryotic cells from yeasts to mammals (Suzuki *et al.*, 2002a). However, the situation seems to be different in filamentous fungi, since data claim that the enzymatic function of the cytosolic PNGase has been abolished in *N. crassa* (Maerz *et al.*, 2010).When free *N*-glycans are created in cytosol they are further catabolized in order to be utilized possibly as a sugar source by cells. In cytosol, two enzymes are responsible for free *N*-glycans degradation: the ENGases and the α-mannosidases (*Figure 4*).

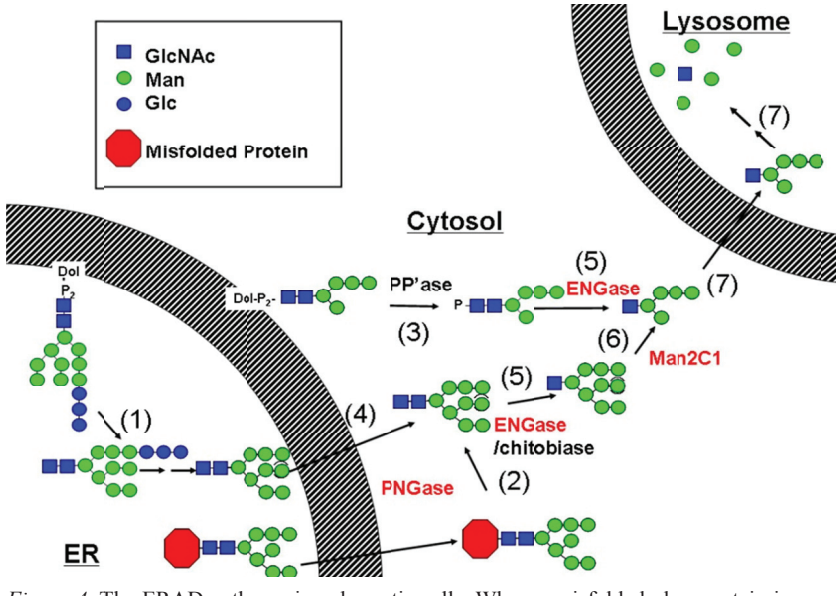

Figure 4. The ERAD pathway in eukaryotic cells. When a misfolded glycoprotein is exported to the cytosol, PNGases cleave the *N*-linked high mannose glycans generating free oligosaccharides with a chitobiose structure at the reducing terminus (fOSs-GN2) (2). Besides PNGases, fOSs-GN2 can be generated by the OST activity (1, 4) and by the hydrolytic activity of the pyrophosphatases (3). Then, fOSs-GN2 are degraded by ENGases responsible for cleaving the β-1.4 bond in the chitobiose core (5) and by the α-mannosidases which trim the mannose chain (6) before the final degradation in lysosomes (7) (by permission of Dr. Tadashi Suzuki).

1.2.3 The functional role of ENGases

ENGases (EC. 3.2.1.96) are enzymes responsible for hydrolysing the β-1.4 bond in the chitobiose core of *N*-linked glycans generating free oligosaccharides with a single GlcNAc residue (GN1) (*Figure 4*) (Suzuki & Funakoshi, 2006). Until now it has not been known if the cytoplasmic ENGases can directly generate free *N*-glycans. The ENGase function seems to be crucial for further trimming of free *N*-glycans by α-mannosidases, since it has been shown that GN1 glycans are better substrate than GN2 for these enzymes (Grard *et al.*, 1994; Haeuw *et al.*, 1991). Finally, trimmed free *N*-glycans ($Man_5GlcNAc$) are transported to lysosome for final degradation (*Figure 4*). In addition to the deglycosylation activity, these enzymes also show significant transglycosylation activity, transferring the released *N*-glycan to an acceptor that forms a new glycosidic linkage (Hamaguchi *et al.*, 2010; Umekawa *et al.*, 2008).

ENGases are classified into GH18 and GH85 (Cantarel *et al.*, 2009). ENGases that belong to GH18 have been characterized in bacterial cells. They are evolutionarily related to chitinases (Stals *et al.*, 2012) and it is thought that they are involved in nutrient acquisition and in defence responses (Collin & Olsen, 2001). Recently, GH18 ENGases have been characterized in different fungal species, for instance, the Endo-T from *T. reesei* (Stals *et al.*, 2010) and the Endo-FV from the basidiomycete *Flammulina velutipes* (Hamaguchi *et al.*, 2010), while a GH85 ENGase has been chacterized in the zygomycycete *Mucor hiemalis* (Fujita *et al*., 2004). These enzymes show low sequence homology with the bacterial ENGases and with fungal chitinases (Stals *et al.*, 2012). While cytosolic ENGases are widely distributed in eukaryotic species, they are absent from some yeast species such as *S. cerevisiae* and *Schizosaccharomyces pombe* (Suzuki *et al.*, 2002b). The role of GH18 ENGases in filamentous fungi seems to be important, since the deletion of the cytosolic ones has a severe impact in fungal phenotype affecting the hyphal growth, conidiation and mycoparasitic ability of *T. atroviride* (Dubey *et al.*, 2012).

2 Objectives of this study

The main objective of this study was to investigate the functional role of GH18 enzymes in different aspects of fungal biology and especially during fungal-fungal interactions. This study focused on the function of the killer toxin-like chitinases, since it is speculated that these enzymes play a crucial role in interspecific interactions. Moreover, the putative GH18 ENGases were enzymatically characterized and their roles in the ERAD process were investigated, since only limited knowledge is available about the function of these enzymes in filamentous fungi.

This thesis is divided into four projects and the specific goals were to:

- Investigate the functional role of GH18 and GH20 genes in *Neurospora crassa*.

- Characterize the enzymatic function of putative GH18 ENGases and investigate the role of these enzymes in the ERAD process in the mycoparasitic species *Trichoderma atroviride*.

- Study the functional role of C-II killer-toxin like chitinases in *Aspergillus nidulans*.

- Identify the GH18 genes and study their regulation patterns in the mycoparasitic species *Clonostachys rosea*.

3 Materials and Methods

3.1 Fungal strains and maintenance conditions

Neurospora crassa wild type (WT) and deletion strains (Colot *et al.*, 2006) were maintained on Vogel's minimal medium (VM) with 1% (w/v) sucrose at 25°C in darkness (Vogel, 1956). *Aspergillus nidulans* WT (strain A4) and auxotrophic strains were maintained on the *Aspergillus* minimal medium (AMM, Melin *et al.*, 2003) and supplemented with 1% (w/v) glucose and 1.5% (w/v) agar, as well as the appropriate supplements (biotin, uridine, pyridoxine, riboflavin, nicotinic acid) when needed and incubated at 28°C in darkness. Additionally, *T. atroviride* (strain IMI206040), *C. rosea* (strain IK726), *B. cinerea* (strain B05.10), *Rhizoctonia solani* (strain SA1), *Fusarium sporotrichoides* (strain J26) and *Fusarium graminearum* (strain 1104-14) were maintained on potato dextrose agar (PDA, Sigma-Aldrich, St. Louis, MO) at 25°C in darkness. Finally, *Phytophthora niederhauserii* (strain P10617) was maintained on diluted Granini juice agar (Hosseini *et al.*, 2012) at 25°C in darkness.

3.2 Gene expression analysis and nucleic acid manipulations

For the chitinase gene expression studies in *N. crassa*, agar plugs from *N. crassa* and *B. cinerea, F. sporotrichoides* or *R. solani* were placed at opposite sides of 9-cm VM plates, and mycelia from the growing front of *N. crassa* were harvested 24 hours after contact. For transcriptional analysis in different carbon sources, VM agar plates were supplemented with 1% (w/v) sucrose (carbon rich), 1% (w/v) colloidal chitin or 0.1% (w/v) sucrose (carbon limitation). They were inoculated with 5 mm agar discs, and mycelia were harvested 24hpi.

The expression patterns of *A. nidulans* C-II chitinase genes were investigated during interspecific interactions. Flasks containing potato dextrose

broth (PDB, Sigma-Aldrich, St. Louis, MO) were inoculated concurrently with *A. nidulans* conidia and with homogenized mycelia from *B. cinerea*, *F. sporotrichoides*, *R. solani* or *P. niederhauserii.* Control samples were inoculated with PDB and mycelia were harvested 24 hpi. The transcription patterns of these genes were also investigated in different carbon sources. Flasks containing AMM supplemented with 0.5% (w/v) colloidal chitin, 0.5% (w/v) *R. solani* cell wall material or 1% (w/v) glucose (control samples), were inoculated with *A. nidulans* conidia and incubated for 24 hours at 25°C in darkness.

Finally, the chitinase gene expression patterns were also investigated under similar conditions in the mycoparasitic species *C. rosea*. Flasks containing PDB were inoculated with *C. rosea* mycelia and with homogenized mycelia from *B. cinerea*, *F. graminearum* or *R. solani* at 25°C for 24 hours. For gene expression analysis in different nutrient conditions, *C. rosea* mycelia were inoculated in flasks containing synthetic minimal salts (SMS) medium (Dubey *et al.*, 2012) and supplemented with 1% (w/v) glucose (carbon rich), 0.1% (w/v) glucose (carbon limitation) or 1% (w/v) colloidal chitin as a sole carbon source at 25°C. Mycelia were harvested 24 hpi.

Frozen mycelia were homogenized and total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). In *A. nidulans*, however, a phenol-chloroform protocol was used (Plumridge *et al.*, 2010). For cDNA synthesis, 1000 ng of total RNA, after DNase I treatment (Fermentas, St-Leon-Rot, Germany), was reversed transcribed with the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) using oligo(dT)18 and random hexamer primers. The transcript levels were quantified by reverse transcriptase PCR (RT-qPCR) in an iQ5 qPCR System (Bio-Rad, Hercules, CA) using the SYBR Green PCR Master Mix (Fermentas, St. Leon-Rot, Germany). Data normalization was conducted with the expression levels of different reference genes (*actin*, *β-tubulin*, *gpdA*), and data analysis was carried out with absolute or relative quantification methods (Livak & Schmittgen, 2001).

3.3 Construction of deletion cassettes and transformation process

In *A. nidulans,* the four C-II chitinase genes (*chiC2-1*, *chiC2-2*, *chiC2-3* and *chiC2-4*) were deleted. In that process, the *pyrG* gene (confers prototrophy to uridine and uracil), the *pyroA* gene (confers prototrophy to pyridoxine) and *riboB* gene (confers prototrophy to riboflavin) were used as marker genes, while the hygromycin resistant gene (*hygB*) was used as a marker gene for the deletion of the single C-II chitinase gene (*chiC2*) in *C. rosea*. Construction of

the deletion cassettes were conducted using either fusion PCR techniques as previously described (Szewczyk *et al.*, 2006) or gateway cloning techniques, according to manufacturer's instructions (Invitrogen, Carlsbad, CA). Each deletion cassette included approximately 1200 bp upstream and downstream flanking sequences together with the appropriate marker genes. Transformations in *A. nidulans* were conducted based on a polyethylene glycol protoplast protocol as described by Melin *et al.* (2003) with minor modifications. In *C. rosea*, transformation was performed with an *Agrobacterium tumefaciens* mediated protocol as described previously (Dubey *et al*. 2013; Utermark & Karlovsky 2008)

3.4 Phenotypic analysis of the deletion strains

3.4.1 Growth, conidiation and protoperithecia production

N. crassa WT and GH18 deletion mutants were grown on solid VM, VM + yeast extract (YE) and *Neurospora* synthetic cross (SC) medium plates. Linear growth rates were measured on solid VM at 25°C using race tubes, while growth on chitin media was investigated on VM plates supplemented with 1% (w/v) colloidal chitin. Moreover, mycelial growth rates in different abiotic stress conditions were measured in *N. crassa*, *A. nidulans* and *C. rosea* (WT and mutants) on plates containing VM or PDA (Sigma-Aldrich, St. Louis, MO) supplemented with NaCl, caffeine or sodium dodecyl sulfate (SDS). Conidiation rates were determined in *N. crassa* and *C. rosea* WT and deletion strains on VM or PDA plates, respectively, using a Bright-Line haemocytometer (Sigma-Aldrich, St. Louis, MO). Protoperithecia production in *N. crassa* was assessed on SC medium supplemented with 0.1% (w/v) sucrose.

3.4.2 Protein secretion assays

Total extracellular protein concentration was measured in *N. crassa* WT and in Δ*gh18-10* strains using the Quick Start Bradford Protein Assay kit (Bio-Rad, Hercules, CA). Fungal strains were grown in PDB (Sigma-Aldrich, St. Louis, MO) at 25°C in darkness for 72 hours. Proteins from culture filtrates were precipitated using an acetone precipitation protocol and dissolved in 1 ml PBS buffer solution. Moreover, the extracellular protease activity was investigated in the same strains using milk powder plates as described by Nygren *et al.* (2007) with minor modifications. Plates were inoculated with a 5 mm agar disc, and the diffusion of extracellular proteases was evaluated measuring the discoloration zone.

3.4.3 Interspecific antagonistic ability assays

For the antagonistic assays, flasks containing PDB were inoculated with conidia of *A. nidulans* strains or mycelia from *C. rosea* strains. The expression of subgroup C-II chitinases in *A. nidulans* was triggered by the addition of a 9 mm diameter *B. cinerea* agar plug, followed by co-cultivation for 48 hours at 25°C. The culture filtrates were filter sterilized and re-inoculated with a 5 mm diameter agar plug from *B. cinerea, R. solani* or *F. graminearum* cultures and dry weight biomass production was recorded seven days after inoculation.

3.5 Expression and characterization of *T. atroviride* ENGases in yeast cells

3.5.1 Plasmid construction and yeast transformation

The Eng18A and Eng18B putative GH18 ENGases from *T. atroviride* were expressed in *S. cerevisiae png1*Δ strain, since no ENGase activity has been observed in this yeast strain (Suzuki *et al.*, 2002b). The entry clones were constructed with the pENTRTM-D-TOPO vector (Invitrogen, Carlsbad, CA) and used for the construction of expression clones in the pYES-DEST52 gateway destination vector using the LR Clonase II reaction (Invitrogen, Carlsbad, CA). A polyethylene glycol-based protocol (Elble, 1992) was used for yeast transformation. Positive colonies were observed three to four days after inoculation and used for further analysis.

3.5.2 Enzymatic activity assay

The deglycosylation activity of *T. atroviride* ENGases was investigated using the S-alkylated RNase B protein as a substrate. Yeast cells, expressing the Eng18A or Eng18B proteins, were grown overnight at 30°C in darkness in yeast SC medium without uracil and histidine, supplemented with 2% raffinose, and expression was induced by 2% galactose. The deglycosylation patterns of S-alkylated RNase B were monitored by Western blot analysis using a rabbit anti-RNase B antibody (Rockland, Gilbertsville, PA) diluted 1:5000 and a donkey horseradish peroxidase-linked anti-rabbit IgG antibody (General Electric, Fairfield, CT) diluted 1:5000. The S-alkylated RNase B substrate incubated with only lysis buffer was used as a negative control, and the S-alkylated RNase B treated with 0.2 µl PNGase F (Roche, Manheim, Germany) was used as a positive control. In order to confirm that any band shifting was attributed to deglycosylation activity, Eng18A and Eng18B samples were also treated with 0.2 µl PNGase F (Roche, Manheim, Germany).

3.5.3 RTL spotting assay

RTL is a membrane protein complex composed of a luminal RTA (ricin A-chain nontoxic mutant), a transmembrane domain and the cytoplasmic Leu2 and has been reported as an ERAD-depended substrate able to be degraded by PNGases (Hosomi *et al*., 2010). By using this assay, an assessment can be made about whether or not the protein of interest is able to degrade this substrate. If a protein has the ability to degrade the RTL, then strains cannot grow in leucine auxotrophic conditions, while no degradation can support growth in leucine auxotrophic environment (*Figure 5*). The *S. cerevisiae png1*Δ strains, expressing this RTL protein system, along with the Eng18A or Eng18B proteins, were grown in liquid yeast SC medium, without uracil and histidine and supplemented with 2% raffinose. A six-fold dilution series of each strain were spotted on SC solid medium without uracil and histidine (complete medium) and SC medium without uracil, histidine and leucine, supplemented with 2% galactose. The *Png1* complement strain (ScPng1) and the strain containing only the empty vector (pRS316), were used as a positive and negative control respectively.

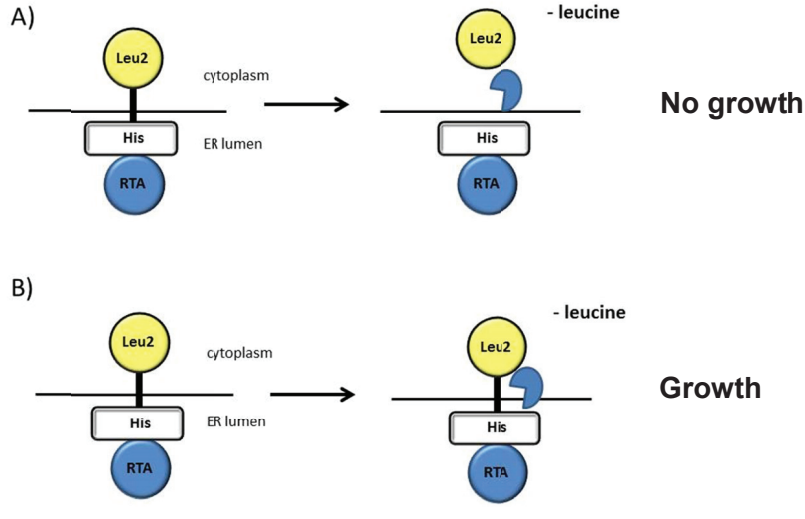

Figure 5. Schematic representation of the RTL protein assay, a PNGase-dependent ERAD substrate. The RTL protein complex consists of the RTA (ricin A-chain nontoxic mutant), a transmembrane domain and the cytoplasmic Leu2 enzyme necessary for leucine biosynthesis. If the protein of interest is able to cleave the RTL, then yeast strains cannot grow on media without leucine (A), while no RTL degradation can support growth on leucine auxotrophic conditions (B).

3.6 Extraction of free *N*-glycans and HPCL analysis

In order to investigate the patterns of free *N*-glycans in *T. atroviride* strains (WT and Δ*Eng18B*), conidia were inoculated in PDB for 5 days at 25°C, and 1 mg dried and homogenized mycelia were suspended in 500µl fOs extraction buffer (Hirayama & Suzuki, 2011). Cell debris was removed by centrifugation. An equal volume of 99% ethanol was added to supernatants and evaporated to dryness, dissolved in water, and then loaded onto AG1-X2 (resin volume, 500 µl; 200-400 mesh; acetate form) and AG50-X8 (resin volume, 500 µl; 200-400 mesh; H+ form) columns (Bio-Rad, Hercules, CA). Flow-through fractions from both columns applied to InertSep GC column (150 mg/3ml; GL-Science). The fOs fraction was absorbed to the column that had been rinsed with water and eluted with acetonitrile. The desalted fOs fraction was lyophilized, and pyridylamination (PA labelling) of *N*-glycans was conducted as previously described (Suzuki *et al.*, 2008) and purified with Concanavalin A agarose (Sigma-Aldrich, St. Louis, MO), followed by treatment with jack bean (JB) α-mannosidases. In order to investigate if peaks derived from GN1 or GN2 oligosaccharides samples were treated with Endoglycosidase H (Endo-H, Sigma-Aldrich, St. Louis, MO).

The size fractionation HPCL analysis was conducted with a Shodex NH2P-50 4E column (4.6 × 150 mm; TOSOH, Tokyo, Japan). Two solvent gradients were used: eluent A (93% acetonitrile in 0.3% acetate, pH 7.0) and eluent B (20% acetonitrile in 0.3% acetate, pH 7.0). The gradient program was set at the flow rate of 0.8 ml/min (expression as the percentage of solvent B): 0–5 min, isocratic 3%; 5–8 min, 3–33%; 8–40 min, 33–71%. PA-oligosaccharides were detected by measuring fluorescence (excitation wavelength, 310 nm; emission wavelength, 380 nm).

4 Results and Discussion

4.1 Functional analysis of group A chitinases

4.1.1 Domain structure and transcription patterns

The analyses of group A chitinases in *N. crassa* and *C. rosea* revealed that, in addition to the GH18 domain, some of group A chitinases contained a signal peptide at the N-termini indicating that these proteins are targeted to the ER, while no CBMs were predicted. This pattern coincides with the situation in *Trichoderma* species group A chitinases (Seidl *et al.*, 2005). Orthologous genes are differentially regulated in different species. In *N. crassa*, the expression of the *gh18-3* gene was induced during interactions with *F. sporotrichoides* compared to the control treatment (*Figure 6A*). In *T. atroviride* the *chi18-3* gene was constitutively expressed (Seidl *et al.*, 2005), and in *C. rosea* the *chiA2* gene was induced by chitin and during carbon starvation conditions (*Figure 6B*). The *T. atroviride* Chi18-3 contains two S-globulin domains (InterPro acc. no: IPR000677) and it is transcribed as two different spliced variants depending on growth conditions (Seidl *et al.*, 2005). Interestingly, it has been reported that all these genes are putatively localized in mitochondria. Additionally, substitutions were observed in their catalytic domain (Karlsson & Stenlid, 2009; Seidl *et al.*, 2005), indicating that their chitinolytic activity might be abolished. Information about the function of the gh18-3 in *N. crassa* is limited, since deletion of this gene in *N. crassa* did not have any impact on the fungal phenotype under the tested conditions.

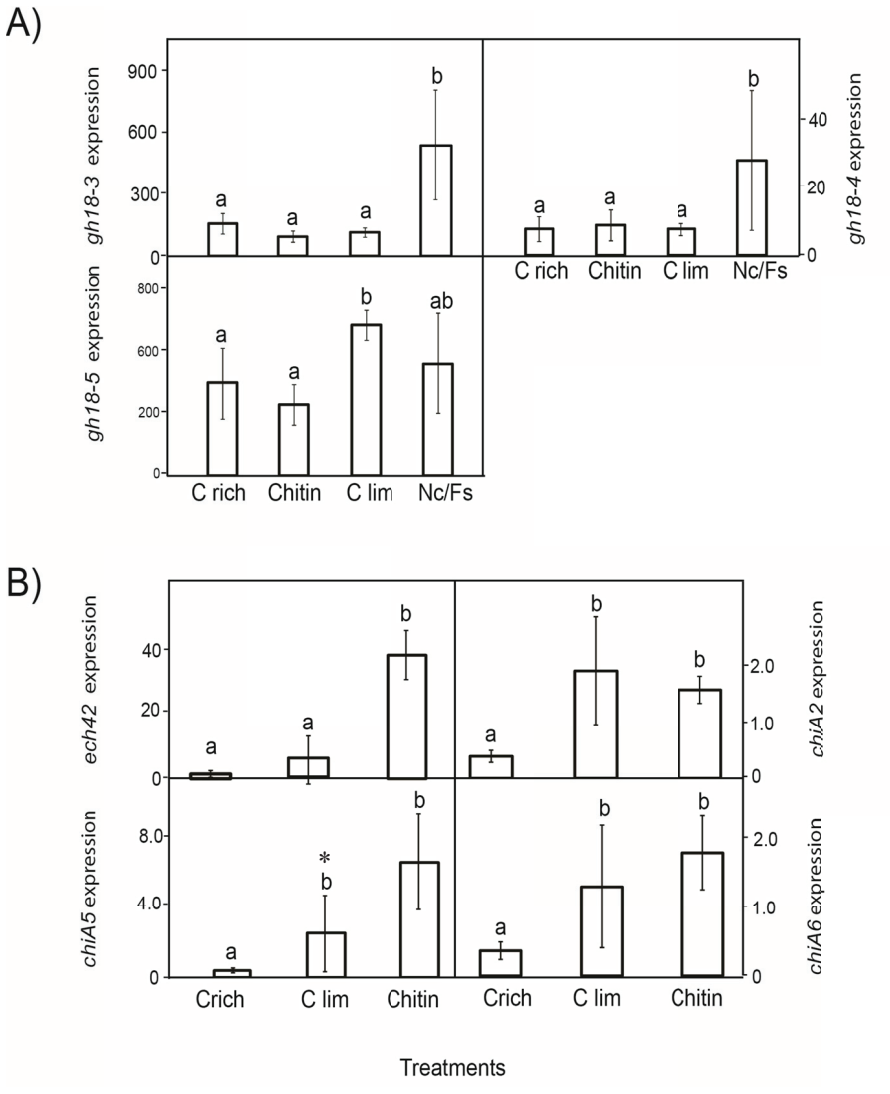

Figure 6. Transcription analysis of group A chitinase genes A) in *Neurospora crassa* after 24 hours of growth on VM supplemented with: 1% (w/v) sucrose (C rich, control), 1% (w/v) colloidal chitin or 0.1 % (w/v) sucrose (C lim). Nc/Fs interaction with *Fusarium sporotrichoides* B) in *Clonostachys rosea* in SMS medium supplemented with 1% (w/v) glucose (Crich, control), 0.1% (w/v) glucose (C lim) or 1% (w/v) colloidal chitin. Data were normalized using the transcripts of the *actin* gene. Different letters (a, b) indicate statistically significant differences according to the Tukey's (A) or Fisher (B) test ($P \leq 0.05$). Error bars represent the standard deviation of at least four biological replicates. Asterisk indicates one outliner measurement.

The *N. crassa* and *C. rosea* genomes contain three and four genes in the A-V subgroup of GH18 respectively. In *N. crassa*, the *gh18-4* gene was induced only during contact with *F. sporotrichoides* mycelia compared to the control treatment (*Figure 6A*), indicating a potential role in fungal-fungal interactions, and the *gh18-5* was induced on carbon limitation conditions. In contrast, the ortholog *ech42* gene in *C. rosea* was not triggered in fungal-fungal interactions (data not shown), while high induction was observed in chitin media (*Figure 6B*), which suggests a role in exogenous chitin degradation. The ortholog to these genes in *Trichoderma* species is the well-known *ech42.* As previously mentioned, the *ech42* gene was highly induced during fungal-fungal interactions and during growth on chitin, and was also involved in autolysis (Seidl 2008; Carsolio *et al.*, 1994). All of these data claim that ECH42 might have multiple and differing roles in the life cycle of different fungal species. Regarding the expression patterns of the other group A genes, the *chiA5* and *chiA6* genes in *C. rosea* were triggered by chitin and in carbon starvation conditions (*Figure 6B*) indicating a role in nutrient acquisition. Finally, deletion of single group A chitinase genes did not show any phenotypic impact in *N. crassa* under the tested conditions, possibly because the function of each deleted gene is substituted by the others.

4.2 Functional analysis of group B chitinases

4.2.1 Modular structure and transcriptional analysis

The phylogenetic GH18 group B contains endo-acting chitinolytic enzymes, while genes in the B-V subgroup encode ENGases (Stals *et al.*, 2010). Regarding the transcription patterns of chitinases in the B-I/B-II subgroups, induction of the *chit-1* gene in *N.crassa* was observed on chitin media compared to the control treatment (*Figure 7A*). The modular structure of this protein revealed the presence of a signal peptide, indicating that it is targeted to the ER and that it contains a GPI anchor implying that it is putatively localized to the cell wall.

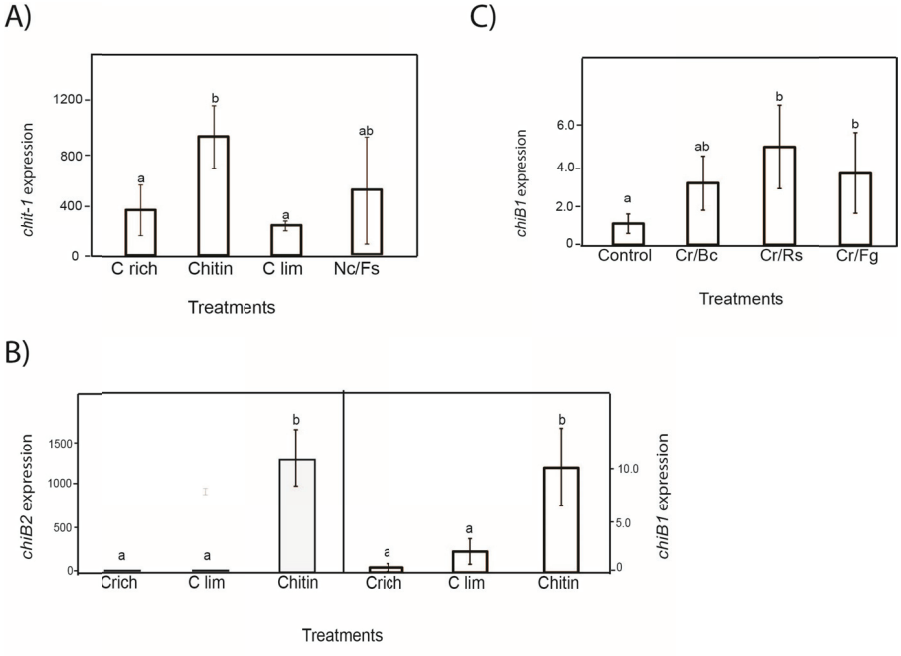

Figure 7. Transcription analysis of group B chitinase genes A) in *Neurospora crassa* after 24 hours growth in VM supplemented with: 1% (w/v) sucrose (C rich) used as a control, 1% (w/v) colloidal chitin, 0.1 % (w/v) sucrose (C lim). Nc/Fs interaction with *Fusarium sporotrichoides,* B) in *Clonostachys rosea* in SMS medium supplemented with 1% (w/v) glucose (Crich, control), 0.1% (w/v) glucose (C lim) or 1% (w/v) colloidal chitin 24hpi C) in *Clonostachys rosea* during interactions with *Botrytis cinerea* (Cr/Bc), *Rhizoctonia solani* (Cr/Rs), *Fusarium graminearum* (Cr/Fg), while *C. rosea* grown only in PBD was used as a control 24 hpi. Data were normalized using the transcripts of the *actin* gene and different letters (a, b) indicate statistically significant differences according to Tukey's (A) or Fisher's (B and C) test ($P \leq 0.05$). Error bars represent the standard deviation of at least four biological replicates.

The *C. rosea* genome contains two chitinase genes in this subgroup (*chiB1* and *chiB2*) and both were highly induced on chitin media (*Figure 7B*). Similarly, the group B chitinase gene *chit33* in *T. harzianum* was also induced by chitin (Dana *et al.*, 2001), implying an involvement of these chitinases in exogenous chitin degradation. In addition, the *chiB1* gene was induced during interactions with *R. solani* and *F. graminearum* (*Figure 7C*). The induction of the *chi18-13* gene, which also belongs to group B, was observed in *T. atroviride* before and during contact with *B. cinerea* (Seidl *et al.*, 2005) indicating that these chitinases are involved in aggressive interactions. In mycoparasitic *Trichoderma* species an increased number of group B chitinases was observed (Ihrmark *et al.*, 2010). This increase suggests that group B chitinases play an important role in mycoparasitism. The fact that the *C. rosea*

genome contains only two genes in this group suggests that endochitinases are not the major factor that contributes to the mycoparasitic interactions in this species.

4.2.2 Phenotypic analysis of group B chitinase deletion strains

The deletion of the *chit-1* gene led to several phenotypic changes in *N. crassa*. Firstly, this deletion strain displayed slower growth rate on VM plates (*Figure 8A*). However, no difference in growth rate between WT and the Δ*chit-1* strain was observed on chitin media (*Figure 8B*), which suggests that the growth reduction is related the to cell wall plasticity. This deletion strain also showed higher protoperithecia production compared to WT (WT = 20 ± 5 (standard deviation) protoperithecia/plate Δ*chit-1* = 90 ± 23, $P = 0.008$). Protoperithecia production is known to be induced by nitrogen starvation (Sommer *et al.*, 1987) or through the osmotic stress mitogen-activated protein kinase (MAPK) pathway (Jones *et al.*, 2007). Higher protoperithecia production was also observed in the inhibition zone between the *chit-1* deletion strain and *A. nidulans,* compared to WT (WT = 62 ± 11, Δ*chit-1* = 181 ± 20 ($P < 0.001$). These data suggests that the deletion of *chit-1* results in a lower threshold of protoperithecia initiation.

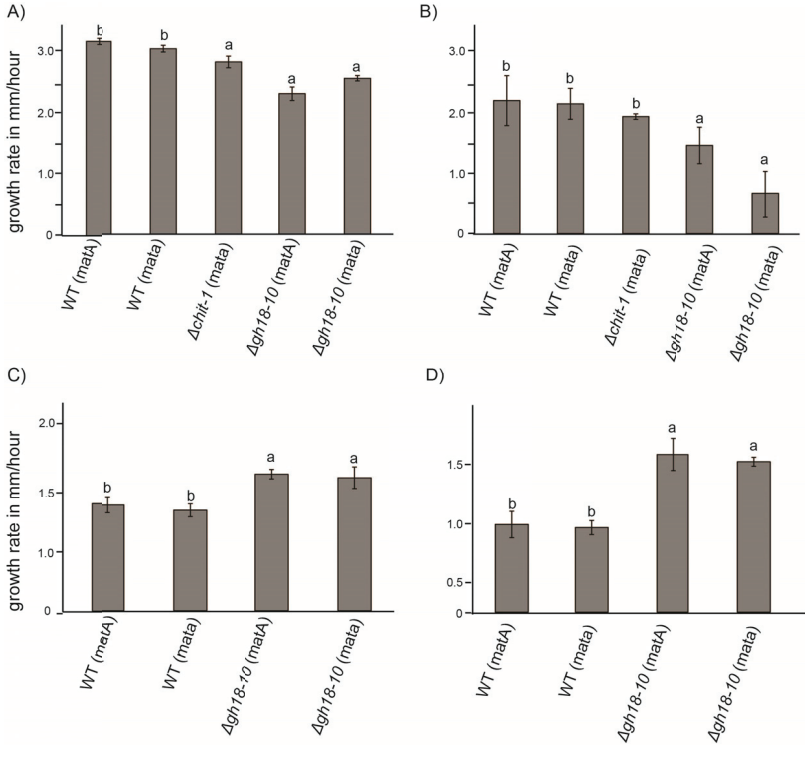

Figure 8. Linear growth rate (mm/hour) of *Neurospora crassa* WT and GH18 gene deletion strains in A) VM + 1% (w/v) sucrose, B) VM + 1% (w/v) colloidal chitin, C) VM + 1% (w/v) sucrose supplemented with 1M NaCl and D) VM + 1% (w/v) sucrose supplemented with 1% (w/v) caffeine. Plates were inoculated with agar plug and incubated at 25°C in darkness. Different letters (a, b) indicate statistically significant differences according to Tukey's test ($P < 0.05$). Error bars represent the standard deviation of three biological replicates. *mata*: mating type gene a, *matA*: mating type gene A.

4.3 Functional role of GH18 ENGases

4.3.1 Phenotypic analysis of the intracellular ENGase deletion strain

ENGases are hydrolytic enzymes responsible for cleaving the *N,N'*-diacetylchiotobiose moiety from high mannose *N*-linked glycans (Karamanos *et al*., 1995). Filamentous fungi usually contain two genes encoding putative GH18 ENGases: One enzyme is putatively cytosolic, and the other is predicted to be targeted to the ER. In *N. crassa*, deletion of the *gh18-10* gene, coding for a putative intracellular GH18 ENGase, had a severe phenotypic impact. The *gh18-10* deletion strains (*mata* and *matA*) displayed a slower growth rate on carbon rich and chitin media compared to WT (*Figure 8A* and *B*). However, they displayed an increased tolerance to different abiotic cell wall stress conditions, since higher growth rate was observed on plates supplemented with NaCl or caffeine compared to WT (*Figure 8C* and *D*). Moreover, the Δ*gh18-10* strains showed higher conidiation rate compared to WT (*Figure 9A* and *B*), whereas no mature perithecia were observed when these deletion strains were mated with WT strains (data not shown). Interestingly, similar results were observed in *T. atroviride* when the *Eng18B* gene (ortholog to *gh18-10*) was deleted (Dubey *et al.*, 2012). The Δ*Eng18B* strain displayed slower growth rate on carbon rich and chitin media, while it was more resistant to abiotic stress conditions (Dubey *et al.*, 2012). Similarly, the conidiation rate was higher in the Δ*Eng18B* strain, while deletion of this gene led to reduced antagonistic ability against *B. cinerea* (Dubey *et al.*, 2012). These data claim that the putative intracellular ENGases perform a conserved function in filamentous ascomycetes.

Additionally, this study, showed that the total amount of secreted proteins in the *gh18-10* deletion strains was significantly lower compared to WT (*Figure 9C*), and that the extracellular protease activity in these deletion strains was significantly reduced (*Figure 9D*). Also, the deletion of this gene resulted in down-regulation of the chitin synthase genes *chs-1*, *chs-2* and *chs-7* (Data not shown). According to these data, one can speculate that improper secretion leads to reduced amount of enzymes localized to the cell wall and involved in cell wall modifications, such as chitinases, proteases and glucanaces, results in

a more rigid cell wall. A possible consequence of this malfunctioned cell wall is reduced hyphal growth and an increased resistance to abiotic stress.

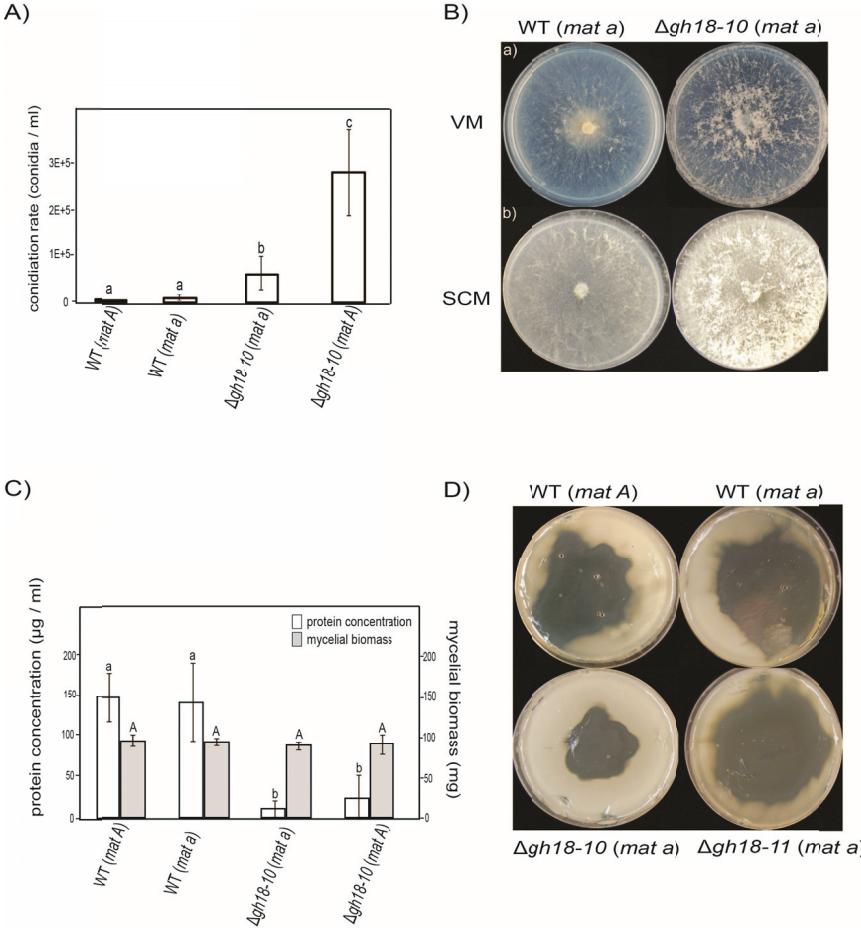

Figure 9. Phenotypic analysis of *Neurospora crassa* GH18 ENGase deletion strains. A) Conidiation rate (conidia/ml) on VM supplemented with 1% (w/v) sucrose 72hpi at 25°C in darkness. Different letters (a, b, c) indicate statistically significant differences according to Tukey's test ($P \leq 0.05$). Error bars represent the standard deviation of three biological replicates. B) Colony morphology on different media: VM + 1% (w/v) sucrose and SCM + 0.1 % (w/v) sucrose. Photographs were taken 4 dpi. C) Total amount of extracellular protein concentration and mycelial biomass. Fungal strains were grown for 72 hours in PDB at 25°C in darkness. Different letters (a, b) indicate statistically significant differences according to Tukey's test ($P \leq 0.05$). Statistical analysis was conducted between columns with the same color. Error bars represent the standard deviation of three biological replicates. D) Protease activity on dextrose media + 1.5 % (w/v) agar covered by milk powder and incubated at 25°C in darkness. Enzymatic activity was determined by measuring the discoloration zone 48hpi. *mata*: mating type gene a, *matA*: mating type gene A.

4.3.2 Heterologous expression of *T. atroviride* GH18 ENGases in yeast cells and enzymatic assays

The severe phenotypic effects resulting from deletion of the putative GH18 cytosolic ENGases show that these enzymes have an important and conserved role in the ascomycete life cycle. The importance of ENGases in fungi is supported by the data published by Maerz *et al.* (2010) show that the cytosolic PNGase in *N. crassa* is not an active deglycosylating enzyme. In *T. atroviride* a single gene encoding a putative cytosolic PNGase was identified. However, an amino acid substitution in its catalytic triad was observed. Specifically, Cys^{250} (corresponding to Cys^{191} in *S. cerevisiae*) was substituted for Val, indicating that this enzyme is not active either. Interestingly, the presence of a second acidic PNGase has been reported in *N. crassa* (Maerz *et al.*, 2010), although the function and the localization of this enzyme remain to be elucidated. Hence, it was decided to study the function of ENGases in *T. atroviride* since this species apparently does not contain any active PNGase, implying that the only deglycosylating activity derives from the ENGases.

The heterologous expression of both *T. atroviride* GH18 ENGases (Eng18A and Eng18B) was successful in the *S. cerevisiae png1*Δ strain, even though the expression level of Eng18B was higher than the Eng18A (*Figure 10A*). Furthermore, this study indicates that Eng18A is a highly *N*-glycosylated protein since a band with lower molecular weight was observed when samples were treated with Endo-H, an enzyme with ENGase activity, derived from *Streptomyces plicatus* (*Figure 10B*). It is known that the *N*-glycosylation process is essential for the trafficking and secretion of variable proteins (Wujek *et al.*, 2004; Helenius & Aebi, 2001; Ray *et al.*, 1998). These data in combination with the presence of a signal peptide at the *N*-terminal indicate that Eng18A is targeted to the ER and it is secreted, a feature that was proven earlier for the ortholog Endo-T in *T. reesei* (Stals *et al*., 2010). In contrast, no difference in Eng18B molecular weight was observed between samples treated with Endo-H and untreated samples (*Figure 10C*), while the expression of the dolichol-phosphate-mannose synthase (Dpm 1) was used as a loading control.

The deglycosylation activity of *T. atroviride* ENGases was investigated using the highly *N*-glycosylated S-alkylated RNase B protein as a substrate. This assay revealed that both ENGases are active deglycosylating enzymes since bands with a similar molecular weight to the positive control were identified (*Figure 10D*). Deglycosylation activity has been reported previously in *T. reesei,* where Endo-T showed similar patterns (Stals *et al*., 2010). In addition, an active GH18 ENGase has also been characterized in the basidiomycete *Flammulina velutipes* (Hamaguchi *et al*., 2010). According to

our knowledge, this study is the first enzymatic characterization of an intracellular GH18 ENGase in filamentous ascomycetes.

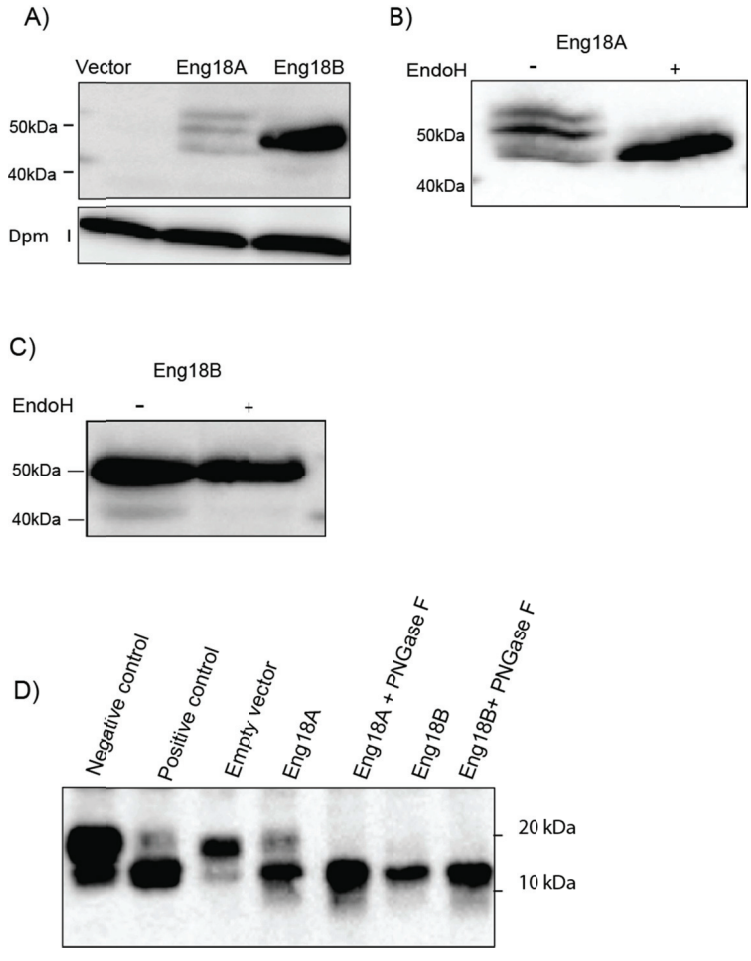

Figure 10. A) Heterologous expression of the *Trichoderma atroviride* Eng18A and Eng18B in the *Saccharomyces cerevisiae png1* deletion strain. B) Treatment of Eng18A with (+) or without (-) Endo-H. C) Treatment of Eng18B with (+) or without (-) Endo-H. Visualization of the bands was conducted by a Western blot analysis with an anti-V5 antibody towards ENGases and anti-Dpm 1 antibody for Dpm 1 expression. D) Deglycosylation activity resulted in decreased molecular weight of S-alkylated RNAse B protein and can be detected by a shift of migration in SDS-PAGE gel. Negative control was treated only with lysis buffer and positive control was treated only with PNGase F. Visualization of proteins was conducted by a Western blot with an anti-RNase B antibody.

It is known that the RTL protein complex is an ERAD-dependent substrate (Hosomi *et al.*, 2010). In order to identify if fungal ENGases are able to

degrade this complex, a spotting assay was conducted. Results revealed that the Eng18B was able to degrade the RTL protein, since no growth was reported in leucine auxotrophic conditions, while the Eng18A could not disrupt the RTL stability (*Figure 11*). In *S. cerevisiae*, different enzymes involved in the ERAD process are able to degrade the RTL complex. For instance the Htm1, an EDEM ortholog responsible for misfolded glycoprotein targeting, (Hosokawa *et al.*, 2001) and the Yos9, a lectin recognizing the α-1.6 mannose exposed by Htm1 action, (Bhamidipati *et al.*, 2005) can degrade the RTL complex as well (Hosomi *et al.*, 2010). In contrast the Mns-1, an α-1.2 mannosidase in *S. cerevisae*, or some ubiquitin-ligase proteins cannot degrade this protein complex (Hosomi *et al.*, 2010) similar to Eng18A. The fact that ENGases displayed different degradation patterns might be attributed to the different localization of these enzymes in yeast cells.

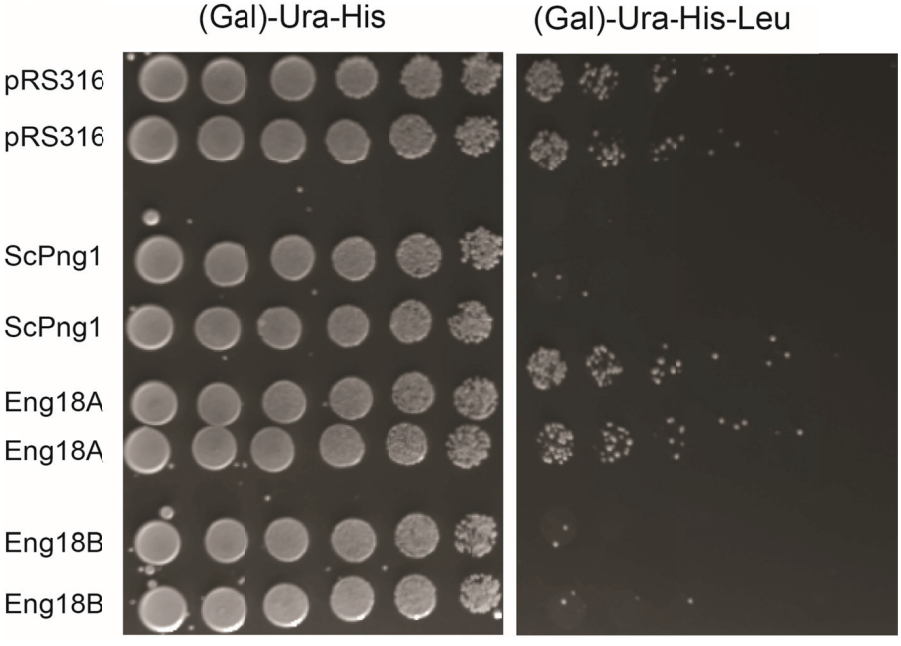

Figure 11. Effect of *Trichoderma atroviride* GH18 ENGases on RTL stability. A six-fold dilution series of each strain were spotted onto a SC solid medium void of uracil and histidine (complete medium) and onto a SC medium without uracil, histidine and leucine supplemented with 2% galactose and were incubated at 30°C in darkness for 72 hours. A yeast strain expressed only the empty vector (pRS316) was used as a negative control, while the *png1* complemented yeast strain (ScPng1) was used as a positive control. Each strain has two technical replicates.

4.3.3 Analysis of free *N*-glycan patterns in the *T. atroviride* cytosol

Cytosolic *N*-glycans were extracted from *T. atroviride* strains (WT, Δ*Eng18* and Δ*Eng18*+ complemented strain) and analyzed using HPLC techniques. Our analysis revealed several peaks sensitive to JB α-mannosidases an indication that they derived from fOSs, since it is hypothesized that filamentous fungi generate high mannose free *N*-glycans similar to yeasts (Hirayama *et al*., 2010) (*Figure 12*). Similar peaks patterns were observed between samples treated with Endo-H and untreated ones, which suggest that *T. atroviride* might form only fOSs-GN1 (*Figure 12)*, because this enzyme is able to cleave the chitobiose residue that exists only in fOSs-GN2. Interestingly, according to the method used in the current study, no peaks were observed in Δ*Eng18B* cytosol (*Figure 12)*. It is possible free *N*-glycans are also generated in the *Eng18B* deletion strain, but the amount was undetectable under the current tested conditions. Finally, similar to WT, peak patterns were observed in the complemented strain (*Figure 12)*, supporting the Eng18B involvement in free *N*-glycans formation. In general, free *N*-glycans in eukaryotic cells can be generated by the PNGase activity, the OST enzymatic complex or the function of pyrophosphatases (Harada *et al.*, 2013). These data confirm that the *T. atroviride* PNGase is not an active enzyme, since only fOSs-GN1were observed. Furthermore, this study showed that Eng18B is the main factor for free *N*-glycans generation in this fungal species. This study is the first to report that free oligosaccharides can be formed directly by the GH18 ENGase activity, indicating that filamentous ascomycetes employ different mechanisms compared to yeasts and mammals to cope with the degradation of misfolded glycoproteins.

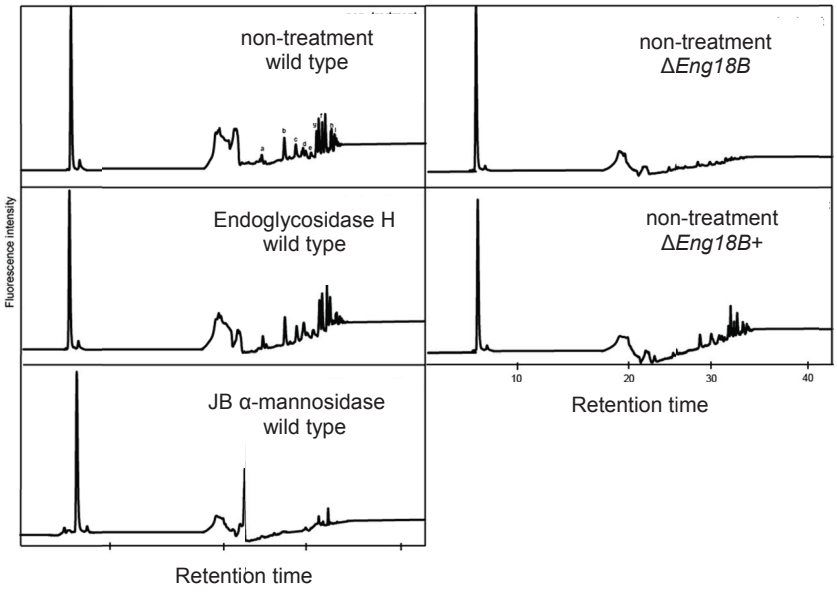

Figure 12. HPLC size fractionation profiles of *Trichoderma atroviride* free *N*-glycans. PA labeled oligosaccharides isolated from WT, Δ*Eng18B*, and Δ*Eng18B+* strains. Peaks were treated with Endo-H or JB mannosidases in order to identify their origin.

4.4 Functional role of group C chitinases

4.4.1 Domain structure and transcription profiles

The chitinases in group C display similarities with the α/β subunit of the killer toxin zymocin produced by the yeast *K. lactis*, and it is hypothesized that these enzymes have a prominent role in fungal-fungal interactions. (Seidl *et al*, 2005). The *N. crassa* genome contains two genes in C-II subgroup, while *A. nidulans* and *C. rosea* contain four and one respectively. Analysis of C-II subgroup chitinases revealed that their modular structures were similar to *Trichoderma* C group chitinases (Seidl *et al.,* 2005), which contain a single GH18 module, a signal peptide at the N-terminus, CBM-50 and CBM-18 modules. Furthermore, the gh18-8 in *N. crassa* and the chiC2-3 and chiC2-4 in *A. nidulans* are putatively localized to the cell wall since transmembrane spanning modules were predicted at the C-termini. Interestingly, Stergiopoulos *et al.* (2012) reported that the Hce2 effector domain is putatively identified in variable fungal proteins, but in chitinases is found exclusively in some killer toxin-like ones. According to their study, only gh18-6, gh18-8 and chiC2-2 contain this effector. Hce2 is homologous to the Ecp2 effector protein which

has been described in different plant pathogens as a virulent factor causing necrosis in plant cells during infection (Stergiopoulos *et al.*, 2010; Lauge *et al.*, 1997).

Similar to group B chitinases, an increased number of killer toxin-like chitinases has been observed in mycoparasitic *Trichoderma* species (Gruber & Seidl-Seiboth, 2012; Ihrmark *et al.*, 2010). In contrast, the current study revealed that the *C. rosea* genome contains only two C group chitinase genes, indicating that these enzymes possibly are not the main determinants in *C. rosea* mycoparasitic interactions.

Regarding the transcription patterns of C-II subgroup chitinases, this study revealed that the *N. crassa gh18-6* gene was induced during interactions with *B. cinerea* and *F. sporotrichoides* but not during contact with *R. solani* compared to self-interactions (*Figure 13A*). These findings suggest a potential role during interactions with ascomycetes, while induction of *gh18-8*, the second member of the C-II subgroup in this species, was observed during self-interactions compared to interactions with *R. solani* or *F. sporotrichoides* mycelia (*Figure 13A*). Induction of C-II chitinases during contact with *B. cinerea* was also observed in *T. atroviride* (Gruber *et al.*, 2011b). However, in *A. nidulans* all C-II genes were induced not only during contact with *B. cinerea* but also during interactions with *R. solani* (*Figure 13B*). Moreover, only *chiC2-2* and *chiC2-3* were induced during contact with *F. sporotrichoides*, and no induction was observed during interactions with *P. niederhauserii* (*Figure 13B*).

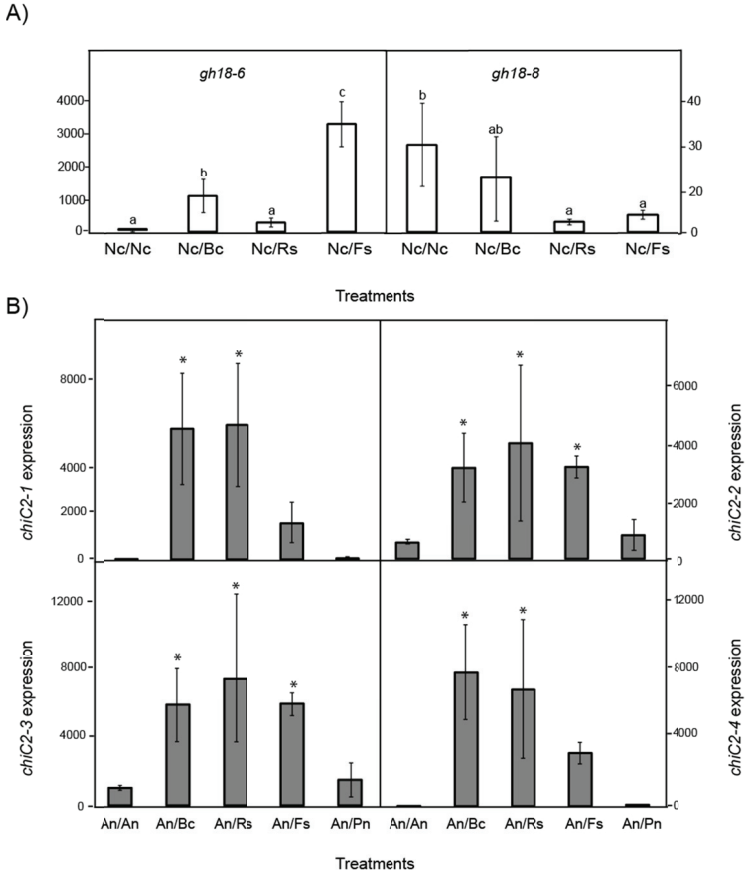

Figure 13. Transcription analysis of C-II subgroup chitinase genes A) in *Neurospora crassa* (Nc) and B) in *Aspergillus nidulans* (An) during interactions with: *Botrytis cinerea* (Bc), *Fusarium sporotrichoides* (Fs), *Rhizoctonia solani*.(Rs) or *Phytopthora niederhauserii* (Pn), while self-interactions (Nc/Nc or An/An) was used as a control. Data were normalized using the transcripts of A) *actin* gene or B) *gpdA* gene and different letters (a, b, c) or asterisks indicate statistically significant differences according to Tukey's (A) or Fisher's (B) test ($P \leq 0.05$). Error bars represent the standard deviation of at least four biological replicates.

In contrast, no induction of the *A. nidulans* C-II genes was observed in colloidal chitin, while they were down-regulated during growth on *R. solani* cell wall material compared to glucose-rich media (data not shown). Thus, these data indicate that inducers derived from living fungal cells walls are able to trigger the expression of these genes. The transcription patterns of the *chiC2* gene, which is the only C-II chitinase gene in *C. rosea*, revealed that it was constitutively expressed in all tested conditions (data not shown). The fact that different C-II subgroup chitinase genes were regulated differentially in diverse fungal species coincides with the transcriptional data derived from

Trichoderma species, where even orthologous chitinase genes in this group displayed different transcription patterns (Gruber *et al.*, 2011a). According to these data, one can speculate that the C-II subgroup chitinases display specificity during interactions with fungal cell walls. Finally, chitinases that belong to the C-I subgroup, revealed different transcription patterns compared to C-II genes. In *N.crassa* the *gh18-9* gene and the *chiC1* in *C. rosea* were both induced by colloidal chitin (data not shown). In *T. atroviride* the group C chitinase genes *tac2, tac6* and *tac 7* were also induced during growth on chitin, which indicates a possible role in exogenous chitin degradation (Gruber *et al.*, 2011b).

4.4.2 Phenotypic analysis of C-II subgroup chitinase deletion strains

Until now, data derived from phenotypic analysis of group C chitinase deletion strains have been limited. In *T. atroviride* deletion of *tac6* resulted in increased growth rate, faster overgrow of fungal prey and changes in conidiation rate (Seidl *et al*., 2013). However, as mentioned earlier, this chitinase contains a mutation in its catalytic motif which is predicted to disrupt the chitinolytic activity (Gruber *et al.*, 2011b). Under our tested conditions, deletion of the C-II chitinase genes *gh18-6* and *gh18-8* did not have any phenotypic impact in *N. crassa* phenotype. However, deletion of C-II chitinase genes in *A. nidulans* altered the responses to abiotic stress (*Figure 14A*). Similarly, cell wall stress influences the expression patterns of group C chitinases in *T. virens* (Gruber *et al.*, 2011a), indicating that these enzymes affect the hyphal growth and the cell wall integrity. An increased biomass production was also observed in liquid cultures (*Figure 14B*).

Interestingly, this study revealed that *B. cinerea* had higher biomass production when it grew in culture filtrates derived from the Δ*chiC2-2* strain, compared to WT and the other deletion strains (*Figure 14B*). The lack of effect on *B. cinerea* growth inhibition in culture filtrates derived from the Δ*chiC2-3* and Δ*chiC2-4* possibly is attributed to the localization of these enzymes, since they are predicted to be localized in cell wall. As previously mentioned, only the ChiC2-2 in *A. nidulans* contains the Hce2 effector domain (Stergiopoulos *et al.*, 2012). This domain is hypothesized to act as an effector protein not only during fungal-plant interactions, as well as during fungal-fungal interactions (Stergiopoulos *et al.*, 2012). Thus, these data indicate that Hce2 probably plays a prominent role in toxin permeability or that ChiC2-2 is proteolytically cleaved to release the Hce2, which may function as a toxin.

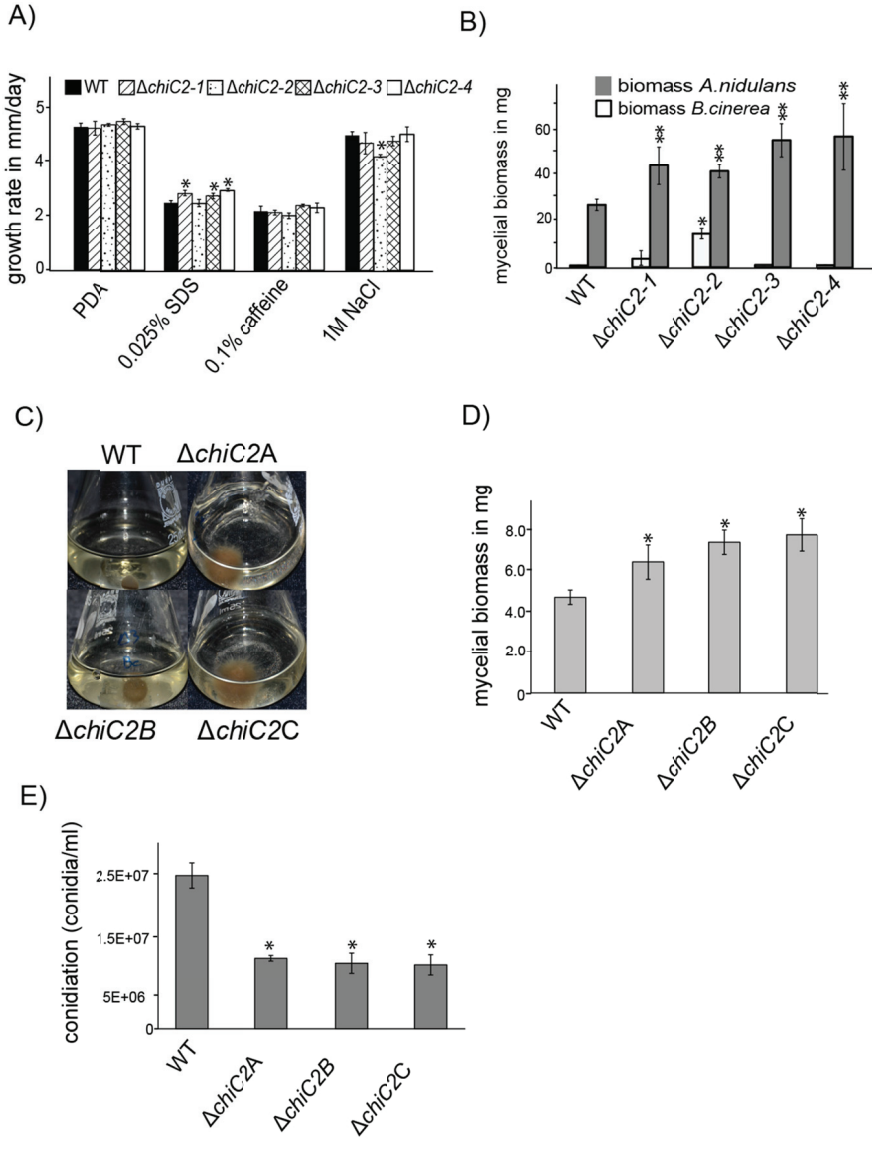

Figure 14. Phenotypic analysis of C-II chitinase gene deletion strains A) Growth rate of *Aspergillus nidulans* wild type (WT) and deletion strains on PDA and on abiotic cell wall stress conditions. B) Biomass production (in mg) of *A. nidulans* WT and deletion strains in PDB cultures (grey columns) and biomass production (in mg) of *Botrytis cinerea* (white columns) grown in culture filtrates derived from *A. nidulans* WT and deletion strains. C) Fungal growth of *B. cinerea* in culture filtrates derived from *Clonostachys rosea* WT and *chiC2* deletion strain. D) Biomass production (in mg) of *Rhizoctonia solani* grown in culture filtrates derived from *C. rosea* WT and *chiC2* deletion strain. E) Conidiation rate (conidia/ml) of *C. rosea* WT and *chiC2* deletion strain on PDA plates. Asterisks indicate statistically significant differences according to Fisher's test ($P \leq 0.05$). Error bars represent the standard deviation of three biological replicates.

The deletion of *chiC2* in *C. rosea* led to similar results. Increased biomasses of *B. cinerea* and *R. solani* were observed when these species grew in *C. rosea* culture filtrates derived from the deletion strain compared to WT (*Figure 14C and D*), suggesting that this killer toxin-like chitinase is involved in fungal-fungal interactions, probably by enhancing the permeability for secondary metabolites into prey cells. Interestingly, these data showed that this deletion did not affect the *C. rosea* growth inhibitory activity against *F. culmorum* (data not shown), supporting the hypothesis about specificity. Finally, this deletion strain displayed lower conidiation rate compared to WT (*Figure 14E*) indicating that this enzyme probably is involved not only in fungal-fungal interactions but also in the sporulation process.

5 Summary

The main conclusions from this study are as follows:

- The modular structures of group A chitinases in *N. crassa* and *C. rosea* were similar to *Trichoderma* species containing only a single GH18 catalytic motif. Moreover, some of these chitinases are targeted to the ER.

- The *C. rosea* genome contains reduced numbers of genes encoding endo-chitinases, as compared to *Trichoderma* mycoparasitic species. This difference indicates that these enzymes possibly do not play the major role during mycoparasitic interactions in *C. rosea*.

- In *Neurospora crassa,* the cell wall localized group B chit-1 chitinase is shown to be involved in the hyphal growth and in protoperithecia production.

- The cytosolic GH18 ENGases play a conserved and important role in filamentous ascomycetes biology, since the deletion of this gene in *N. crassa* affects hyphal growth, abiotic stress tolerance, protein secretion, as well as sexual and asexual reproduction, similar to *T. atroviride*.

- The *T. atroviride* cytosolic PNGase contains a mutation at the catalytic motif and its enzymatic activity is possibly disrupted.

- *T. atroviride* contains two GH18 ENGase active enzymes, while the Eng18B is the main factor for free *N*-glycans generation.

- The cytosolic Eng18B ENGase in *T. atroviride* was able to degrade the RTL protein complex, indicating that this enzyme is involved in the ERAD process.

- The group C killer toxin-like chitinases were induced during fungal-fungal interactions in all tested fungal species, indicating a potential role in interspecific interactions even in saprophytes.

- Different transcription patterns of C-II chitinase genes were observed during interactions with different fungal species, implying possible specificity.

- Deletion of the *ChiC2-2* C-II chitinase in *A. nidulans* reduced the inhibitory growth activity of culture filtrates against *B. cinerea,* suggesting a role in fungal-fungal interactions.

- The ChiC2-2 chitinase was predicted to contain the Hce2 effector domain, implying that this domain plays a prominent role in fungal-fungal interactions.

- C-II subgroup chitinases possibly play a role in cell wall modification since deletion of these genes in *A. nidulans* altered its tolerance to the abiotic stress environment.

- The chiC2 killer toxin-like chitinase in *C. rosea* is involved in fungal-fungal interactions, since the deletion of this gene affects the *C. rosea* antifungal activity of culture filtrates against *B. cinerea* and *R. solani.*

- Deletion of the chiC2 chitinase in *C. rosea* did not affect its *in vitro* antifungal activity against *F. graminearum*, supporting our hypothesis about specificity.

- An additional role of the chiC2 C-II chitinase in *C. rosea* conidiation was revealed, implying that these chitinases play multiple roles in the fungal life cycle.

6 Future perspectives

The functional roles of GH18 enzymes in filamentous ascomycetes were investigated in this study. Previous knowledge about the specific function of chitinases was limited. Regarding the GH18 ENGases, besides the data characterizing their enzymatic function in filamentous fungi, no more information was available. Although several aspects regarding the function and regulation of these enzymes were disclosed by this study, the following areas need further exploration.

Interestingly, the intracellular GH18 ENGases seem to have a conserved role in filamentous ascomycetes. Since this enzyme is involved in the ERAD process and PNGases are not active, we can speculate that deletion of cytosolic ENGases might cause accumulation of misfolded glycoproteins in fungal cytosol. Thus, it would be valuable to quantify and compare the total amount of misfolded glycoproteins in the cytosol of WT and ENGase deletion strains. It would also be worthwhile to study the cell wall structure of the ENGase deletion strains using electron microscopy or by quantifying the amount of cell wall compounds in order to identify if this deletion causes any cell wall modification. Furthermore, it would be interesting to investigate the role of the cytosolic ENGases in pathogenic fungi virulence. Moreover, no data are available about the function of the secreted ENGases, other than the enzymatic characterization in some fungal species, so the deletion of these genes may provide further information about the role in fungi. Furthermore, the hypothesis that secreted fungal ENGases act against alien glycoproteins for nutrient purposes could be further investigated.

N. crassa contains an acidic PNGase with unknown enzymatic activity and function. It would be interesting to investigate if this PNGase is an active deglycosylating enzyme, where it is localized, and if it is involved in free *N*-glycans generation. Moreover, it is known that in mammalian cells, free *N*-glycans can be formed by the enzymatic function of the OST complex and

additionally, pyrophosphatases can generate free oligosaccharides from the cytosolic side of the ER. Hence, it is challenging to investigate if these pathways can generate free *N*-glycans in filamentous ascomycetes.

The specific function of the group C chitinases needs to be elucidated. It would be worthwhile to investigate if these enzymes cause any modification in antagonist cell wall. Interestingly, the data showed that these genes were not induced by chitin or by *R. solani* cell wall material, indicates that molecules produced by living cells are responsible for this induction. Thus, an analysis could be conducted in order to investigate the potential inducers of these genes. Finally, further investigation can be conducted about the role of CBM-18 and CBM-50 modules, by deleting these domains from the C group chitinases and studying the impact in interspecific interactions.

Furthermore, some views about the potential practical uses of the findings arose. The data showed that blocking the deglycosylation process leads to reduced protein secretion. Since pathogenic fungi are relied on secreted proteins in order to establish a successful infection, deactivating the deglycosylation process could be a potential target in control of diseases caused by pathogenic fungi not only in plants but also in humans. Moreover, since chitin is not present in mammalian and plant cells it has been considered as a potential target for antifungal treatment. This study showed that certain killer toxin-like chitinases play an important role in fungal-fungal interactions. Thus, overexpressing these enzymes in mycoparasites could improve their biocontrol efficacy against plant pathogens and pests. Furthermore, transgenic plants overexpressing killer toxin-like chitinases could show increased resistance to pathogens. Finally, since killer toxin-like chitinases are supposed to facilitate the permeabilization of toxins to antagonistic cells it would be interesting to purify these enzymes and to utilize them as antifungal agent enhancers against mycoses.

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