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Chitin catabolism in filamentous fungi

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„Erfolg hat drei Buchstaben: TUN!“

Johann Wolfgang von Goethe

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Kurzfassung

Chitin ist ein Polysaccharid, das aus *N*-Acetylglucosamineinheiten (GlcNAc) aufgebaut ist. Die *N*-Acetylglucosamineinheiten sind durch β-1,4-glycosidische Bindungen verknüpft. Chitin ist neben Cellulose, das am weitesten verbreitete Polysaccharid. Es kommt im Exoskelett von Insekten und Krustentieren vor und ist auch ein wichtiger Bestandteil in Pilzzellwänden. Da sich Chitin in der Biosphäre nicht sichtbar anhäuft, müssen die großen Mengen die jährlich an Chitin produziert werden, auch effizient abgebaut werden. Bakterien und Pilze sind hauptverantwortlich für den Chitinabbau in der Natur, sie können Chitin als Nährstoffquelle nutzen. Der erste Schritt des Chitinabbaus wird von extrazellulären Enzymen ausgeführt, den Chitinasen. Chitinasen spalten das Polymer Chitin in kleinere Oligomere, bis hin zu Dimeren (Chitobiose). Danach wandeln *N*-Acetylglucosaminidasen das Dimer in 2 *N*-Acetylglucosaminmonomere um. Der Transport von GlcNAc in die Zelle und die katabolische Umwandlung von GlcNAc zu Fruktose-6-Phosphat ist bis jetzt nur in der humanpathogenen Hefe *Candida albicans* untersucht worden.

In dieser Doktorarbeit wurde der GlcNAc-Katabolismus in filamentösen Pilzen erforscht. Der erste Teil dieser Ergebnisse ist in einer Publikation veröffentlicht bei der wir zeigen konnten, dass die Gene, die am GlcNAc Abbau beteiligt sind, in einem Cluster vorkommen. Wir entdeckten, dass dieser Cluster zusätzlich einen Transkriptionsfaktor enthält, der starke Homologie zu Ndt80 aus *S. cerevisiae* aufweist. Wir nannten diesen Transkriptionsfaktor, der in *C. albicans* nicht vorkommt, RON1 (regulator of *N*-acetylglucosamine catabolism 1). Wir konnten zeigen, dass RON1 essentiell für den GlcNAc Abbau in *Trichoderma reesei* ist. Eine Literatursuche ergab, dass *Neurospora crassa* einen Transkriptionsfaktor besitzt, der eventuell auch in der Regulierung des GlcNAc-Katabolismus involviert ist. Dieser heißt GRHL (grainy head like protein), sein Ortholog in *T. reesei* ist CSP2. Untersuchungen dieses Transkriptionsfaktors ergaben, dass CSP2 in *T. reesei* nicht im GlcNAc Abbau beteiligt ist. In einer zweiten Studie, die in dieser Arbeit vorgestellt wird, lag der Fokus auf dem GlcNAc-Katabolismus in *N. crassa.* Wachstumstests zeigten, dass im Gegensatz zu anderen Pilzen, z.B. *Trichoderma,* GlcNAc eine schlechte Kohlenstoffquelle für *N. crassa* ist. Es stellte sich heraus, dass GlcNAc sogar in der Gegenwart einer zusätzlichen Kohlenstoffquelle wachstumshemmend ist. Überraschenderweise war *N. crassa* aber in der Lage auf dem Polymer Chitin zu wachsen. Auf Chitin waren die GlcNAc-abbauenden Gene jedoch nicht exprimiert, obwohl GlcNAc starke Induktion der Katabolismusgene bewirkte. Unsere Daten lassen darauf schließen, dass der Zwischenschritt, der zur Produktion von Glucosamine-6-Phopshat führt und von DAM1 durchgeführt wird, einen Engpass im GlcNAc-Stoffwechsel in *N. crassa* darstellen

könnte. Analysen von Knockout-Mutanten der GlcNAc-abbauenden Gene zeigten, dass der Deletionsstamm des GlcNAc-6-phosphat-Deaminase-Gens (*dam1*) den größten Wachstumsdefekt auf Medium mit GlcNAc aufweist. Überexpression des *T. reesei dam1* Gens in dem *N. crassa* Wildtyp und dem Δ*dam-1* Stamm ermöglichte die teilweise Wiederherstellung des Wachstums von *N. crassa* auf Medium mit GlcNAc. Das Wachstum war aber nicht vollständig wieder hergestellt, was darauf hindeutet, dass andere Aspekte oder Kombinationen aus mehreren Schritten verantwortlich für die beobachteten Effekte von GlcNAc in *N. crassa* sind.

Ein weiteres Thema dieser Doktorarbeit waren Chitin-bindende Proteine die zur Familie der Cerato-Platanine (CPPs) gehören. CPPs sind kleine, sezernierte Proteine, die vier konservierte Cysteine besitzen. Diese Proteine kommen nur in filamentösen Pilzen vor und werden leicht von anderen Organismen erkannt, was zu Wechselwirkungen von Pilzen mit anderen Organismen führt, z.B. regen diese Wechselwirkungen die Induktion von Abwehrreaktionen in Pflanzen an. Es ist aber noch nicht bekannt, ob die Hauptfunktion der CPPs diese Wechselwirkungen sind, oder ob sie eher eine Rolle im Pilzwachstum spielen. CPPs haben mehrere biochemische Eigenschaften, die in dieser Kombination nicht in anderen Proteinen zu finden sind. Einerseits sind CPPs Kohlenhydrat-bindende Proteine und sind in der Lage Chitin und GlcNAc-Oligosaccharide zu binden. Andererseits können sie an hydrophoben/hydrophilen Oberflächen akkumulieren und formen Proteinschichten, z.B. auf der Oberfläche von wässrigen Lösungen und verändern dadurch die Lösungen und Oberflächen. *Trichoderma atroviride* und *Trichoderma virens* werden in der Landwirtschaft als biologisches Schädlingsbekämpfungsmittel verwendet. CPPs sind Schlüsselfaktoren für die Interaktion zwischen *Trichoderma* und Pflanzen. *T. atroviride* and *T. virens* sind Mykoparasiten, das heißt sie sind in der Lage andere Pilze anzugreifen und zu töten. Sie können aber auch direkt mit den Pflanzen interagieren, wodurch deren Abwehrreaktionssystem stimuliert wird und die Pflanzen dadurch widerstandsfähiger gegen Krankheitserreger werden. Nur zwei der drei CP-Gene sind stark exprimiert in *Trichoderma* (*sm1*/*epl1* and *sm2*/*epl2*). Knockoutstämme dieser Gene wurden auf ihr Wachstum und ihre Entwicklung untersucht und auch auf ihre *Trichoderm*a-Pflanzen Interaktionen getestet. Das Fehlen der Proteine SM1/ EPL1 und SM2/ EPL2 in *T. virens* und auch *T. atroviride* reduzierte die systemische Resistenz von Maiskeimlingen gegenüber dem pflanzenpathogenen Pilz *Cochliobolus heterostrophus* drastisch. Die Ergebnisse zeigten, dass *T. virens* im Allgemeinen einen effektiveren Pflanzenschutz als *T. atroviride* ermöglicht. Zusätzlich wurde festgestellt, dass obwohl die CP-Gene *sm1*/*epl1* wesentlich stärker als *sm2*/ epl2 während Pilzwachstum induziert wurden, SM2/ EPL2 wichtiger als SM1/ EPL1 zur Förderung der Pflanzenabwehr ist.

Summary

The polysaccharide chitin consists of β-(1-4) linked *N*-acetylglucosamine (GlcNAc) subunits. It is the second most abundant biopolymer after cellulose with a natural turnover of at least 10^9 tons of chitin per year. In the biosphere, it is not only present in fungal cell walls, but also in the exoskeletons of protists and arthropods, e.g. insects and shrimps. Nonetheless, chitin does not visibly accumulate in the biosphere which is indicative for its efficient natural recycling by microbes. Bacteria and fungi are mainly responsible for the degradation of chitin in soil and can utilize chitin as nutrient source. The first step of chitin degradation is performed by extracellular enzymes called chitinases, which decompose the chitin polymer into shorter oligomers down to the level of dimers (chitobiose). Subsequently, *N*-acetylglucosaminidases convert the dimer chitobiose into GlcNAc monomers. Transport of GlcNAc into the cell and catabolic conversion of GlcNAc into fructose-6-phosphate has so far only been studied in the human pathogenic yeast *Candida albicans*, but had not been investigated in filamentous fungi yet.

In this doctoral thesis, GlcNAc-catabolism was studied in filamentous fungi. The first part of these results contributed to a publication in which we could show that in filamentous fungi the genes involved in GlcNAc-catabolism are clustered. Importantly, this cluster contains an Ndt80-like transcription factor, which we called RON1 (regulator of *N*-acetylglucosamine catabolism 1) that is not present in *C. albicans*. We could show that RON1 is essential for GlcNAc-catabolism in the filamentous fungus *Trichoderma reesei*. A survey of the literature showed that the *Neurospora crassa* GRHL (grainy head like protein) transcription factor could also be involved in the regulation of GlcNAc-metabolism. Investigation of CSP2, the ortholog of GRHL in *T. reesei*, indicated that this transcription factor has other functions in the fungus *T. reesei*.

In a second study that is presented in this work, I focused on GlcNAc-catabolism in *N. crassa*. Growth assays showed that, in contrast to other fungi, such as *Trichoderma* spp., GlcNAc is surprisingly a very poor carbon source for *N. crassa*. GlcNAc turned even out to be growth-inhibiting in the presence of other carbon sources. Nonetheless *N. crassa* was surprisingly still able to grow on chitin. In agreement with this puzzling finding, the genes encoding the enzymes for GlcNAc-catabolism were not expressed on chitin, but induced by GlcNAc. Our data suggested that the glucosamine-6-phophate-deaminase gene could be a bottleneck in GlcNAc-catabolism in *N. crassa.* Analysis of knockout mutants of the GlcNAc-catabolism cluster genes showed that the lack of GlcNAc-6-phosphate deaminase gene (*dam1*) completely abolished residual growth of *N. crassa* on GlcNAc containing medium, which can probably be attributed to deleterious effects of GlcNAc-6-phosphate. Overexpression of *T. reesei dam1* in *N. crassa* wild-type and Δ*dam-1* strains

under control of the strong *N. crassa* promoter *ccg1* restored growth on GlcNAc-containing medium only to the (low) *N. crassa* wild-type level, but not beyond that, which suggests that other aspects or combinations of several steps are rate-limiting for the failure of *N. crassa* to metabolize GlcNAc efficiently.

Another topic of this doctoral thesis were chitin-binding proteins belong to the cerato-platanin protein family. Cerato-platanin proteins (CPPs) are small, secreted proteins with four conserved cysteines that are abundantly produced by filamentous fungi with all types of life-styles. These proteins appear to be readily recognized by other organisms and are therefore important factors in interactions of fungi with other organisms, e.g. by stimulating the induction of defense responses in plants. However, it is not known yet whether the main function of CPPs is associated with these fungal interactions or rather plays a role in fungal growth and development. CPPs seem to unify several biochemical properties that are not found in this combination in other proteins. On one hand CPPs are carbohydrate-binding proteins and are able to bind to chitin and *N*-acetylglucosamine oligosaccharides, on the other hand they are able to self-assemble at hydrophobic/hydrophilic interfaces and form protein layers e.g. on the surface of aqueous solutions, thereby altering the polarity of solutions and surfaces. In the fungal species *Trichoderma atroviride* and *Trichoderma virens*, which are used as biocontrol agents in agricultural applications, CPPs have been shown to be key factors for the beneficial *Trichoderma*-plant interaction. *T. atroviride* and *T. virens* are potent mycoparasites, i.e. they are able to attack and kill other fungi, but can also directly interact with plants, thereby stimulating their defense response systems and making them more resistant against pathogens. Since only two (*sm1*/*epl1* and *sm2*/*epl2*) of the three genes encoding CPPs in *T. virens* and *T. atroviride* were found to be strongly expressed, gene knockout strains were analyzed with respect to fungal growth and development as well as in *Trichoderma*-plant interactions. The effect of the lack of SM1/EPL1 and SM2/EPL2 in *T. virens*/*T. atroviride* on inducing systemic resistance in maize seedlings, challenged with the plant pathogen *Cochliobolus heterostrophus*, was tested. The results showed that *T. virens* was in general a more effective plant protectant than *T. atroviride* and further, although interestingly the CP genes *sm1*/*epl1* were substantially stronger induced than *sm2*/*epl2* during fungal growth, SM2/EPL2 turned out to be more important than SM1/EPL1 for the promotion of plant protection.

Introduction

The fungal genus Trichoderma

Trichoderma are filamentous fungi belonging to the phylum ascomycota and the class sordariomycetes. The fungal genus *Trichoderma* (teleomorph *Hypocrea*) was first recognized by Persoon in 1794 (Persoon, 1794). However, it took approximately 200 years from the discovery on that this genus was taxonomically correctly classified. The problems in identification of *Trichoderma* species were mis- and reidentifications of several isolates. In the past nearly all strains of *Trichoderma* were identified as *T. viride* (Bisby, 1939). Due to the fact that the identification of species was mainly based on morphological characteristics, the classification of *Trichoderma* was extremely difficult. Only within the last decades new developments in DNA-based phylogenetic analysis enabled re-evaluation of the genus *Trichoderma*. By 2009, already 75 species were identified in temperate Europe (Jaklitsch, 2009). The first sequenced *Trichoderma* genome was *Trichoderma reesei* QM6a (Martinez et al., 2008) followed by the genomes of *Trichoderma atroviride* IMI206040 and *Trichoderma virens* Gv29-8 (Kubicek et al., 2011). Nowadays there are about 20 fully sequenced *Trichoderma* genome sequences available on the website of the Joint Genome Institute (JGI) of the US Department of Energy (http://genome.jgi.doe.gov/programs/fungi/index.jsf). The sequencing of these species has shed new light on the ecology and the evolution of these species. Members of the genus *Trichoderma* are widely distributed across diverse geographical zones and various habitats (Migheli et al., 2009; Samuels, 2006; Schuster and Schmoll, 2010). They are usually found in root, soil and foliar environments (Gams and Bissett, 1998). They are among the most widely distributed and common fungi in nature, probably due to their diverse metabolic capability and aggressively competitive nature (Kubicek et al., 2011). Species of *Trichoderma/ Hypocrea* can grow saprotrophically on decaying wood, but many *Trichoderma* spp. are also opportunistic, necrotrophic mycoparasites (Druzhinina et al., 2011). Species of the genus *Trichoderma* are also economically important organisms. Some are industrial producers of enzymes such as cellulases and hemicellulases (*T. reesei*) (Seiboth et al., 2012). Many species of the genus are being used for the production of secondary metabolites or used as mycoparasitic biocontrol

agents and bio-fertilizers as they were shown to promote plant growth and induce plant defense responses (*T. atroviride*, *T. harzianum*, *T. virens, T. asperellum*) (Harman et al., 2004; Mukherjee et al., 2013). Furthermore, *Trichoderma* species, similar to other filamentous fungi, act as decomposers of e.g. plant material and therefore are essential for recycling of nutrients in the environment (Carreras-Villasenor et al., 2012).

One human pathogen of the genus *Trichoderma* is *T. longibrachiatum,* which has been detected in sputum and sinus ethmoidalis of healthy humans (Kredics et al., 2003). In contrast, *T. pleuroticola* and *T. pleurotum* are pathogenic on commercial mushroom species like *Pleurotus ostreatus* (Komoń-Zelazowska et al., 2007; Park et al., 2006).

The Trichoderma *species:* T.reesei, T. atroviride *and* T. virens

T. reesei is a filamentous, saprotroph fungus. During World War II the first isolate, *T. reesei* QM6a (Quarter Master 6a), was found on the Solomon Islands in the South Pacific. Later on it received major attention, when it became known that this fungus was responsible for the textile degradation of the cotton canvas of US army tents (Reese, 1976). *T. reesei* is widely used for biotechnological application due to its remarkable cellulolytic potential. It is more amenable to molecular-genetic manipulations than other *Trichoderma* spp., and a range of different selection markers for gene deletion and other molecular biological tools are readily available for *T. reesei* (Bischof and Seiboth, 2014; Guangtao et al., 2009; Seiboth et al., 2011).

T. atroviride is a filamentous, mycoparasitic fungus. Mycoparasitism is the phenomenon where one fungus is parasitic on another fungus. This species occurs in soil, on decaying wood and on plant debris (Kubicek et al., 2008). *T. atroviride* was first described in 1892 by Karsten, but this species has been frequently mistaken in literature with the superficially similar species *T. harzianum,* which also has smooth, globose to subglobose conidia (Karsten, 1892). Bissett and Samuel uncovered the mistake and described the differences between the two species. In contrast to *T. harzianum, T. atroviride* has a characteristic coconut aroma (Bissett, 1991; Samuels et al., 2002). The fully sequenced, annotated genome of *T. atroviride* is available. The genome size is 36.1 Mbp and contains 11865 annotated gene models.

T. virens is a filamentous, mycoparasitic fungus like *T. atroviride*. The genome size is 38.8 Mbp, and the genome database of *T. virens* contains 12428 annotated gene models. Interestingly, the two *Trichoderma* species, *T. virens* and *T. atroviride*, share 1273

orthologues that are not found in the genome of the saprotrophic species *T. reesei*. Based on this finding this gene pool might be associated with the strong mycoparasitic potential of *T. atroviride* and *T. virens*. The genome of *T. reesei* is smaller, 43.1Mbp, than the genomes of the two mycoparasitic fungi (Kubicek et al., 2011; Schmoll et al., 2016).

The model organism **Neurospora crassa**

Neurospora crassa is a filamentous fungus belonging to the phylum ascomycota and is commonly known as orange bread mold. It first received major attention in 1843, when there was a *N. crassa* infestation in French bakeries (Payen, 1843; Perkins, 1991). For the discovery of one-gene one enzyme hypothesis, which was based on first investigations in *Neurospora,* Beadle and Tatum were awarded with Nobel Prize for Biology in 1958. *N. crassa* is an extensively studies model fungus (Davis and Perkins, 2002), because of its various advantages for experimental research, for example, *N. crassa* exhibits rapid growth and large hyphae, both of that making it highly suitable for cytological examinations. Cell biological and biochemical studies revealed molecular details of *N. crassa* establishment and maintenance of cell polarity, cell recognition and cell fusion. *N. crassa* is haploid throughout the majority of its life cycle, mating is facilitated and it is easy to cross them (Zelter et al., 2004).

Furthermore, *N. crassa* possesses a wide variety of epigenetic phenomena, most importantly repeat induced point mutation (RIP), an effective defense against duplicated sequences such as those arising from the multiplication of transposons (Cambareri et al., 1989; Cambareri et al., 1991; Selker et al., 2002). Numerous research programs focused on *N. crassa* including formal, population, and molecular genetics, biochemistry, physiology, and molecular cell biology and more recent studies of development, photobiology, circadian rhythms, gene silencing, ecology, and evolution. Substantial contribution to genetic and molecular information achieved David Perkins, who has sampled natural isolates from all over the world (Turner et al., 2001). In 2003 *N. crassa* became the first filamentous fungus to have its genome sequenced and annotated. The total genome size is 43 Mbp and in the *N. crassa* genome database 9826 open reading frames (ORFs) have been annotated (Borkovich et al., 2004; Galagan et al., 2003).

The degradation of chitin and its monomer N-*acetylglucosamine*

Chitin is a polysaccharide composed of β-(1-4) linked residues of *N*-acetylglucosamine (GlcNAc; 2-acetamido-2-deoxy-D-glucopyranose) units. It is one of the world`s most abundant biopolymers, but in contrast to cellulose its potential is rather underrated, so far. Compared to cellulose, which is composed of glucose monomers, chitin features an additional acetyl-amino-group on the hexose sugar ring of its GlcNAc subunits that renders it a promising raw material for functional biopolymers in biomedical applications as well as biochemical synthesis of specialty and fine chemicals (Muzzarelli, 1999; Tharanathan and Kittur, 2003). Chitin can be found in the exoskeleton of insects and shrimps, but also in the cell walls of fungi. In filamentous fungi chitin is located in the inner layers of the cell wall, close to the plasma membrane (Ruiz-Herrera, 1991) and forms together with β-(1,3 1,6) glucan the structural scaffold of the fungal cell wall (Dumitriu and Dekker, 1998; Latgé, 2007). Chitin does not visibly accumulate in the biosphere, which shows that the huge amounts of annually produced chitin, at least 10 gigatons, also are degraded each year (Muzzarelli, 1999). The main degraders of chitin are bacteria and fungi, which are able to use chitin as nutrient source. Fungi with specialized life styles, such as mycoparasites and entomopathogenic fungi, have evolved a diversified machinery to utilize chitin derived from other fungi and arthropods, respectively, and are interesting targets for studying and developing novel methods to exploit chitin from these sources. Therefore, fungal chitin degradation and chitin synthesis have been subject to extensive investigation in the past years to gain insights in these intricately regulated processes, which render fungi capable of harnessing the recalcitrant chitin polymer as nutritional source and building block for growth. For the design of new products and their sustainable production via biotechnological processes through enzymatic action, the production and performance of chitinolytic pathways still need to be improved.

Chitin can be degraded by two main enzymes, namely chitinases (EC.3.2.1.14) belonging to the glycoside hydrolase (GH) 18 family and members of the GH 20 family, called *N*-acetylglucosaminidases (NAGases) (Davies and Henrissat, 1995; Henrissat and Bairoch, 1996). Fungal chitinases can be divided into three different subgroups, A, B and C based on the amino acid sequences of their GH 18 modules. Filamentous fungi have usually between 10 and 20 different chitinases and more than 30 chitinases can be found in fungi with

specialized life styles such as insect-pathogenic fungi or mycoparasites (fungi that parasitize other fungi) (Karlsson and Stenlid, 2009; Seidl, 2008; Seidl et al., 2009). However, fungal chitinases are not only important for external chitin degradation for nutritional purposes, but are also involved in internal chitin degradation during cell wall remodelling and recycling. Subgroup C chitinases contain several carbohydrate binding domains that can solely be found in this type of enzymes in fungi and possibly makes these enzymes particularly effective for the degradation of insoluble polysaccharides containing chitin (Gruber and Seidl-Seiboth, 2012; Hartl et al., 2012). Chitinases decompose the chitin polymer into shorter oligomers with a minimal chain length of two sugar subunits. Although fungi have umpteen chitinases, for the next step of degradation they possess only one or two enzymes, (the NAGases) which convert the chitin dimer chitobiose into two GlcNAc monomers (Seidl, 2008).

GlcNAc plays an important role in a broad range of mechanisms throughout all kingdoms of life (Chen et al., 2010). GlcNAc function can act as potent inducer of the yeast-to-filament transition in dimorphic yeasts such as *C. albicans*, *Histoplasma capsulatum* and *Blastomyces dermatitidis* (Gilmore et al., 2013; Simonetti et al., 1974). In bacteria, extracellular GlcNAc triggers the production of CURLI (= curled pili) fibers that stimulate biofilm formation (Barnhart and Chapman, 2006). GlcNAc occurs also in heterogeneous polysaccharides, such as peptidoglycan/murein found in bacterial cell walls. A β- (1, 6) linked poly-*N*-acetylglucosamine is the major component of bacterial biofilms (Itoh et al., 2008; Roux et al., 2015). In humans, GlcNAc can be found in hyaluronic acid, which is an essential element of the connective tissue such as cartilage, as well as epithelial and neuronal tissue (Ashry and Aly, 2007). In some cases, GlcNAc can be found in free form, e.g. in human milk (Kobata et al., 1969; Miller et al., 1994).

In *C. albicans* the genes encoding the enzymes involved in GlcNAc-catabolism are clustered (Konopka, 2012). The GlcNAc-catabolism cluster contains genes encoding a sugar kinase that phosphorylates GlcNAc to create GlcNAc-6-phosphate, a deacetylase that splits it into acetate and glucosamine-6-phosphate, and a deaminase that subsequently converts glucosamine-6-phosphate to ammonium and fructose-6-phophate, which can enter glycolysis. A GlcNAc transporter can be found outside the cluster (Kumar et al., 2000; Naseem et al., 2011). The GlcNAc-catabolism has not been studied in filamentous fungi so far.

Cerato-platanin proteins – a family of chitin-binding proteins

Proteins belonging to the cerato-platanin protein (CPP) family are only found in filamentous fungi. The name giving protein for this family was cerato-platanin (CP) from the plant pathogenic fungus *Ceratocystis platani*, which infects plane trees. CP was first described in 1999 (Pazzagli et al., 1999). Since then, CPPs have been reported from many different filamentous fungi, and it has been recognized that genes encoding CPPs can be found in the genomes of fungi with all kinds of lifestyles, including biotrophic and necrotrophic plant pathogens, human pathogens, mycoparasites, plant-beneficial fungi and saprotrophs (Chen et al., 2013; Frischmann et al., 2013). CPPs are small proteins (12 kDa) that are abundantly secreted into the culture filtrate, but remain also partially bound in the fungal cell wall (Boddi et al., 2004; González-Fernández et al., 2014; Seidl et al., 2006). Over the last decade, several studies revealed that CPPs are important players in interactions of fungi with other organisms. Many of the so far reported CPPs are from plant pathogenic fungi, and these proteins are able to act as virulence factors in fungal-plant interactions (Frías et al., 2011; Jeong et al., 2007; Scala et al., 2004). However, in fungi that positively interact with plants, e.g. *Trichoderma* spp., which are used as biocontrol fungi in agricultural applications, they act in a positive way as elicitors of plant defence responses (Djonovic et al., 2006). Further, a member of the CPP family from the human pathogen *Coccidioides immitis* has been described as an antigen (Pan and Cole, 1995). Thus, CPPs are readily perceived by other organisms and signal them the presence of a fungus.

CPPs seem to unify several biochemical properties that are not found in this combination in other proteins. On one hand, cerato-platanins are carbohydrate-binding proteins and are able to bind to chitin and *N*-acetylglucosamine oligosaccharides (Baccelli et al., 2014; de Oliveira et al., 2011); on the other hand, they are able to self-assemble at hydrophobic/ hydrophilic interfaces and form protein layers, e.g. on the surface of aqueous solutions, thereby altering the polarity of solutions and surfaces (Frischmann et al., 2013). It had been suggested, in previous studies, that CP might have similar functions as hydrophobins, which are amphiphilic fungal proteins that self-assemble at hydrophobic-hydrophilic interfaces and invert the polarity of surfaces (Boddi et al., 2004; Seidl et al., 2006). However, this hypothesis has turned out to be incorrect because of the recent findings in our research group that CPPs increase the polarity effects of solutions and

surfaces and this is the opposite effect of what is observed for hydrophobins, due to this effect CPPs are not hydrophobin-like proteins (Frischmann et al., 2013).

CPPs are strongly conserved throughout the fungal kingdom. Fungal genomes analysis showed that filamentous fungi have typically two or three gene encoding CPPs (Frischmann et al., 2013). The structure of CP from *C. platani*, revealed that the fold of these proteins is similar to Barwin-like endoglucanases and plant expansins. Indeed, Bacelli *et al* (Baccelli et al., 2014) reported that CPPs exhibited expansin-like properties on cellulosic materials. Expansins are small, extracellular proteins predominantly found in plants that are associated with carbohydrate-binding and loosening of the cellulose scaffolds in plant cell walls (Sampedro and Cosgrove, 2005). However, CPPs are not cellulose-binding proteins, but instead have a binding site for GlcNAc oligomers and indeed exhibit $(GlcNAc)_X$ and chitin-binding properties. Recent studies focussed on various aspects related to the surface-activity altering properties (Frischmann et al., 2013) as well as the carbohydrate-binding properties (de Oliveira et al., 2011). In addition, it was reported for CP that it can exhibit loosen cellulose fibres, but no loosening of chitin was detected (Baccelli et al., 2014). The first CPPs in *Trichoderma* were published in 2006 and in *T. atroviride* they were called eliciting plant-response-like proteins (EPLs) (Seidl et al., 2006), whereas their orthologues in *T. virens* were termed small proteins (SMs) (Djonovic et al., 2006).

Fungal genome analysis showed that *T. atrovirdie* and *T. virens* have each three genes encoding CPPs, namely, *epl1*, e*pl2*, *epl3* in *T. atroviride* and s*m1*, *sm2*, s*m3*, in *T. virens* respectively (Frischmann et al., 2013). EPL1 and SM1 have been shown to be elicitors of plant defense-responses, but this ability is far weaker for EPL1 than for SM1 (Vargas et al., 2008). The reason for that is that EPL1 is readily able to form dimers, but only the monomeric form is able to efficiently induce plant defense responses. SM1 has a single glycosylation site that is not present in EPL1 and is predominantly found in its monomeric form and therefore induces plant defense responses more potently (Vargas et al., 2008).

References

Ashry, E.S.H.E. and Aly, M.R.E., 2007. Synthesis and Biological Relevance of *N*-Acetylglucosamine-containing Oligosaccharides. Pure Appl. Chem., 12: 2229-2242.

Baccelli, I., Luti, S., Bernardi, R., Scala, A. and Pazzagli, L., 2014. Cerato-platanin shows expansin-like activity on cellulosic materials. Appl Microbiol Biotechnol, 98(1): 175-84.

Barnhart, M.M. and Chapman, M.R., 2006. Curli biogenesis and function. Annu Rev Microbiol, 60: 131-47.

Bisby, G.R., 1939. *Trichoderma viride* Pers ex ries, and notes *on Hypocrea*. Trans. Br. Mycol. Soc., 23: 149-168.

Bischof, R. and Seiboth, B., 2014. Molecular tools for strain improvement of *Trichoderma* spp. In: V.K. Gupta et al. (Editors), Biotechnology and Biology of *Trichoderma*. Elsevier, Oxford, UK, pp. 179-192.

Bissett, J., 1991. A revision of the genus *Trichoderma*. II. Infrageneric classification. Canadian Journal of Botany, 69(11): 2357-2372.

Boddi, S., Comparini, C., Calamassi, R., Pazzagli, L., Cappugi, G. and Scala, A., 2004. Cerato-platanin protein is located in the cell walls of ascospores, conidia and hyphae of *Ceratocystis fimbriata* f. sp. *platani*. FEMS Microbiol Lett, 233(2): 341-346.

Borkovich, K.A., Alex, L.A., Yarden, O., Freitag, M., Turner, G.E., Read, N.D. et al., 2004. Lessons from the genome sequence of *Neurospora crassa*: tracing the path from genomic blueprint to multicellular organism. Microbiol Mol Biol Rev, 68(1): 1-108.

Cambareri, E.B., Jensen, B.C., Schabtach, E. and Selker, E.U., 1989. Repeat-induced G-C to A-T mutations in *Neurospora*. Science, 244(4912): 1571-5.

Cambareri, E.B., Singer, M.J. and Selker, E.U., 1991. Recurrence of repeat-induced point mutation (RIP) in *Neurospora crassa*. Genetics, 127(4): 699-710.

Carreras-Villasenor, N., Sanchez-Arreguin, J.A. and Herrera-Estrella, A.H., 2012. *Trichoderma*: sensing the environment for survival and dispersal. Microbiology, 158(Pt 1): 3-16.

Chen, H., Kovalchuk, A., Keriö, S. and Asiegbu, F.O., 2013. Distribution and bioinformatic analysis of the cerato-platanin protein family in *Dikarya*. Mycologia, 105(6): 1479-88.

Chen, J.K., Shen, C.R. and Liu, C.L., 2010. *N*-acetylglucosamine: production and applications. Mar Drugs, 8(9): 2493-2516.

Davies, G. and Henrissat, B., 1995. Structures and mechanisms of glycosyl hydrolases. Structure, 3(9): 853-9.

Davis, R.H. and Perkins, D.D., 2002. Timeline: *Neurospora*: a model of model microbes. Nat Rev Genet, 3(5): 397-403.

de Oliveira, A.L., Gallo, M., Pazzagli, L., Benedetti, C.E., Cappugi, G., Scala, A. et al., 2011. The structure of the elicitor cerato-platanin (CP), the first member of the CP fungal protein family, reveals a double ψβ-barrel fold and carbohydrate binding. J Biol Chem, 286(20): 17560-17568.

Djonovic, S., Pozo, M.J., Dangott, L.J., Howell, C.R. and Kenerley, C.M., 2006. Sm1, a proteinaceous elicitor secreted by the biocontrol fungus *Trichoderma virens* induces plant defense responses and systemic resistance. Mol Plant Microbe Interact, 19(8): 838-853.

Druzhinina, I.S., Seidl-Seiboth, V., Herrera-Estrella, A., Horwitz, B.A., Kenerley, C.M., Monte, E. et al., 2011. *Trichoderma*: the genomics of opportunistic success. Nat Rev Microbiol, 9(10): 749-759.

Dumitriu, S. and Dekker, M., 1998. Polysaccharides: Structural Diversity and Functional Versatility. 2nd ed., New York: CRC Press.

Frías, M., González, C. and Brito, N., 2011. BcSpl1, a cerato-platanin family protein, contributes to *Botrytis cinerea* virulence and elicits the hypersensitive response in the host. New Phytol, 192(2): 483-95.

Frischmann, A., Neudl, S., Gaderer, R., Bonazza, K., Zach, S., Gruber, S. et al., 2013. Self-assembly at air/water interfaces and carbohydrate binding properties of the small secreted protein EPL1 from the fungus *Trichoderma atroviride*. J Biol Chem, 288(6): 4278-4287.

Galagan, J.E., Calvo, S.E., Borkovich, K.A., Selker, E.U., Read, N.D., Jaffe, D. et al., 2003. The genome sequence of the filamentous fungus *Neurospora crassa*. Nature, 422(6934): 859-868.

Gams, W. and Bissett, J., 1998. Morphology and Identification of *Trichoderma*. In: C.P. Kubicek and G.E. Harman (Editors), *Trichoderma* and *Gliocladium*: Basic biology, taxonomy and genetics. Taylor & Francis, London.

Gilmore, S.A., Naseem, S., Konopka, J.B. and Sil, A., 2013. *N*-acetylglucosamine (GlcNAc) triggers a rapid, temperature-responsive morphogenetic program in thermally dimorphic fungi. PLoS Genet, 9(9): e1003799.

González-Fernández, R., Aloria, K., Valero-Galván, J., Redondo, I., Arizmendi, J.M. and Jorrín-Novo, J.V., 2014. Proteomic analysis of mycelium and secretome of different *Botrytis cinerea* wild-type strains. J Proteomics, 97(0): 195-221.

Gruber, S. and Seidl-Seiboth, V., 2012. Self versus non-self: fungal cell wall degradation in *Trichoderma*. Microbiology, 158(Pt 1): 26-34.

Guangtao, Z., Hartl, L., Schuster, A., Polak, S., Schmoll, M., Wang, T. et al., 2009. Gene targeting in a nonhomologous end joining deficient *Hypocrea jecorina*. J Biotechnol, 139(2): 146-151.

Harman, G.E., Howell, C.R., Viterbo, A., Chet, I. and Lorito, M., 2004. *Trichoderma* species - opportunistic, avirulent plant symbionts. Nat Rev Microbiol, 2(1): 43-56.

Hartl, L., Zach, S. and Seidl-Seiboth, V., 2012. Fungal chitinases: diversity, mechanistic properties and biotechnological potential. Appl Microbiol Biotechnol, 93(2): 533-43.

Henrissat, B. and Bairoch, A., 1996. Updating the sequence-based classification of glycosyl hydrolases. Biochem J, 316 (Pt 2): 695-696.

Itoh, Y., Rice, J.D., Goller, C., Pannuri, A., Taylor, J., Meisner, J. et al., 2008. Roles of pgaABCD genes in synthesis, modification, and export of the *Escherichia coli* biofilm adhesin poly-beta-1,6-*N*-acetyl-D-glucosamine. J Bacteriol, 190(10): 3670-3680.

Jaklitsch, W., 2009. European species of *Hypocrea* Part I. The green-spored species. Stud Mycol, 63: 1-91.

Jeong, J.S., Mitchell, T.K. and Dean, R.A., 2007. The *Magnaporthe grisea* snodprot1 homolog, MSP1, is required for virulence. FEMS Microbiol Lett, 273(2): 157-165.

Karlsson, M. and Stenlid, J., 2009. Evolution of family 18 glycoside hydrolases: diversity, domain structures and phylogenetic relationships. Journal of molecular microbiology and biotechnology, 16(3-4): 208-223.

Karsten, P.A., 1892. Finlands mögelsvamper (*Hyphomycetes fennici*), Helsinki, 192 pp.

Kobata, A., Ginsburg, V. and Tsuda, M., 1969. Oligosaccharides of human milk. I. Isolation and characterization. Arch Biochem Biophys, 130(1): 509-513.

Komoń-Zelazowska, M., Bissett, J., Zafari, D., Hatvani, L., Manczinger, L., Woo, S. et al., 2007. Genetically closely related but phenotypically divergent *Trichoderma* species cause green mold disease in oyster mushroom farms worldwide. Appl Environ Microbiol, 73(22): 7415-7426.

Konopka, J.B., 2012. *N*-acetylglucosamine (GlcNAc) functions in cell signaling. Scientifica (Cairo).

Kredics, L., Antal, Z., Doczi, I., Manczinger, L., Kevei, F. and Nagy, E., 2003. Clinical importance of the genus *Trichoderma*. A review. Acta Microbiol Immunol Hung, 50(2-3): 105-117.

Kubicek, C., Herrera-Estrella, A., Seidl-Seiboth, V., Martinez, D., Druzhinina, I., Thon, M. et al., 2011. Comparative genome sequence analysis underscores mycoparasitism as the ancestral life style of *Trichoderma*. Genome Biol, 12(4): r40.

Kubicek, C.P., Komon-Zelazowska, M. and Druzhinina, I.S., 2008. Fungal genus *Hypocrea/Trichoderma*: from barcodes to biodiversity. J Zhejiang Univ Sci B, 9(10): 753-763.

Kumar, M.J., Jamaluddin, M.S., Natarajan, K., Kaur, D. and Datta, A., 2000. The inducible *N*-acetylglucosamine catabolic pathway gene cluster in *Candida albicans*: discrete *N*-acetylglucosamine-inducible factors interact at the promoter of *NAG1*. Proc Natl Acad Sci U S A, 97(26): 14218-23.

Latgé, J.P., 2007. The cell wall: a carbohydrate armour for the fungal cell. Mol Microbiol, 66(2): 279-290.

Martinez, D., Berka, R.M., Henrissat, B., Saloheimo, M., Arvas, M., Baker, S.E. et al., 2008. Genome sequencing and analysis of the biomass-degrading fungus *Trichoderma reesei* (syn. *Hypocrea jecorina*). Nat Biotechnol, 26(5): 553-560.

Migheli, Q., Balmas, V., Komon-Zelazowska, M., Scherm, B., Fiori, S., Kopchinskiy, A. et al., 2009. Soils of a Mediterranean hot spot of biodiversity and endemism (Sardinia, Tyrrhenian Islands) are inhabited by pan-European, invasive species of *Hypocrea/Trichoderma*. Environ Microbiol, 11: 35 - 46.

Miller, J.B., Bull, S., Miller, J. and McVeagh, P., 1994. The oligosaccharide composition of human milk: temporal and individual variations in monosaccharide components. J Pediatr Gastroenterol Nutr, 19(4): 371-6.

Mukherjee, P.K., Horwitz, B.A., Herrera-Estrella, A., Schmoll, M. and Kenerley, C.M., 2013. *Trichoderma* research in the genome era. Annu Rev Phytopathol, 51: 105-129.

Muzzarelli, R.A., 1999. Native, industrial and fossil chitins. EXS, 87: 1-6.

Naseem, S., Gunasekera, A., Araya, E. and Konopka, J.B., 2011. *N*-acetylglucosamine (GlcNAc) induction of hyphal morphogenesis and transcriptional responses in *Candida albicans* are not dependent on its metabolism. J Biol Chem, 286(33): 28671-28680.

Pan, S. and Cole, G.T., 1995. Molecular and biochemical characterization of a *Coccidioides immitis*-specific antigen. Infect Immun, 63(10): 3994-4002.

Park, M.S., Bae, K.S. and Yu, S.H., 2006. Two new species of *Trichoderma* associated with green mold of oyster mushroom cultivation in Korea. Mycobiology, 34(3): 111-3.

Payen, A., 1843. Extrain d'un rapport addresse' a' M. Le Marechal Duc de Dalmatie, Ministre de la Guerre, President du Conseil, sur une alteration extraordinaire du pain du munition. Ann. Chim. Phys., 3: 5-21.

Pazzagli, L., Cappugi, G., Manao, G., Camici, G., Santini, A. and Scala, A., 1999. Purification, characterization, and amino acid sequence of cerato-platanin, a new phytotoxic protein from *Ceratocystis fimbriata* f. sp. *platani*. J Biol Chem, 274(35): 24959-24964.

Perkins, D.D., 1991. The first published scientific study of *Neurospora*, including a description of photoinduction of carotenoids. Fungal Genet. Newsl., 38: 64-65.

Persoon, C., 1794. Disposito methodica fungorum in classes, ordines, familias adn generas. Neues Magazin für die Botanik. Ziegler und Söhne, Zürich.

Reese, E.T., 1976. History of the cellulase program at the U.S. army Natick Development Center. Biotechnol Bioeng Symp(6): 9-20.

Roux, D., Cywes-Bentley, C., Zhang, Y.F., Pons, S., Konkol, M., Kearns, D.B. et al., 2015. Identification of Poly-*N*-acetylglucosamine as a Major Polysaccharide Component of the *Bacillus subtilis* Biofilm Matrix. J Biol Chem, 290(31): 19261-72.

Ruiz-Herrera, J., 1991. Fungal cell wall: structure, synthesis and assembly. CRC Press, Boca Raton.

Sampedro, J. and Cosgrove, D.J., 2005. The expansin superfamily. Genome Biol, 6(12): 242.

Samuels, G.J., 2006. *Trichoderma*: systematics, the sexual state, and ecology. Phytopath, 96(2): 195-206.

Samuels, G.J., Dodd, S.L., Gams, W., Castlebury, L.A. and Petrini, O., 2002. *Trichoderma* species associated with the green mold epidemic of commercially grown *Agaricus bisporus*. Mycologia, 94(1): 146-70.

Scala, A., Pazzagli, L., Comparini, C., Santini, A., Tegli, S. and Cappugi, G., 2004. Cerato-platanin, an early-produced protein by *Ceratocystis fimbriata* f.sp. *platani*, elicits phytoalexin synthesis in host and non-host plants. J Plant Pathol, 86(1): 27-33.

Schmoll, M., Dattenböck, C., Carreras-Villasenor, N., Mendoza-Mendoza, A., Tisch, D., Aleman, M. et al., 2016. The Genomes of Three Uneven Siblings: Footprints of the Lifestyles of Three *Trichoderma* Species. Microbiol Mol Biol Rev, in press.

Schuster, A. and Schmoll, M., 2010. Biology and biotechnology of *Trichoderma*. Appl Microbiol Biotechnol, 87(3): 787-99.

Seiboth, B., Herold, S. and Kubicek, C.P., 2012. Metabolic engineering of inducer formation for cellulase and hemicellulase gene expression in Trichoderma reesei. Subcell Biochem, 64: 367-90.

Seiboth, B., Ivanova, C. and Seidl-Seiboth, V., 2011. *Trichoderma reesei:* A fungal enzyme producer for cellulosic biofuels. In: dos Santos Bernardes MA, Eds. Biofuel production-Recent developments and prospects. In Tech, pp. 309-340.

Seidl, V., 2008. Chitinases of filamentous fungi: a large group of diverse proteins with multiple physiological functions. Fungal Biol Rev, 22: 36-42.

Seidl, V., Druzhinina, I.S. and Kubicek, C.P., 2006. A screening system for carbon sources enhancing β-*N*-acetylglucosaminidase formation in *Hypocrea atroviridis* (*Trichoderma atroviride*). Microbiology, 152(Pt 7): 2003-2012.

Seidl, V., Song, L., Lindquist, E., Gruber, S., Koptchinskiy, A., Zeilinger, S. et al., 2009. Transcriptomic response of the mycoparasitic fungus *Trichoderma atroviride* to the presence of a fungal prey. BMC Genomics, 10: 567.

Selker, E.U., Freitag, M., Kothe, G.O., Margolin, B.S., Rountree, M.R., Allis, C.D. et al., 2002. Induction and maintenance of nonsymmetrical DNA methylation in *Neurospora*. Proc Natl Acad Sci USA, 99 Suppl 4: 16485-16490.

Simonetti, N., Strippoli, V. and Cassone, A., 1974. Yeast-mycelial conversion induced by *N*-acetyl-D-glucosamine in *Candida albicans*. Nature, 250(464): 344-6.

Tharanathan, R.N. and Kittur, F.S., 2003. Chitin-the undisputed biomolecule of great potential. Crit Rev Food Sci Nutr, 43(1): 61-87.

Turner, B.C., Perkins, D.D. and Fairfield, A., 2001. *Neurospora* from natural populations: a global study. Fungal Genet Biol, 32(2): 67-92.

Vargas, W.A., Djonovic, S., Sukno, S.A. and Kenerley, C.M., 2008. Dimerization controls the activity of fungal elicitors that trigger systemic resistance in plants. J Biol Chem, 283(28): 19804-19815.

Zelter, A., Bencina, M., Bowman, B.J., Yarden, O. and Read, N.D., 2004. A comparative genomic analysis of the calcium signaling machinery in *Neurospora crassa*, *Magnaporthe grisea*, and *Saccharomyces cerevisiae*. Fungal Genet Biol, 41(9): 827-841.

Aims of this thesis

In this doctoral research study we wanted to provide new insights into the *N*-acetylglucosamine catabolism and one chitin-binding protein family, called cerato-platanins.

Chitin is after cellulose the second most abundant polymer on earth. Chitin and its monomer *N*-acetylglucosamine can be used for numerous biotechnological applications. Therefore, it is of major importance to understand the enzymatic degradation of this polymer and to find new environmentally friendly ways of chitin degradation. Although chitin is present in fungi, the industrial production from these sources is still in fledgling stages due to higher costs and still remarkably low knowledge of the interplay and regulation of chitin degrading and processing enzymes. Based on data from genome sequencing programs, we are just beginning to understand the large variety of chitin-degrading enzymes and chitin-binding proteins that can be found in fungi.

The aims of this work were to fill in several of the missing pieces of the jigsaw that will aid in understanding enzymatic degradation and processing of chitin in fungi. One focus of this study was the *N*-acetylglucosamine catabolism in filamentous fungi. The functional analyses were performed with *Neurospora crassa* and *Trichoderma reesei* in order to investigate gene expression and gene deletion strains of those genes that we identified as essential for *N*-acetylglucosamine catabolism in fungi during this thesis.

The second focus topic of this study was the cerato-platanin protein family, a group of proteins that exhibit the unique combination of chitin-binding as well as surface-activity altering properties. The biological functions, as well as biochemical properties of cerato-platanin proteins are only poorly understood so far. In this thesis various aspects were investigated including the role of the cerato-platanins from *Trichoderma atroviride* and *Trichoderma virens* with respect to the induction of plant defense responses and investigations of their self-assembly and surface activity-altering features of cerato-platanin proteins.

Scientific goals and most important results

1. *GlcNAc catabolism in filamentous fungi (covering the research papers shown in chapter 1)*

Chapter 1.1

Chitin and *N*-acetylglucosamine metabolism in fungi - a complex machinery harnessed for the design of chitin-based high value products. Romana Gaderer, Verena Seidl-Seiboth and Lisa Kappel. (2015). Review. Curr Biotech. Under review.

Chitin is one of the world's most abundant biopolymers, but in contrast to cellulose its potential has been fairly underrated, so far. Compared to cellulose chitin features an additional amino-group on the hexose sugar ring that renders it a promising substrate for functional biopolymers in biomedical applications as well as biochemical synthesis of specialty and fine chemicals (Muzzarelli, 1999; Tharanathan and Kittur, 2003). Fungi with specialized life styles, such as mycoparasites and entomopathogenic fungi, have evolved an effective machinery to utilize chitin derived from other fungi and arthropods, respectively (Seidl, 2008). Therefore, fungal chitin degradation and chitin synthesis have been subject to extensive investigation in the past years to gain insights in these intricately regulated processes, which render fungi capable of harnessing the recalcitrant chitin polymer as nutritional source and building block for growth (Cosgrove, 2010; Lenardon et al., 2010; Muzzarelli et al., 2012). For the development of methods to exploit chitin from these origins, the design of new products and their sustainable production through enzymatic action, identifying the regulators of the chitin anabolic and catabolic pathways will be essential.

Chapter 1.2

The *N*-acetylglucosamine catabolic gene cluster in *Trichoderma reesei* is controlled by the Ndt80-like transcription factor RON1. Lisa Kappel, Romana Gaderer, Michel Flipphi & Verena Seidl-Seiboth (2015). *Mol Microbiol*. doi: 10.1111/mmi.13256

Although filamentous fungi possess a multitude of different chitinases, catabolism of GlcNAc had previously not been studied in filamentous fungi but only in the dimorphic yeast *Candida albicans*. We therefore used the results available for *C. albicans* as starting point to perform a detailed analysis of GlcNAc-catabolism in filamentous fungi. Our experimental approaches included the genomic organisation of the GlcNAc catabolic gene cluster across the phylum Ascomycota, followed by more detailed analyses of the transcriptional regulation of the respective genes as well as their functional characterization in gene knockout strains in the two species *T. reesei* and *N. crassa*.

The enzymatic steps of GlcNAc-catabolism are shown in Fig.1. GlcNAc is phosphorylated by GlcNAc hexokinase (*Trichoderma*: HXK3; *C. albicans:* Nag5/Hxk1), then GlcNAc-6-phosphate is deacetylated by GlcNAc-6-phosphate deacetylase (*Trichoderma*: DAC1, *C. albicans*: Nag2/Dac1), and subsequently, GlcN-6-phosphate deaminase converts GlcN-6-phosphate into fructose-6-phosphate (*Trichoderma*: DAM1; *C. albicans:* Nag1). Acetate and ammonium are the other end products of the GlcNAc-catabolism.

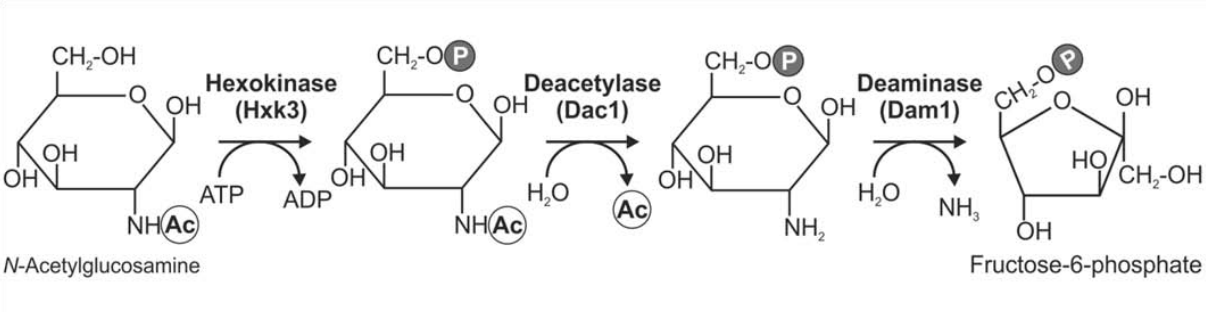

Fig.1: Schematic representation of the GlcNAc catabolism pathway.

With respect to genome organization of the GlcNAc catabolic genes in filamentous fungi, we found that the genes encoding the three enzymes responsible for stepwise conversion of GlcNAc to fructose-6-phosphate are clustered in filamentous Ascomycota. In most cases the gene encoding a putative GlcNAc transporter is located outside the cluster.

In contrast to C*. albicans*, in filamentous fungi a gene encoding a GH family 3 protein can often be found in the GlcNAc gene cluster, which we have annotated as *nag3*. NAG3 exhibits similarity to bacterial GH3 β-*N*-acetylhexosaminidases ((Cheng and Li, 2000; Li et al., 2002; Litzinger et al., 2010; Mayer et al., 2006; Tsujibo et al., 1994) and http://www.cazy.org/GH3.html)). In addition, our analysis showed that the GlcNAc cluster in

filamentous fungi in many cases also contains a gene for a transcription factor with an Ndt80-like DNA-binding domain (PFAM family PF05224). The transcription factor was designated RON1 (**r**egulator **o**f ***N***-acetylglucosamine catabolism 1) (Chu and Herskowitz, 1998; Fingerman et al., 2004; Lamoureux and Glover, 2006; Xu et al., 1995). We could show that RON1 is the key activator of the GlcNAc catabolic genes and therefore essential for this degradation pathway. The key findings from this paper are summarized in the graphical abstract of this publication (Kappel et al., 2015) that is shown in Fig.2. For more detailed information about enzymatic chitin degradation and GlcNAc metabolism in fungi see our review in chapter 1.1.

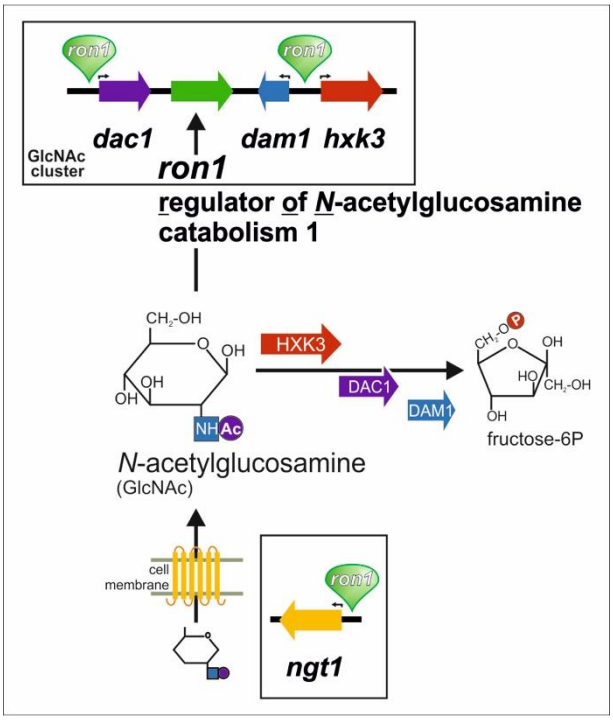

Fig.2: Graphical abstract modified from our paper (Kappel et al., 2015). Overview of the GlcNAc-catabolism pathway in filamentous fungi. Transport of GlcNAc is mediated by Ngt1. Conversion of GlcNAc by GlcNAc-hexokinase (*hxk3*) into GlcNAc-6-phosphate, which is deacetylated by GlcNAc-6-phopsphate deacetylase (*dac1*) and subsequently GlcN-6-phosphate deaminase (*dam1*) converts GlcN-6-phosphate into fructose-6-phosphate. *ron1* act as transcriptional regulator of this pathway.

In addition to our findings on RON1, we performed a survey of the literature for transcription factors that might also be involved in the regulation of GlcNAc metabolism and

found that in *N. crassa*, the transcriptional regulator termed GRHL (grainy head like protein) could be involved in the regulation of GlcNAc metabolism in aerial hyphae (Pare et al., 2012). GRHL proteins belong to the CP2 superfamily of transcription factors (PFAM family PF04516), and in the *T. reesei* genome database, only one predicted member can be found (NCBI accession number: XP_006965082), which we named CSP2. We found that *T. reesei csp2* is expressed under various growth conditions, but we were not able to detect any morphological defects in our Δ*csp2* strains. Further, no phenotype was evident for growth on GlcNAc and chitin. CSP2 is not involved in the regulation of GlcNAc genes during vegetative growth or under carbon starvation conditions (Fig.3) (chapter 1.2).

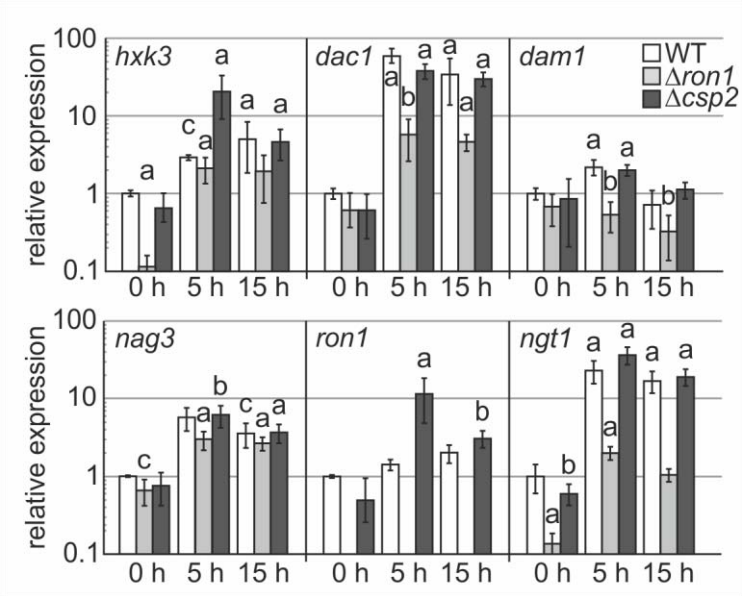

Fig.3: qRT-PCR of the parental strain (WT), and two Δ*ron1* and Δ*csp2* strains. Mycelium was pre-cultivated for 16 h in MA medium containing 1% glucose, and biomass samples were taken at 0, 5 and 15 h after a shift to MA medium lacking added carbon sources. All measured values were normalised to *tef1* expression and compared with the time point [WT, 0 h], which was set at 1. Bars indicate the SEM (standard error of the mean). 'a', 'b' and 'c' indicate significance at $P < 0.001$, 0.01 and 0.05 respectively.

Chapter 1.3

***N*-acetylglucosamine, the monomeric building block of chitin, inhibits growth of *Neurospora crassa*.** Romana Gaderer, Verena Seidl-Seiboth and Lisa Kappel. (2016). Manuscript in preparation for submission.

The GlcNAc catabolic gene cluster of *N. crassa* is very similar to that of *T. reesei* and other Ascomycota. It contains the three catabolic genes, a *nag3* gene, and Ndt80-like transcription factor (*ron1*) and again the gene encoding the putative GlcNAc transporter is located elsewhere in the genome. In addition to RON1, *N. crassa* has two other Ndt80-like transcription factors, VIB1 and FSD1, whose biological functions seem to be unrelated to GlcNAc-catabolism. FSD1 plays an important role in timing and development of female reproductive structures and ascospore maturation (Hutchison and Glass, 2010) and VIB1 controls the production of proteases upon nutrient starvation, is involved in the regulation of vegetative incompatibility and programmed cell death, as well as plant cell wall degradation by repressing glucose signalling and carbon catabolite repression (CCR) (Dementhon et al., 2006; Hutchison and Glass, 2010; Xiong et al., 2014).

Since *Trichoderma* species are readily able to utilize GlcNAc as carbon source and the GlcNAc-catabolic cluster is strongly conserved between *Trichoderma* spp. and *N. crassa,* we anticipated that *N. crassa* would grow equally well on GlcNAc as sole carbon source. However, when we compared the growth of *N. crassa* 74A on VM medium containing different carbon sources (glycerol, D-glucose, cellulose, D-fructose, chitin and GlcNAc), the results (Fig.4) (chapter 1.3) showed surprisingly that *N. crassa* was unable to grow on GlcNAc.

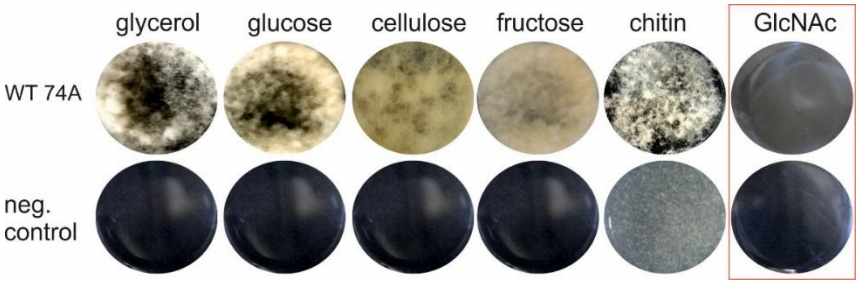

Fig.4: Growth behavior of *N. crassa* wild-type 74A on VM with different carbon sources, such as glycerol, glucose, cellulose, fructose, chitin and GlcNAc.

Somewhat in contrast to that finding, *N. crassa* was able to grow on medium containing purified chitin powder from crab shells and colloidal chitin, a better accessible, acid-pre-treated, purified form of chitin. In order to verify that the observed growth defect was not

specific for *N. crassa* strain 74A, we tested three additional wild-type (WT) strains (Chilton (FSGC1691), Lindegren25a (FSGC353), Fast growth (FSGC5729)). All tested *N. crassa* WT strains were unable to grow on GlcNAc and it turned out to be even growth-inhibiting in the presence of a non-repressive carbon source such as glycerol.

We therefore investigated growth of all GlcNAc-catabolism gene knockout strains on GlcNAc in order to test whether a defect in the GlcNAc-catabolism pathway at certain steps would have an effect on growth on GlcNAc alone or in the presence of other carbon sources. However, the results were similar as those that we found for the WT and our findings again showed that growth was also inhibited in the presence of glycerol.

In order to get a first insight into gene expression levels of the GlcNAc-catabolic cluster genes i.e. *hxk-3*, *dac-1*, *dam-1*, *ron-1*, *nag-3*, as well as the transporter-encoding gene *ngt-1*, we analysed their expression in *N. crassa* WT strain 74A on medium containing chitin or GlcNAc as carbon source (Fig.5) (chapter 1.3). The results showed that the genes of the GlcNAc-catabolism cluster are interestingly inducible by GlcNAc although *N. crassa* is not able to grow on this carbon source. When chitin serves as carbon source they do not appear to be strongly induced, which is actually in agreement with the rather puzzling finding that *N. crassa* can grow rather well on chitin and that it does not seem to be growth inhibiting.

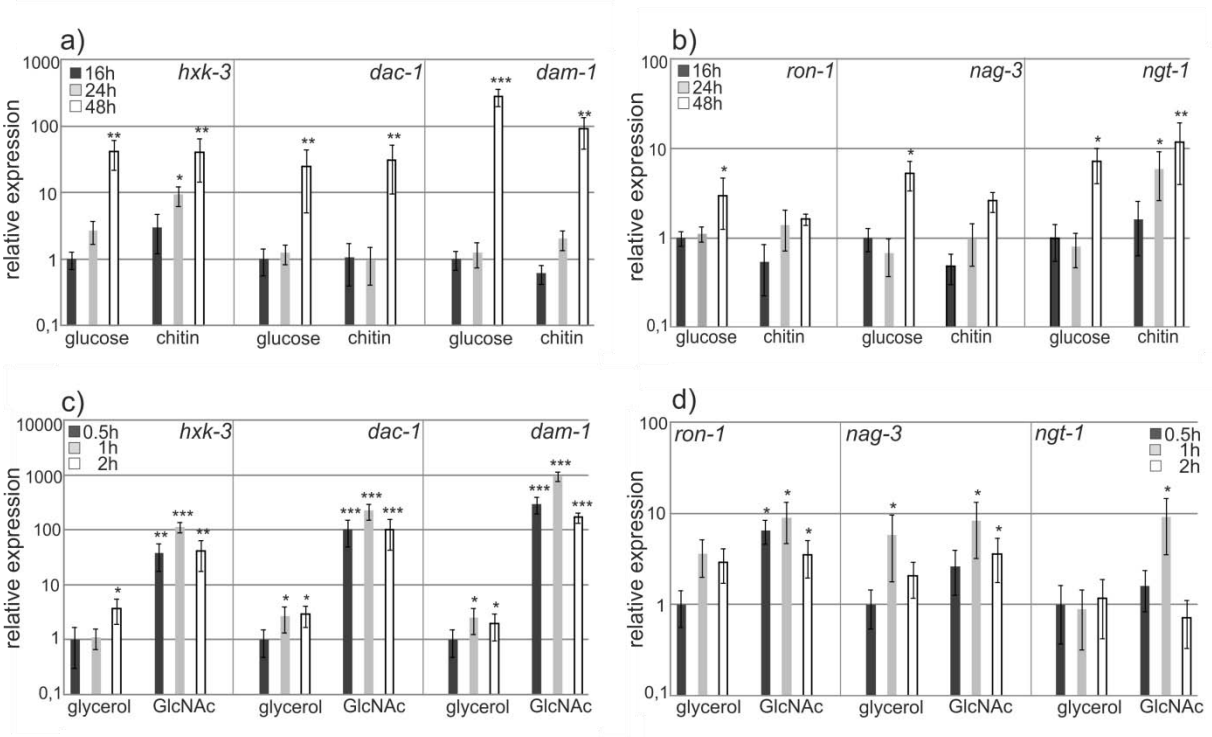

Fig.5: Gene expression (RT-qPCR) of GlcNAc-catabolism cluster genes in the WT 74A strain. **a-b)** Samples were taken after 16 h, 24 h and 48 h from liquid standing cultivations with glucose or chitin as carbon source. All samples were normalized to the 16 h glucose sample. **c-d)** Samples were taken after 0.5 h, 1 h and 2h after replacing from 1% glycerol into 1% glycerol (control) or 1% GlcNAc. All samples were normalized to the 0.5 h glycerol sample. *act1* was used as reference gene. The standard deviation of the mean expression values from at least two independent biological replicates is shown. Bars indicate the SEM (*, ** and *** indicate significance at $P < 0.05$, 0.01 and 0.001).

Since we observed the strongest growth defect on GlcNAc in Δ*dam1* and the findings in recent studies corroborate the importance of Dam1 in GlcNAc-catabolism, we hypothesised that the glucosamine-6-phophate-deaminase gene is a bottleneck in the GlcNAc utilisation process of *N. crassa. T. reesei* deaminase gene *dam1,* which has already been shown to be important and functional in *T. reesei* (Kappel et al., 2015), was heterologously overexpressed in *N. crassa* WT and Δ*dam-1* background to see if high expression by another functional deaminase gene can alleviate the observed severe growth defects. Investigation of the overexpression strains showed that growth of the WT::*ccg1*:*Trdam1* and Δ*dam-1*::*ccg1*:*Trdam1* strains was partially restored on medium containing both, glycerol and GlcNAc. This suggests that overexpression of *T. reesei dam1* indeed seems to slightly restore *N. crassa* growth on GlcNAc in the presence of glycerol. On medium containing GlcNAc as sole carbon source the inhibitory effect of GlcNAc was still present (Fig.6). Thus, overexpression of *T. reesei dam1* in *N. crassa* restored growth on GlcNAc to a certain extent, but did not completely recover the growth defect of *N. crassa* on this carbon source (chapter 1.3).

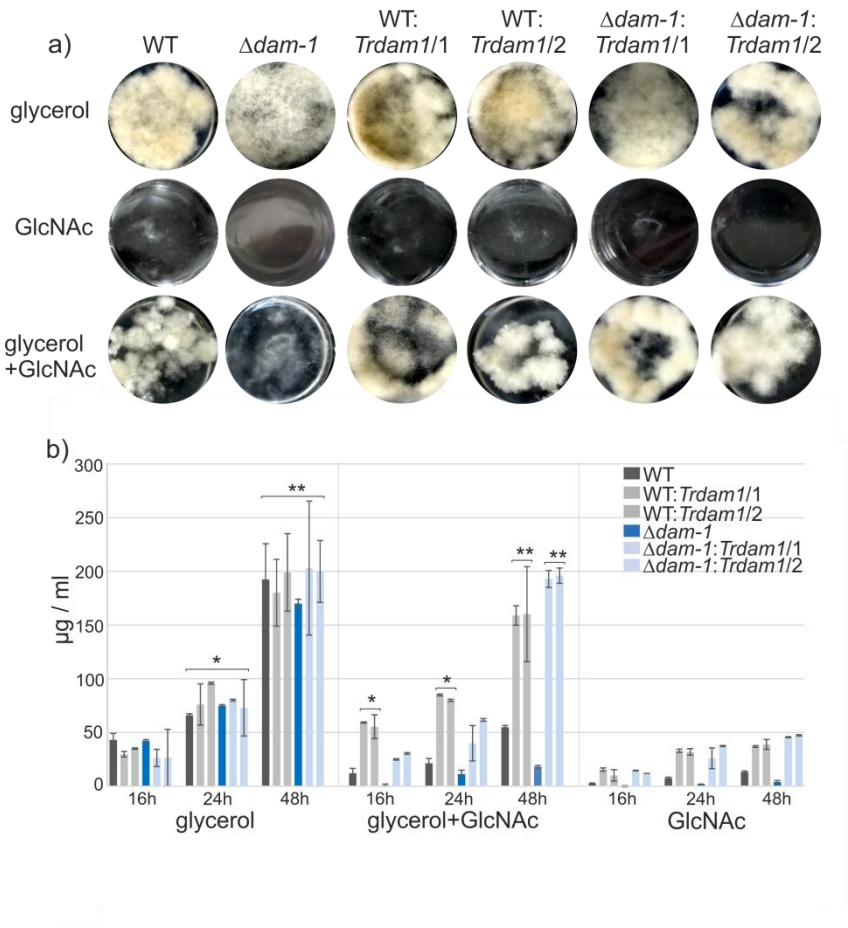

Fig.6: Growth tests and biomass formation of *N. crassa* WT 74A and overexpression strains (WT::*ccg1*:*Trdam1* and Δ*dam-1*::*ccg1*:*Trdam1*). a) WT and overexpression strains were grown for 12 days on VM containing 1% glycerol, 1% GlcNAc and 1% glycerol + 1% GlcNAc as carbon source. Negative control was VM with the corresponding carbon source without spores. **b)** For biomass measurements, strains were grown in liquid standing VM medium, containing 1% glycerol or 1% gylcerol+1% GlcNAc or 1% GlcNAc and total protein concentration was determined after 16 h, 24 h and 48 h after inoculation.

2. *Functional and biochemical studies of members of the cerato-platanin protein family (covering the research papers shown in chapter 2)*

Chapter 2.1

Cerato-platanins: a fungal protein family with intriguing properties and application potential. Gaderer, R., Bonazza, K., & Seidl-Seiboth, V. (2014). *Appl Microbiol Biotechnol, 98*(11), 4795-4803. doi: 10.1007/s00253-014-5690-y

Cerato-platanin proteins are small, secreted proteins with four conserved cysteines that are abundantly produced by filamentous fungi with all types of lifestyles (Chen et al., 2013; Frischmann et al., 2013; Pazzagli et al., 1999). These proteins appear to be readily recognized by other organisms and are therefore important factors in interactions of fungi with other organisms, e.g. by stimulating the induction of defence responses in plants (Djonovic et al., 2006). However, it is not known yet whether the main function of cerato-platanin proteins is associated with these fungal interactions or rather a role in fungal growth and development. Cerato-platanin proteins seem to unify several biochemical properties that are not found in this combination in other proteins. On one hand, cerato-platanins are carbohydrate-binding proteins and are able to bind to chitin and *N*-acetylglucosamine oligosaccharides (Baccelli et al., 2014; de Oliveira et al., 2011); on the other hand, they are able to self-assemble at hydrophobic/ hydrophilic interfaces and form protein layers, e.g. on the surface of aqueous solutions, thereby altering the polarity of solutions and surfaces (Frischmann et al., 2013).

Chapter 2.2

Sm2, a paralog of the *Trichoderma* cerato-platanin elicitor Sm1, is also highly important for plant protection conferred by the fungal-root interaction of *Trichoderma* with maize. Gaderer, R., Lamdan, N. L., Frischmann, A., Sulyok, M., Krska, R., Horwitz, B. A., & Seidl-Seiboth, V. (2015). *BMC microbiol, 15*, doi:10.1186/s12866-014-0333-0

The proteins Sm1 and Sm2 from the biocontrol fungus *Trichoderma virens* belong to the cerato-platanin protein family. Sm1 was previously shown to be important for the induction of plant defense responses by *T. virens* in plants for the protection of plants against pathogenic fungi (Djonovic et al., 2006). While this has in the meantime already been shown for orthologs of Sm1 in other fungi, not much information about other members of this protein family is available. In order to test whether in *T. virens* Sm2 is also involved in the interaction of *Trichoderma* with plants we analyzed whether the lack of *sm2* leads to an altered potential to protect plants against fungal pathogens. In the maize-*Cochliobolus heterostrophus* pathosystem used in our study, knock-out of *sm1* reduced the plant

protection potential of *T. virens*, but interestingly the lack of *sm2* had an even greater effect. As can be seen in Fig.7 (chapter 2.2) and lesion size of maize leaves in *sm2* knockout strains was statistically not different from the control, i.e. infected plants without *Trichoderma*.

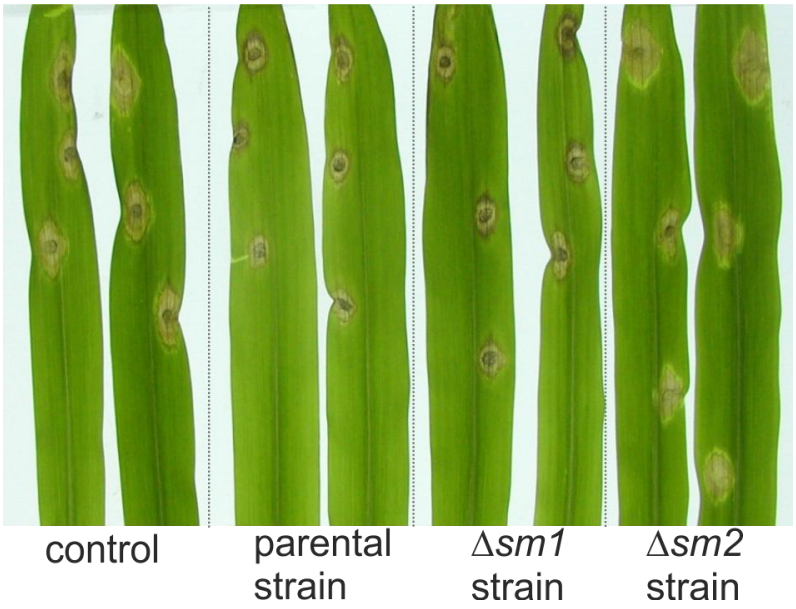

Fig.7: Effect of *T. virens* (parental strain, Δ*sm1* and Δ*sm2*) on plant protection in maize seedlings challenged with the, maize pathogen *Cochliobolus heterostrophus*. Lesion development in leaves of *T. virens*-induced maize, two days after pathogen challenge.

Thus, our results showed that, although the cerato-platanin gene *sm1* is more abundantly expressed than *sm2* during fungal growth, Sm2 is, interestingly, more important than Sm1 for the promotion of plant protection conferred by *T. virens* in the maize-*C. heterostrophus* pathosystem (Chapter 2.2). The same assay was performed with *T. atroviride epl1* and *epl2* knockout strains. The results showed that *T. atroviride* was overall less effective in plant protection than *T. virens*, but the same trend was observed for the respective *epl2* and *epl1* knockout strains. These findings advance our understanding of the diversified functions of CPPs in fungi and of the pool of molecules that are involved in the beneficial interaction of *Trichoderma* with plants.

Chapter 2.3

The fungal cerato-platanin protein EPL1 forms highly ordered layers at hydrophobic/hydrophilic interfaces. Bonazza, K., Gaderer, R., Neudl, S., & Friedbacher, G. (2015). *Soft Matter, 11*(9), 1723-1732. doi: 10.1039/c4sm02389g

T. atroviride protein EPL1 has additional intriguing features. Detailed biochemical analysis of EPL1 revealed that CPPs are not hydrophobine-like proteins, because EPL1 enhanced the polar/apolar properties of solutions and surfaces, instead of inverting them, as hydrophobins do. EPL1 readily self-assembles at air/water interfaces and forms highly ordered monolayers at a hydrophobic surface/liquid-interface, furthermore we could recently show that a combination of hydrophobins and EPL1 results a previously unknown hybrid layer. This mixed layer combined features of both proteins, hydrophobins and EPL1. The enhancing of the hydrophobicity of HOPG (highly oriented pyrolytic graphite) is typical for EPL1, and the stability and water insolubility of the layer are properties of hydrophobins (Fig.8) (chapter 2.3).

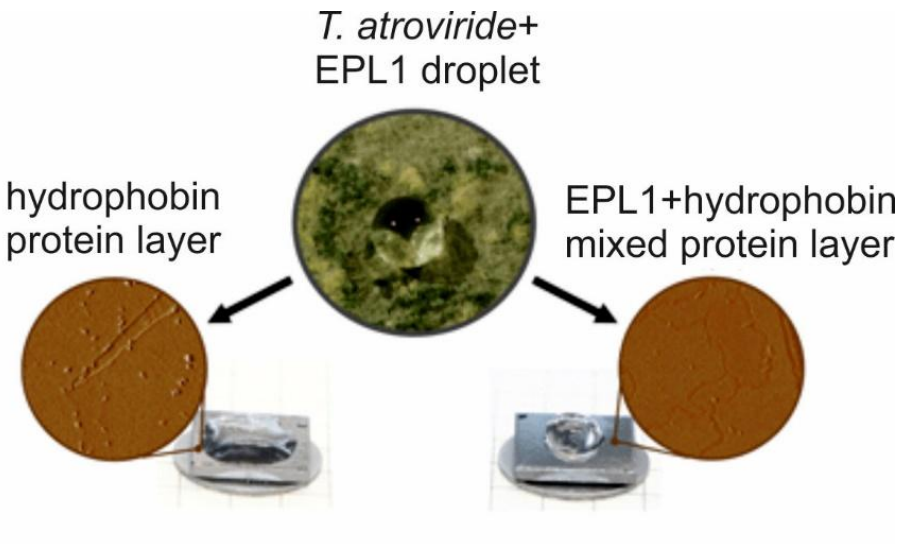

Fig.8: Graphical abstract from our paper (Bonazza et al., 2015). Overview of the properties of hydrophobines (left) compared to the combination of hydrophobine and EPL1 (right). AFM amplitude error images produced by the "drop method" imaged under PBS buffer. Photographs of 50µl water drops deposited on hydrophobine layer (left) and hydrophobine/EPL1 hybrid layer (right).

References

Baccelli, I., Luti, S., Bernardi, R., Scala, A. and Pazzagli, L., 2014. Cerato-platanin shows expansin-like activity on cellulosic materials. Appl Microbiol Biotechnol, 98(1): 175-84.

Bonazza, K., Gaderer, R., Neudl, S. and Friedbacher, G., 2015. The fungal cerato-platanin protein EPL1 forms highly ordered layers at hydrophobic/hydrophilic interfaces. Soft Matter, 11(9): 1723-1732.

Chen, H., Kovalchuk, A., Keriö, S. and Asiegbu, F.O., 2013. Distribution and bioinformatic analysis of the cerato-platanin protein family in *Dikarya*. Mycologia, 105(6): 1479-88.

Cheng, C.Y. and Li, Y.K., 2000. An *Aspergillus* chitosanase with potential for large-scale preparation of chitosan oligosaccharides. Biotechnol Appl Biochem, 32 (Pt 3): 197-203.

Chu, S. and Herskowitz, I., 1998. Gametogenesis in yeast is regulated by a transcriptional cascade dependent on Ndt80. Mol Cell, 1(5): 685-696.

Cosgrove, J., 2010. Chitin & Chitosan: A Global Strategic Business Report.

de Oliveira, A.L., Gallo, M., Pazzagli, L., Benedetti, C.E., Cappugi, G., Scala, A. et al., 2011. The structure of the elicitor cerato-platanin (CP), the first member of the CP fungal protein family, reveals a double ψβ-barrel fold and carbohydrate binding. J Biol Chem, 286(20): 17560-17568.

Dementhon, K., Iyer, G. and Glass, N.L., 2006. VIB-1 is required for expression of genes necessary for programmed cell death in *Neurospora crassa*. Eukaryot Cell, 5(12): 2161-2173.

Djonovic, S., Pozo, M.J., Dangott, L.J., Howell, C.R. and Kenerley, C.M., 2006. Sm1, a proteinaceous elicitor secreted by the biocontrol fungus *Trichoderma virens* induces plant defense responses and systemic resistance. Mol Plant Microbe Interact, 19(8): 838-853.

Fingerman, I.M., Sutphen, K., Montano, S.P., Georgiadis, M.M. and Vershon, A.K., 2004. Characterization of critical interactions between Ndt80 and MSE DNA defining a novel family of Ig-fold transcription factors. Nucleic Acids Res, 32(9): 2947-2956.

Frischmann, A., Neudl, S., Gaderer, R., Bonazza, K., Zach, S., Gruber, S. et al., 2013. Self-assembly at air/water interfaces and carbohydrate binding properties of the small secreted protein EPL1 from the fungus *Trichoderma atroviride*. J Biol Chem, 288(6): 4278-4287.

Hutchison, E.A. and Glass, N.L., 2010. Meiotic regulators Ndt80 and ime2 have different roles in *Saccharomyces* and *Neurospora*. Genetics, 185(4): 1271-1282.

Kappel, L., Gaderer, R., Flipphi, M. and Seidl-Seiboth, V., 2015. The *N*-acetylglucosamine catabolic gene cluster in *Trichoderma reesei* is controlled by the Ndt80-like transcription factor RON1. Mol Microbiol.

Lamoureux, J.S. and Glover, J.N., 2006. Principles of protein-DNA recognition revealed in the structural analysis of Ndt80-MSE DNA complexes. Structure, 14(3): 555-565.

Lenardon, M.D., Munro, C.A. and Gow, N.A., 2010. Chitin synthesis and fungal pathogenesis. Curr Opin Microbiol, 13(4): 416-23.

Li, H., Morimoto, K., Katagiri, N., Kimura, T., Sakka, K., Lun, S. et al., 2002. A novel beta-*N*-acetylglucosaminidase of *Clostridium paraputrificum* M-21 with high activity on chitobiose. Appl Microbiol Biotechnol, 60(4): 420-7.

Litzinger, S., Fischer, S., Polzer, P., Diederichs, K., Welte, W. and Mayer, C., 2010. Structural and kinetic analysis of *Bacillus subtilis N*-acetylglucosaminidase reveals a unique Asp-His dyad mechanism. J Biol Chem, 285(46): 35675-35684.

Mayer, C., Vocadlo, D.J., Mah, M., Rupitz, K., Stoll, D., Warren, R.A. et al., 2006. Characterization of a beta-*N*-acetylhexosaminidase and a beta-*N*-acetylglucosaminidase/beta-glucosidase from *Cellulomonas fimi*. FEBS J, 273(13): 2929-41.

Muzzarelli, R.A., 1999. Native, industrial and fossil chitins. EXS, 87: 1-6.

Muzzarelli, R.A.A., Boudrant, J., Meyer, D., Manno, N., DeMarchis, M. and Paoletti, M.G., 2012. Current views on fungal chitin/chitosan, human chitinases, food preservation, glucans, pectins and inulin: A tribute to Henri Braconnot, precursor of the carbohydrate polymers science, on the chitin bicentennial. Carbohyd Polym, 87(2): 995-1012.

Pare, A., Kim, M., Juarez, M.T., Brody, S. and McGinnis, W., 2012. The functions of grainy head-like proteins in animals and fungi and the evolution of apical extracellular barriers. PLoS One, 7(5): e36254.

Pazzagli, L., Cappugi, G., Manao, G., Camici, G., Santini, A. and Scala, A., 1999. Purification, characterization, and amino acid sequence of cerato-platanin, a new phytotoxic protein from *Ceratocystis fimbriata* f. sp. *platani*. J Biol Chem, 274(35): 24959-24964.

Seidl, V., 2008. Chitinases of filamentous fungi: a large group of diverse proteins with multiple physiological functions. Fungal Biol Rev, 22: 36-42.

Tharanathan, R.N. and Kittur, F.S., 2003. Chitin-the undisputed biomolecule of great potential. Crit Rev Food Sci Nutr, 43(1): 61-87.

Tsujibo, H., Fujimoto, K., Tanno, H., Miyamoto, K., Imada, C., Okami, Y. et al., 1994. Gene sequence, purification and characterization of *N*-acetyl-beta-glucosaminidase from a marine bacterium, *Alteromonas* sp. strain O-7. Gene, 146(1): 111-5.

Xiong, Y., Sun, J. and Glass, N.L., 2014. VIB1, a link between glucose signaling and carbon catabolite repression, is essential for plant cell wall degradation by *Neurospora crassa*. PLoS Genet, 10(8): e1004500.

Xu, L., Ajimura, M., Padmore, R., Klein, C. and Kleckner, N., 1995. *NDT80*, a meiosis-specific gene required for exit from pachytene in *Saccharomyces cerevisiae*. Mol Cell Biol, 15(12): 6572-6581.

Contribution to the papers

Chapter 1:

- **Chapter 1.1:** Chitin and *N*-acetylglucosamine metabolism in fungi - a complex machinery harnessed for the design of chitin-based high value products.

  Romana Gaderer, Verena Seidl-Seiboth and Lisa Kappel. (2015). Curr Biotech. Under review.

  RG co-drafted and revised the manuscript.

- **Chapter 1.2:** The *N*-acetylglucosamine catabolic gene cluster in *Trichoderma reesei* is controlled by the Ndt80-like transcription factor RON1.

  Lisa Kappel, Romana Gaderer, Michel Flipphi & Verena Seidl-Seiboth (2015). *Mol Microbiol*. doi: 10.1111/mmi.13256.

  RG performed the experiments of the CSP2 transcription factor, co-drafted and revised the manuscript.

- **Chapter 1.3**: *N*-acetylglucosamine, the monomeric building block of chitin, inhibits growth of *Neurospora crassa*.

  Romana Gaderer, Verena Seidl-Seiboth and Lisa Kappel. (2016). Manuscript in preparation for submission.

  RG performed the experiments, prepared the figures, interpreted the results and drafted the manuscript.

Chapter 2:

- **Chapter 2.1:** Cerato-platanins: a fungal protein family with intriguing properties and application potential.

  Gaderer, R., Bonazza, K., & Seidl-Seiboth, V. (2014). *Appl Microbiol Biotechnol, 98*(11), 4795-4803. doi: 10.1007/s00253-014-5690-y.

  RG co-drafted and revised the manuscript.

- **Chapter 2.2:** Sm2, a paralog of the *Trichoderma* cerato-platanin elicitor Sm1, is also highly important for plant protection conferred by the fungal-root interaction of *Trichoderma* with maize.

  Gaderer, R., Lamdan, N. L., Frischmann, A., Sulyok, M., Krska, R., Horwitz, B. A., & Seidl-Seiboth, V. (2015). *BMC microbiol, 15*, doi:10.1186/s12866-014-0333-0

  RG did the phenotypic analysis and performed the gene expression experiments and prepared the corresponding figures. RG co-drafted the manuscript and interpreted the results.

- **Chapter 2.3:** The fungal cerato-platanin protein EPL1 forms highly ordered layers at hydrophobic/hydrophilic interfaces.

  Bonazza, K., Gaderer, R., Neudl, S., & Friedbacher, G. (2015). *Soft Matter, 11*(9), 1723-1732. doi: 10.1039/c4sm02389g

  RG produced and purified the protein, co-drafted and revised the manuscript.

Chapter 1: GlcNAc-catabolism in filamentous fungi

Chapter 1.1

Chitin and *N*-acetylglucosamine metabolism in fungi - a complex machinery harnessed for the design of chitin-based high value products.

Romana Gaderer, Verena Seidl-Seiboth and Lisa Kappel. (2015). Curr Biotech. Under review.

Chitin and *N*-acetylglucosamine metabolism in fungi - a complex machinery harnessed for the design of chitin-based high value products

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Abstract

Chitin is one of the world's most abundant biopolymers, but in contrast to cellulose its potential is rather underrated, so far. Compared to cellulose chitin features an additional amino-group on the hexose sugar ring that renders it a promising substrate for functional biopolymers in biomedical applications as well as biochemical synthesis of specialty and fine chemicals. Fungi with specialized life styles, such as mycoparasites and entomopathogenic fungi, have evolved an effective machinery to utilize chitin derived from other fungi and arthropods, respectively, and are an interesting target to study and develop methods to exploit chitin from these origins. Therefore, fungal chitin degradation and chitin synthesis have been subject to extensive investigation in the past years to gain insights in these intricately regulated processes, which render fungi capable of harnessing the recalcitrant chitin polymer as nutritional source and building block for growth. For the design of new products and their sustainable production through enzymatic action, identifying the regulators of the chitin anabolic and catabolic pathways will be essential. In this review our current knowledge on chitin metabolism in fungi is presented, new sources for chitin production and new products derived from enzymatic processing will be introduced.

Keywords: chitin, chitosan, *N*-acetylglucosamine, filamentous fungi, regulation, transcription factor, bio-based chemicals, biopolymer

1. Chitin – a biopolymer of unexhausted potential

Chitin is a linear amino-polysaccharide that is present in high amounts in arthropods, crustaceans and cell walls of fungi and is composed of β - (1, 4) linked *N*-acetylglucosamine (GlcNAc; 2-acetamido-2-deoxy-D-glucopyranose) subunits. The GlcNAc chains adopt a 2-fold helical conformation, similar to that observed in other homopolymers such as cellulose [1]. These helical chains of chitin can be arranged in antiparallel (α-chitin) or parallel (β-chitin) order to form fibers of a high tensile strength and crystallinity. α-chitin, the prevalent form, is more rigid and strong due to extensive hydrogen bonding [2], whereas β-chitin has a higher affinity to solvents due to its weaker hydrogen-bonding and has so far only been identified in squid pens and other specialized parts of mollusks and arthropods [3-5]. A third form, γ-chitin, is assembled in stacks of two parallel and one anti-parallel chains, and seems to occur very rarely e.g. in the cocoon fibers of *Ptinus* beetle and the stomach of *Loligo* squid [3, 6]. Chitosan is a partially deacetylated derivative of chitin, that occurs in high amounts only in some specialized fungi (see also section 2 and [7]) or can be generated via chemical or enzymatic deacetylation. The partial deacetylation leads to free amino groups with positive charges at slightly acidic pH values, rendering it soluble in dilute aqueous acidic solutions and the only known polycationic natural polysaccharide. Further deacetylation increases solubility and antibiotic effectivity [8] and references therein.

Chitin is the second most abundant polysaccharide after cellulose [9, 10] and probably the most abundant biopolymer in the aquatic environment [11, 12]. Six to eight million tons of chitinous waste are produced annually only from the fishing industries, of which 1.5 million tons are generated in south Asia alone [13]. This corresponds to roughly 75% of total weight of shellfish, such as shrimp, crab and krill shells, currently disposed of in landfills were uncontrolled decomposition by microbes, leads to environmental and human health concerns and thus increases costs for waste processing. This shellfish `waste´ contains around 20-58% chitin [14], but only little more than 100, 000 tons of chitin are estimated to be obtained from these marine by-products annually by 2018 [13, 15]. Considering the still underexploited use of

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chitin, it is interesting to note that the global market for chitin and its derivatives was estimated to reach up to US$ 63 billion by end of 2015, with Japan being the biggest player in chitin processing worldwide. Moreover, chitin derivatives, such as chitosan, *N*-acetylglucosamine and glucosamine (GlcN; the monomers of chitin and chitosan, respectively) were considered to have the greatest potential on the global market by 2010 [16].

Conventional production of chitin and chitosans from shellfish waste is carried out in a chemical process with a deproteination and a demineralization step by harsh treatment with NaOH (around 50%) at high temperatures (up to 160 °C) followed by an acid treatment step with organic acids. This creates large amounts of hazardous waste byproducts and for chitosan production a further alkali treatment results in ill-defined deacetylation grades. Poor reproducibility is another drawback of this process for chitin-based products. Further research on chitin properties, structure-function relationships and evolving strategies to analyze chitin and chitosan polymerization and deacetylation grades lead to fairly reproducible and also marketable chitin derivatives in the last years (for a review on current procedures for preparation of chitin and chitosan from marine sources see also [5]). Although chitin and chitosan are also present in fungi (basidiomycetes and ascomycetes), the industrial production from these sources is still in fledgling stages due to higher costs and still remarkably low knowledge of the interplay and regulation of chitin degrading and processing enzymes. Major efforts are currently underway to increase the knowledge in chitin and its enzymatic decay and to develop new methods for the production of well-defined chitin (-oligomers) and chitosans, to be able to harness their qualities for the generation of high value products.

Fungi have proven to be feasible tools for a variety of biotechnological applications in the past decades. An economically important field that evolved is secondary metabolite production, such as citric acid derived from *Aspergillus niger*, antibiotics from *Penicillium* spp. or carotenes from yeasts [17]. Moreover, recombinant enzymes/proteins for the food (food additives), medical (e.g. antibodies) and textile (e.g. cellulases) industry are produced with fungi. More recently filamentous fungi have proven to be potent producers for enzymes involved in biomass degradation (advanced biofuels from cellulosic waste material; [18, 19]) and production/modification of new biorefinery products. In the following sections the fascinating possibilities upcoming with the new knowledge on enzymatic chitin processing and generation in fungi are matched with ideas and strategies to produce chitin from alternative sources and to design new products. We first give an overview on the current knowledge on chitin degradation, including the last steps for chitin catabolism, as well as a brief overview of chitin synthesis in fungi and then illustrate how these insights can be exploited to design biochemical approaches for chitin hydrolysis and new high end products of great biotechnological potential.

2. Chitin and chitosan in fungi

Chitin is a crystalline, extremely strong biopolymer, with a tensile strength that exceeds that of many artificial materials which results from extensive hydrogen bonding along the chitin chains [20]. These appealing properties on the one hand have furthered the biopolymer research on chitin and made it attractive for a variety of applications, but on the other hand harvesting chitin and byproducts thereof is impeded by its recalcitrant nature.

Chitin is an important constituent of fungi that confers strength and rigidity to the fungal cell wall. Chitosan is a partially deacetylated (DA-grade: less than 50%, [21]) derivative of chitin, that occurs in high amounts predominantly in the order of *Mucorales*, and is produced by chitin-deacetylases in the fungal cell wall [7, 22]. Chitosan has also been found in some fungi at certain growth stages, such as in e.g. Zygomycota [23, 24], *Cryptococcus neoformans* [25], and even low amounts in *Saccharomyces cerevisiae* [26]. However, due to the presence of genes encoding for chitin-deacetylases in the genomes of a wide range of filamentous fungi (www.cazy.org), chitosan presumably also occurs in most fungi at certain growth-stages. The fungal cell wall consists of an alkali-insoluble fraction, which contains chitin and β -(1, 3) glucan, and an alkali-soluble fraction composed of α-glucans, galactomannans and other carbohydrate polymers. Chitin and chitosan are therefore not easily accessible from the outer side of the cell wall, since they are masked by layers of other carbohydrates and proteins. The harsh chemical treatments used to extract the sugars from the fungal cell wall for compositional analysis, also lead to partial de-acetylation of GlcNAc. Therefore, the detected amounts of GlcNAc and GlcN that are reported in the literature cannot always be reliably related to the occurrence of chitin or chitosan in the fungal cell wall. So far evidence for targeted deacetylation of chitin within fungal cell walls is missing, although it has been speculated that chitin deacetylation may increase the elasticity of the polymer and protect it from hostile chitinases [27, 28]. In fungi, the amount of chitin and/or chitosan differs considerably among various classes of fungi. Whereas yeasts contain

only 0.5-5% chitin mainly in septa, constriction rings and budding scars, filamentous fungi harbor up to 20% chitin in the inner layers of the cell wall, located close to the plasma membrane [29, 30], which interestingly is also reflected in the number of chitinases (see section 3.1).
These findings show that especially mycoparasitic filamentous fungi were required to evolve a very diverse network of enzymes and auxiliary proteins to attack and nourish on other fungal cell walls and at the same time need to be able to distinguish between self- and non-self and remodel chitin during certain growth stages.

3. Enzymatic decomposition of chitin and chitosan in fungi

3.1 Chitinases and chitosanases

Chitin has an estimated natural turnover of 10^9 tons per year so that it is very efficiently decomposed by microorganisms. It can be degraded by two main enzyme groups designated chitinases and *N*-acetylglucosaminidases (NAGases). In analogy to that, chitosan, the *N*-deacetylated derivative of chitin, can be degraded by chitosanases and glucosaminidases, but both have so far been less studied than chitinases. These enzymes hydrolyse the glycosidic bonds between the complex sugars and thus fall into the enzyme class of glycoside hydrolases (GH) [31, 32]. Chitinases can be found in organisms in all kingdoms of life, such as bacteria [33], fungi [34], plants [35], viruses [36] and animals including insects [37] and vertebrates [38]. Low numbers of chitinases are found in yeast and yeast-like fungi, for example *Saccharomyces serevisiae* and the dimorphic basidiomycete *Ustilago maydis* have two and *Candida albicans* four [39]. By contrast, filamentous fungi usually possess 10 to 20 chitinases and the number of chitinases can rise up to 35 in fungi that have adapted to a particular lifestyle, i.e. mycoparasitic (fungi that parasitize other fungi) and insect-pathogenic fungi (entomopathogens)[40, 41].
Chitinases (EC.3.2.1.14) are hydrolytic enzymes, which catalyze the hydrolysis of the β- (1, 4) linkages in chitin and chitooligomers, releasing short-chain chitooligosaccharide products with a minimum chain length of 2 GlcNAc molecules [42]. In general, chitinases can be found in GH 18 and 19 and while in bacteria and plants chitinases from both of these families are present, in fungi they belong exclusively to GH 18. They further can be phylogenetically divided into subgroup A, B and C, based on the amino acid sequences of their GH 18 modules [39, 43, 44]. These subgroups differ in their modular protein architecture as well as in the shape of their substrate binding clefts and thus their predicted enzymatic activities. According to their cleavage patterns, they can be classified as endochitinases, which cleave chitin at random positions within the chitin chain and have a more shallow and open substrate-binding region (subgroup B) or as exochitinases, which act on either end of the polymer and have deep, or even tunnel-shaped catalytic clefts (subgroups A and C) and more likely cleave chitobiose $(GlcNAc)_2$ from the ends of chitin chains [42, 45-47]. Furthermore, the chitinases from different subgroups can contain carbohydrate binding modules (CBMs), that are characteristic for individual subgroups and can influence enzymatic processivity (i.e. consecutive cleavage steps on the polymer chain, for reviews on this topic see [28, 48]). In general, subgroup A chitinases are devoid of CBMs, subgroup B chitinases may contain a CBM (Species depent; CBM 1,5,19 etc.) at the C- terminus and subgroup C chitinases possess multiple CBMs (CBM 18 and CBM 50). An extraordinary group of GH 18 proteins that can be attributed to subgroup B are the so called endo-β-*N*-acetylglucosaminidases (ENGase, EC.3.2.1.96). These enzymes cleave the $(GlcNAc)_2$ linkages that anchor N-glycan antennae to proteins in glycosylated proteins and can therefore act as deglycosylation enzymes [49, 50]. Many fungal species harbor two ENGases, of which one, *T. reesei* Eng18A/Endo T, has been shown to be secreted and the second one, Eng18B, to be intracellularly localized and suggested to be associated with ER-associated degradation (ERAD) [49]. This process describes the retro-translocation of carrier proteins from ER to the cytosol for degradation by the proteasome, the cut glycans serve as degradation signal [51]. The exact role for the secreted ENGase, Eng18A and its orthologs lacks evidence, but due to its enzymatic mechanism it is suggested to be involved in post-secretional modification of glycan structures on endogenous or foreign glycoproteins [50]. Furthermore, Cfcl from the fungus *A. niger*, which is produced during autolysis, is an interesting extension of the enzymatic repertoire of GH 18 proteins because it releases monomers from chitin oligosaccharides, corresponding to an Exo-*N*-acetylglucosaminidase activity [52]. This can be explained by its protein architecture since a CBM 18 is inserted in the GH 18 module of CfcI. Bacterial chitinases have been shown to exhibit enzymatic activity on chitosan and most likely this finding can also be extrapolated to fungal chitinases, but this activity is strongly dependent on the degree of acetylation due to the requirement of chitinases for the acetyl residue at the GlcNAc in the -1 subsite.

In contrast to chitinases, which prefer highly acetylated chitin (higher than 50%, [21]), chitosanases favor highly deacetylated chitosan.

Chitosanases (EC 3.2.1.132) are glycosyl hydrolases that cleave the β- (1, 4) glycosidic linkage of GlcN-GlcN and GlcNAc-GlcN, but only chitinases are able to split GlcNAc-GlcNAc linkages. To date, many chitosanases have been found in a variety of microorganisms, including fungi [53-56], bacteria [57-60], plants [61], and viruses [62]. In fungi in general chitosanases belong to GH family 75 [63-66]. Chitosanases act on the reducing end of the chitosan molecule and thereby produce glucosamine oligosaccharides and they can be classified according to their ability to hydrolyze various types of linkages in the chitosan molecule, as explained below [67, 68]. Enzymes belonging to subclass I are able to split the linkages of GlcNAc-GlcN and GlcN-GlcN, whereas subclass II chitosanases are characterized by their ability to cleave only GlcN-GlcN linkages. Subclass III enzymes are able to hydrolyze GlcN-GlcNAc and GlcN-GlcN linkages [63, 69].

Classification into the chitinases and chitosanases groups and subgroups alone is not necessarily indicative for the biological function of the chitinases. Furthermore, chitinases may play a role in more than one process, they are not only important for degradation of extracellular chitin, but also in self-digestion, such as cell wall remodeling during fungal growth, as well as in cell wall degradation during autolysis and apoptosis [70].
As already stated above the number of chitinases reflects the fungal lifestyle. For example the mycoparasites *T. atroviride* and *T. virens* have 29 and 36 GH 18 proteins, while the unicellular yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe,* which harbor chitin only in their bud scars, have only 2 and 1 [70, 71]. A versatile array of chitinases on the other hand might not simply be an indicator for higher chitinolytic efficiency, but may rather mirror the competitive environment that the fungus encounters and which might render it less susceptible to chitinase inhibitors or proteases that are secreted from other organisms in this habitat [70]. Furthermore, the high degree of variability among different chitinases seems to reflect the adaption to various environmental stimuli, leading to the diversification of some subgroups, e.g. the subgroup C chitinases in mycoparasites. With the 1-2 GH 20 *N*-acetylglucosaminidases present in fungi, the 2-5 GH 75 chitosanases and the chitin deacetylases, that both unfortunately have not been studied extensively in fungi yet, this confusing bundle of chitin active enzymes certainly must be subject to tight and purposeful regulation that so far lacks any evidence of transcriptional activators. On the other hand external factors regulating the expression of these enzymes have earned a lot of attention and a general overview of the current findings will be given below.

The conditions tested for chitinase expression can roughly be grouped into (myco-) parasitism, nutrition on chitin as carbon source, or cell wall remodeling related stimuli. *Trichoderma atroviride* Chi18-5/Ech42 (*T. reesei* Chi18-5, *T. harzianum* Chit42 and *T. virens* Cht42/Tv-Ech1, *Neurospora crassa* gh18-4, *Aspergillus nidulans* ChiB, *A. fumigatus* ChiB1) is one of the best studied chitinases which belongs to subgroup A and is secreted during the mycoparasitic attack [43, 72-74]. However, Chi18-5/ECH42 is not a mycoparasitism specific chitinase, but is also involved in self- and non-self cell wall degradation, since it has been found expressed during autolysis and starvation The role of Chi18-5/ECH42 seems also to be conserved in other species and even genera with a similar lifestyle [75-79]. Subgroup C chitinases have been found to be partially associated with (parasitic) fungal-fungal interactions. In *T. atroviride* for example all subgroup C chitinases were induced during interactions with *B. cinerea*, but not *R. solani* or itself, except for *tac6* that does not have an intact active site [80]. In contrast, in the closely related *T. virens* the regulation of the subgroup C chitinases could not clearly be assigned to only one fungal host or growth condition [81]. In *A. nidulans* expression of subgroup C-II chitinases was in general strong upon contact with some (including *B. cinerea* and *R. solani*), but not all of the tested fungal interactors, however no response to intra-species interactions or chitin was observed [82]. From the available data the subgroup C chitinases have the strongest implication in inter-species interactions, due to the fact that many chitinases are upregulated under mycoparasitic growth conditions. Also the strong diversification and the increase in number in fungi with mycoparasitic lifestyle hint at their involvement in the attack [28, 39, 48, 70, 83]. On the other hand chitinases that are expressed during mycoparasitic growth may also react to other stimuli, such as starvation and autolysis, or may also be induced during growth on chitin as carbon source which contradicts the clear pattern. Chitinases involved in processes generally associated with cell wall remodeling (including starvation and autolysis) can come from all three subgroups, but for A or B so far more examples have been found [39, 47, 79, 84-86]. Some subgroup B chitinase-encoding genes, such as *T. atroviride* chi18-13/ech30, show a carbon source-inducible regulation, hinting rather at a catabolic role. Moreover, Chi18-13/ECH30 has an unusual substrate binding cleft underlining the diversity and biotechnological potential of chitinases [39, 48]. In addition, the presence of subgroup B5, the so called

ENGases, which seem to be involved mainly in post-secretional processes or ERAD [49, 50] and the presence of one subgroup B GPI-anchored chitinase per species, such as *N. crassa* Chit-1 or *Aspergillus nidulans* ChiA, that seems to be involved in cell wall synthesis and remodeling at the tips and branching points of hyphae, again make the subgroup B chitinases a very diverse group [28, 87, 88].
Thus, despite the categorization into the three architectonically different subgroups A, B and C and the further classification into smaller phylogenetic groups, which helps to allocate the vast number of chitinases present in fungal species, their regulation can only partially be inferred from GH18 module-based phylogenetic clustering analyses.

3.2 *N*-acetylglucosaminidases

As mentioned above, fungi have a multitude of chitinases, which convert chitin into chitobiose. Interestingly, for the next step of the chitin-degrading pathway - splitting the dimer chitobiose into two GlcNAc monomers - fungi possess only one or two *N*-acetylglucosaminidases which belong to the GH 20 family proteins [89]. *N. crassa* has one NAGase gene, *nag1*, which is located in the cell wall and has been found associated with fungal-fungal interactions [90]. Furthermore, the *Aspergillus nidulans nagA* gene encodes a NAGase which plays an important role during autolysis [77, 91]. The yeast *Candida albicans* also harbors only one NAGase, Hex1, and *S. cerevisiae* does not possess any [92]. *T. atroviride* contains two NAGases that were designated NAG1 and NAG2 and a very distantly related third gene encoding a GH 20 protein that lacks detailed characterization (JGI accession number: 293914), but is rather related to *N*-acetylgalactosaminidases. Although the extracellular localization of NAG1 and NAG2 is different - NAG1 is secreted into the medium, whereas NAG2 stays (non-covalently) attached to the cell wall [93]. The presence of either of these enzymes is sufficient but essential for the use of chitin or chitobiose as carbon source in *T. atroviride*. Although *nag1* and *nag2* were found to be strongly inducible by GlcNAc, the double knockout strain could still utilize GlcNAc as carbon source indicating that the GlcNAc monomer could still enter the cell to be metabolized in absence of the NAGases [93]. In addition, NAG1 was shown to be involved in autolytic processes, and was also inducible by mycoparasitic growth conditions and by chitin [72, 74, 94]. NAGase activity has been reported from a number of other fungi, but a clear transcriptional regulator had not been identified up to now ([70, 90, 95], see section 4.1 for new findings on that topic).

The presented knowledge on chitin degrading enzyme regulation already reveals the flaw in the expression studies performed so far. It seems there is quite some interplay of growth conditions, lifestyle and growth stage/age, which makes it seemingly impossible to look at the expression of a single chitin degrading enzyme at a certain condition. From all the data available, including the phylogenetic analysis only a rough categorization with notable exceptions can be drawn. Since evidence is lacking for regulation by transcriptional or post-transcriptional regulators so far, a complete characterization is not possible at the moment. Identifying regulators of genes encoding chitinolytic enzymes and understanding their role in chitin degradation therefore represents a major future objective to be able to reprogram chitin degradation for optimized biotechnological processes.

3.3 Auxiliary proteins /carbohydrate binding modules

Carbohydrate-active enzymes such as cellulases or chitinases often harbor not only a catalytic domain but also one or more carbohydrate binding modules. CBMs are devoid of any catalytic activity, consist of up to 200 amino acids and multiples of the same or different CBMs can appear in one protein in tandem. The topography of the CBMs is designed such that it facilitates tight binding to an insoluble substrate and thereby supports the penetration of a crystalline structure. The efficient recognition and binding to the substrate is facilitated by an arrangement of conserved residues in the binding site of the CBMs which mirror the specific polysaccharide structure. Minute changes in the topology of the binding sites alter the specificity towards the ligand. CBMs thus increase specific binding of an enzyme to and can prevent the dissociation from the substrate after successful cleavage, enabling the protein to slide along the carbohydrate chain for the next cleavage step. Thus, CBMs have a positive effect on the processivity of enzymes [96, 97].

Due to their substantial variation in substrate specificity CBMs have been classified to date into 55 classes (www.cazypedia.org). Chitin-binding properties have been described for a number of different CBM families that are found in fungi, including CBM 1 and 18, which usually occur as separate protein domains in enzymes. CBM family 1 typically exhibits cellulose-binding properties, but chitin-binding characteristics have also been reported [98]. CBMs of family 18 are often found in fungal chitinases (GH family 18), chitin synthases (GT family 2) and chitin deacetylases (CE family 4) and therefore members of CBM

family 18 are probably typical chitin-binding modules.

LysM motifs belonging to the CBM family 50 have general GlcNAc binding properties and therefore bind to chitin, chito-oligosaccharides and peptidoglycan. LysM motifs can be found in prokaryotes and eukaryotes, they occur in fungal subgroup C chitinases and interestingly, in contrast to other CBMs, also in secreted fungal proteins that contain multiple LysMs, but lack a catalytic domain (effector proteins). In fungi, LysM motifs can be phylogenetically divided into two clades, a fungal-specific and a fungal/bacterial subclass, based on their amino acid sequences [99]. LysMs of the latter class are involved in interactions between fungi and plants. Members of the fungal/bacterial subclass are reported from the tomato pathogen *Cladosporium fulvum*, *Magnaporthe grisea,* and the hemi-biotrophic wheat pathogen *Mycosphaerella graminicola*. These LysM effector proteins are specifically upregulated during the plant-pathogen interaction and associated with suppression of chitin-induced plant immune responses and protection against plant chitinases [100-103]. A representative for a member from the fungal-specific subclass is *T. atroviride* TAL6, which inhibits germination from *Trichoderma* spp., but not from other fungi, however, the main function is still unknown [70, 104]. It will be interesting to test if addition of LysM effectors to chitinous substrates enhances or decreases the accessibility for chitinases.

Another auxiliary protein family with chitin-binding properties is the cerato-platanin protein (CPP) family, which is exclusively found in fungi [105-109]. Previous studies for several CPPs (for *T.atroviride* EPL1, *C. platani* CP, *Ceratocysis populicola* Pop1 and *M. perniciosa* MpCP1-5) showed that they bind polymeric chitin and/or chitin oligomers [107, 110, 111]. CPPs are small secreted proteins, which are important factors in interaction of fungi with other organisms and they are able to alter the polarity of solutions [107-109, 112]. It has been reported that CPPs can exert expansin-like features on cellulosic materials. Expansins are small proteins which show carbohydrate binding and loosening properties, which is similar to the recent findings for CPPs, and there is also a structural similarity between CPPs and plant expansins [105-107]. Expansins contain an N-terminal (D1) and a C-terminal (D2) domain. The expansin D1 domain forms a double β-barrel fold, which is similar to the CPPs single domain named certo-platanin domain [106, 110]. Some CPPs were found to have weakening activity on cellulose materials without enzymatic activity, interestingly no such effect was detected on chitin, however, the binding affinity of CPPs to chitin is stronger than to cellulose [107, 110, 111, 113]. CPPs therefore are an attractive target for future studies to assist the industrial chitinolytic processes. It will be interesting to see if CPPs can help to make chitin more accessible due to their amphiphilic character or if they can stabilize chitin such that chitinases can more easily access the chitin chains for catalytic cleavage.

A more recently described group of enzymes are lytic polysaccharide monooxygenases (LPMOs) that introduce random chain breaks into cellulose, hemicellulose and chitin. In contrast to the hydrolytic cleavage by GH enzymes the copper-dependent LPMOs insert molecular oxygen into the C-H bonds adjacent to the glycosidic linkage which results in oxidative cleavage of the substrate [114-116]. LPMOs have been shown to exhibit increased enzymatic cellulose degradation properties, but are also increasingly important in the utilization of chitin. They offer an alternative to typical chitinases, due to their ability to cleave polysaccharides oxidatively and may also be used to increase chitinolytic activity of chitinases [114, 115, 117-119]. LPMOs are registered as “Auxiliary Activities” (AAs) family 9 (formerly GH61) in the CAZy database (www.cazy.org). The first LPMO that was reported, CBP21 [114], is actually a chitin-active enzyme, but it is a bacterial protein from *Serratia marcescens* (AA family 10; formerly CBM 33) that does not seem to exist in fungi. However, recently the first chitin-active LPMO, AoAA11 from the fungus *A. oryzae* was detected and is now listed as AA family 11 [120].
Testing these LPMOs in regard to chitin degradation with respect to synergistic effects with chitinases represents a promising task to increase chitooligomer production from the recalcitrant chitin sources.

4. Chitin and GlcNAc - nutritional source or building block for growth

Besides its role in chitin and chitosan, GlcNAc plays an important role in a broad range of mechanisms throughout all kingdoms of life [113]. It occurs also in heterogeneous polysaccharides, such as peptidoglycan/murein. A β- (1, 6) linked poly-*N*-acetylglucosamine is the major constituent of bacterial biofilms [121, 122]. Apart from its important role in cell structure GlcNAc itself may mediate cellular signaling. In dimorphic yeasts, e. g. *C. albicans,* GlcNAc induces the switch from budding to filamentous growth [123, 124]. In bacteria, extracellular GlcNAc provokes production of CURLI (= curled pili) fibers that promote biofilm formation [125]. Furthermore GlcNAc can be found as N- and O-linked attachment on glycoproteins or in GPI (glycosylphosphatidylinositol) anchors on plasma membrane attached proteins. Even in humans GlcNAc plays a pivotal role. Hyaluronic acid (HA)

is an indispensible component of the connective, epithelial and neuronal tissue and consists of repeating D-glucuronic acid and GlcNAc residues [126]. In some cases GlcNAc exists in free form e. g. in human milk (at 600–1500 mg/mL) [127, 128]. Thus, GlcNAc can serve as a carbon source, but can also be recycled and used as building block to generate new cell material or contribute to cell signaling in free form. Regulating the fate of GlcNAc in the fungal cell by coordinating its distribution must be subject to tight control. The balance between the anabolic and catabolic pathways is critical since disproportional coordination of the metabolism can severely impair growth [129, 130]. In the following section the catabolism pathway for chitin breakdown and anabolism to generate UDP-GlcNAc, as well as chitin synthesis, will briefly be introduced and the mechanisms regulating these processes will be discussed.

4.1 *N*-acetylglucosamine catabolism

The monomer of chitin, GlcNAc, can serve as carbon and nitrogen source due to the presence of the acet-amido group linked to the hexose ring structure. Microorganisms using chitin as nutritional source have evolved a simple, but effective three step process to feed GlcNAc into glycolysis with the benefit of ammonium sequestration. GlcNAc catabolism gained considerable attention mostly in bacteria due to the potential to produce enough enzymes to effectively degrade GlcNAc [131, 132]. In eukaryotes, however GlcNAc catabolism so far was investigated mainly in *C. albicans* in the past years [95, 133]. In *S. cerevisiae*, which lacks the enzymes for GlcNAc catabolism, introduction of the GlcNAc catabolic genes from *C. albicans* was tested for bioethanol production [134].

C. albicans is a dimorphic ascomycete that can switch between a unicellular budding yeast form and a multicellular, pathogenic filamentous form, which show different characteristics like a white versus opaque growth phenotype at certain temperatures, respectively. The switch to filamentous growth is induced by human/animal serum and interestingly also by the presence of GlcNAc and renders *C. albicans* a highly pathogenic threat for transplant patients as well as a cumbersome nuisance as oral and vaginal candidiasis [129, 135, 136]. This switch can also be induced by GlcNAc in other yeasts, e.g. *C. lusitaniae* [137] and *Yarrowia* liplytica [124] and only recently also the thermally dimorphic yeast *Histoplasma capsulatum* has been shown to efficiently switch to filamentous form upon concurrent induction by GlcNAc and a temperature switch to room temperature [138]. In *C. albicans* the mechanism of GlcNAc signaling has earned substantial attention and lead also to the discovery of a GlcNAc:H^+ symporter from the major facilitator group of transporters (MFS), Ngt1, and a cluster of three catabolic genes that are involved in the well-defined GlcNAc catabolism pathway [139]. Via consecutive action of Hxk1 (GlcNAc-hexokinase; EC 2.7.1.59), Dac1 (GlcNAc-6-PO_4-deacetylase; EC 3.5.1.33) and Nag1 (GlcN-6-PO_4-deaminase; EC 3.5.99.6) GlcNAc is transformed to fructose-6-PO_4 which can enter glycolysis [129, 140]. This catabolic pathway was found to be dispensable for the switch to filamentous growth in *C. albicans*. Deletion of either of the catabolic enzymes rendered *C. albicans* incapable of utilizing GlcNAc, but the switch to filamentous growth could still be performed [129, 133]. Moreover it could be shown that in a homozygous *hxk1* knockout strain signaling was still induced by GlcNAc [95], in fact it is believed that GlcNAc serves as signal rather than GlcNAc-6-PO_4 since the cell thereby can distinguish between the external GlcNAc, that enters the cell, and the native GlcNAc-6-PO_4 generated by anabolic processes in the cell (see also section 4 b). It was further shown that GlcNAc needs to enter the cell to signal availability of the carbon source for nutritional purposes and that Ngt1 is the major and high affinity transporter for GlcNAc [139]. In contrast to a *hxk1* knockout strain, deletion of *dac1* or *nag1* caused severe growth defects when GlcNAc served as sole carbon source, even in the presence of other sugars (D-glucose, D-fructose, D-galactose), suggesting that excess GlcNAc-6-phosphate is deleterious [129]. In a recent study analysis of a broad taxonomic range of ascomycetous fungi (Pezizomycotina) identified the presence of a GlcNAc cluster in many lineages, although with variations in cluster organization [141]. The three catabolic genes (designated *hxk3, dac1* and *dam1*) are conserved in filamentous fungi and were shown to be essential for growth of *T. reesei* on GlcNAc as sole carbon source and to some extent also on chitin. The GlcNAc transporter NGT1 identified in this study is also important for growth on GlcNAc [141]. Another gene identified in the clusters was a GH3-family gene of yet unclassified function. The gene, which was termed *nag3,* exhibits similarities to bacterial GH 3 β-*N*-acetylhexosaminidases ([142-146] and www.cazy.org/GH3.html). In fungi, *N*-acetylglucosaminidase function has so far only been assigned to GH family 20 [39, 93], but the gene has been found essential for growth on GlcNAc [141]. Recently a GH3 family protein, Nag3, from *Cellulomonas fimi* was found to be a GlcNAc-phosphorylase using phosphate rather than water as nucleophile [147]. Importantly, in many filamentous fungi a transcription factor with an Ndt80-like DNA-binding domain (PFAM family PF05224) is also included in the cluster, which was

designated RON1 (**r**egulator **o**f ***N***-acetylglucosamine catabolism 1) in *Trichoderma* spp. [141]. With the discovery of this transcription factor in the GlcNAc cluster of many filamentous fungi, the first positive regulator of GlcNAc catabolism genes has been pinpointed that was found to be an essential transcriptional activator of the GlcNAc gene cluster [141]. RON1 is a member of a rare family of exclusively fungal transcription factors, which were first described in *S. cerevisiae* [148-151]. In *Trichoderma* spp. RON1 highly induces transcription of all three (*hxk3*, *dac1*, *dam1*) catabolic genes in the presence of GlcNAc as well as the GlcNAc transport protein NGT1 [141]. In *C. albicans* a gene for an orthologous Ndt80-like transcription factor, *REP1* (orf 19.7521) has been described that has been found to play a role in the regulation of a multiple drug resistance efflux pump [152]. In filamentous fungi, Ndt80-family proteins were also studied extensively and XprG from *Aspergillus* spp. [153] and NCU04729 from *N. crassa* [154] have been identified as the closest orthologs in our study but so far an involvement in GlcNAc catabolism of neither of the three was tested.

Importantly, in *T. reesei*, induction of gene expression of all GlcNAc cluster genes and *ngt1* was completely abolished upon deletion of *ron1*. Furthermore, biomass formation on GlcNAc as sole carbon source was suppressed in knockout strains in submerged cultivations. Thus RON1 is essential for growth on GlcNAc. Remarkably expression of the genes encoding the two NAGases, NAG1 and NAG2, was also found to be dependent on RON1 [141]. RON1 therefore is the first identified positive regulator of chitin degradation for nutritional purposes.

4.2 *N*-acetylglucosamine anabolism – The chitin biosynthesis pathway

From the available data generated over the past years it can be expected that the intracellular GlcNAc pool not only undergoes catabolic recycling, but also feeds into anabolism as UDP-GlcNAc, which is a building block for *de novo* chitin synthesis. For chitin synthesis as well as glycosylation and anchoring of proteins in the cell wall UDP-GlcNAc has been found to be detrimental, and therefore also the enzymatic machinery needed to produce these universally present structures are conserved in eukaryotes down to yeast *S. cerevisiae* and *Sz. pombe* as well as prokaryotes. Gene deletion of any of the involved enzymes is deleterious unless GlcN or GlcNAc are present in the growth medium, emphasizing their detrimental role in growth processes [155-157], but also pointing at possible redundancies with the catabolic enzymes.

Hyphae of *C. albicans* have been shown to harbor around five-fold amounts of chitin (with around 4-5% chitin in their cell walls) compared to the yeast like form, while in the thermally dimorphic *H. capsulatum* the opposite is the case [158, 159]. The anabolic enzymes for chitin synthesis have been extensively studied mainly in the two yeasts, *C. albicans* and *S. cerevisiae*, and a brief overview of the generation of UDP-GlcNAc and chitin is given here (for more details on that topic the reader is referred to [158]). For other GlcNAc modifications the reader is referred to [160, 161] for an overview on these topics. The conversion of sugar into sugar nucleotides was discovered by Luis F. Leloir in the 1950s and therefore the biosynthetic reaction to create UDP-GlcNAc represents one version of the Leloir pathway. The first two steps are an exact reversion of the last two catabolic steps conferred by CaNag1/TrDAM1 and CaDac1/TrDAC1, respectively. Fructose-6-PO_4 is converted to GlcN by Glucosamine 6-PO_4 synthase (EC 2.6.1.16), that is designated Gfa1 in *S. cerevisiae* and *C. albicans*. Gfa1 catalyses the transfer of ammonium from L-glutamine to Fructose-6-PO_4. In homozygous deletions of *gfa1* in diploid *C. albicans* strains growth was only possible upon supplementation with GlcNAc [155]. The second step is generation of GlcNAc-6-PO_4 by GlcN-6-PO_4 acetyltransferase/Gna1 (EC 2.3.1.4), that has been characterized in detail in *S. cerevisiae*, *C. albicans* and *Sz. pombe* and involves AcetylCoA as a co-factor. *S. cerevisiae gna1* deficient mutants cannot grow even in the presence of GlcNAc [157], but homozygous *C. albicans gna1* null mutants may grow when supplemented with GlcNAc, although they dramatically swell upon growth and are not able to undergo cell separation any more [162]. The differences in response to *gna1* deletion in the two yeasts can be explained by the lack of GlcNAc-deacetylase and -kinase in *S. cerevisiae*. Considerable effort has been made to identify regulators of the anabolic enzyme Gfa1, although in contrast to GlcNAc catabolism (RON1) so far evidence for the transcriptional regulator(s) of this pathway is missing. Cell wall stress and mating have been found as triggers for *S. cerevisiae GFA1* expression [163-166]. Cell wall stress, as an inducer of *gfaA* (the *GFA1* ortholog) expression and chitin deposition in the cell wall, was confirmed in *A. niger* [167] and in *C. albicans* during the switch from the yeast-like to hyphal form of growth [168]. The catalytic activity of Gfa1 can further be post-transcriptionally increased nearly five-fold upon phosphorylation by protein kinase A, as has been demonstrated for CaGfa1 [169]. A relatively strong and specific inhibitor of Gfa1 enzymatic activity is feedback inhibition by UDP-GlcNAc, the endproduct of the anabolic

pathway which can be alleviated when Glucose-6-PO_4 is absent.
The last two synthesis steps are carried out by phosphoacetylglucosamine mutase, Agm1 (EC 5.4.2.3), which accounts for the isomerization of GlcNAc-6-PO_4 to GlcNAc-1-PO_4 and UDP-GlcNAc pyrophosphorylase ScQri1/ CaUap1 (EC 2.7.7.23), that catalyzes the exchange of phosphate with *Uridine-5'-diphosphate* (UDP). For both anabolic enzymes only limited data is available regarding their regulation [169, 170]. For *ScAGM1* three putative pheromone-responsive elements have been identified [171] and the Uap1 enzyme was effectively inhibited *in vitro* by uridine [172].

UDP-GlcNAc may then be fed into chitin biosynthesis were again many of the studies about chitin synthases (CHS, Chitin-UDP acetyl-glucosaminyl transferase**,** EC 2.4.1.16) and their regulation come from *S.cerevisiae* and *C. albicans*. There are 3 CHS enzymes in *S. cerevisiae* and 4 in *C. albicans* and in filamentous fungi the number increases up to 8 [20]. By search in genomes with the catalytic motif Q(R/Q)XRW more than 150 synthases have been identified and are grouped in 7 classes. Class III seems to be important for the bulk chitin synthase activity in *S. cerevisiae* and *C. albicans* accounting for 80-90% of total cellular chitin production. Simultaneous deletion of all three chitin synthases in *S. cerevisiae* causes a lethal phenotype [173]. Class V and VII have been shown to be highly conserved 1500 amino acid long chitin synthases where most of them harbor a myosin-motor like domain [20, 174]. In *N. crassa* 7 chitin synthases have been identified, each for every class, of which NcChs-3 (class I) seems to be responsible for the majority of chitin production [175, 176]. In *A. fumigatus* and *A. nidulans,* which have 8 chitin synthases, AfCHSE and AnCHSB (class III) have been found essential for growth [177, 178]. Chitin synthases transfer UDP-GlcNAc in an inverting mechanism onto the non-reducing end of the growing acceptor oligosaccharide [179]. The large enzymes are an integral part of the membrane with multiple domains important for activation and subcellular localization. They are believed to form a transport channel through the outer membrane, and deposit chitin at the outer surface, similar to the cellulose synthases [180]. In *C. albicans* they have been shown to synthesize different forms of chitin (long microfibrils or short chitin rodlets), depending on their localization and the life cycle [181]. Several competitive natural chitin synthase inhibitors have been identified, among them nikkomycin and polyoxins and their chemically modified derivatives, but they seem not to be sufficiently effective to treat mycoses, yet [182].
Regulation of chitin synthesis is tightly linked to the life cycle. In yeast *S. cerevisiae CHS1* (class I) transcription is increased during mating as well as after activation of the salvage pathway, i.e. the nucleotide re-usage after recombination [183, 184]. In *A. nidulans* and *N. crassa* differential expression of chitin synthases during sexual and asexual development was also reported [174, 185]. For the class I homolog in *C. albicans* induction of transcription was found after the switch to filamentous growth [186, 187]. The activity of *ScCHS2* (class II) is highest before cytokinesis, whereas it is strongly decreased during mating and sporulation [188, 189]. For the homolog *CaCHS1* a low, but constant expression has so far been determined [187]. *ScCHS3*, that was found responsible for the bulk chitin synthesis, seems to lack transcriptional regulation but is expressed throughout the whole cell cycle. Instead of transcriptional regulation, chaperones are implicated in directing *ScCHS3* chitin synthase activity to sites of action. Chs7 (close homologs have been identified in *C. albicans, A. fumigatus, A. gossipyi* and *N. crassa* [20]) and Chs6/5 are involved in recruiting the chitin synthase from the ER or chitosome to the cell membrane. Another protein, Chs4, could act as a direct activator of Chs3, since its overexpression increases the activity of Chs3 during septum synthesis ([190, 191] and reviewed in [20]). *CHS4* has a functional homolog *SHC1,* which is alternatingly expressed only during ascospore formation when the chitosan layer is produced and expression of *CHS4* is turned off, so that the proteins are functionally redundant, but biologically compartmentalized by differential expression. *C. albicans* seems to have the ortholog of *CHS4* despite its disability to sporulate [192]. A recent finding also suggests an involvement of *GFA1* in regulation of chitin synthase activity/expression since its expression levels seem to directly alter chitin synthesis and *vice versa*: the need for chitin synthesis can increase *GFA1* levels [166].

5. Recent approaches for mining chitin and the generation of new high value products

5.1 New sources

Over the past decades chitin has proven to be present - if not as major constituent, but still in measurable amounts - in every eukaryotic kingdom except for plants and higher vertebrates. Interestingly, the main source of chitin to enter production processes remains crustacean shells derived from the high amounts of crustacean wastes from the fishing industries. Moreover, the prevailing method used to extract chitin is chemo-catalytic leaching which not only results in poorly defined end-products, but also accumulates considerable amounts of unhealthy wastes.

In order to reduce the hazardous wastes many projects[1] were initiated to exploit the current knowledge about chitinases and chitosanases (e.g. produced from filamentous fungi such as *Trichoderma*, *Aspergillus* but also from bacteria e.g. *Bacillus* and *Serratia*) to assist the chemo-catalytic methods in mining GlcNAc or chitooligomers form crude chitinous sources in the last years [193]. From the Antarctic fungus Lecanicillium muscarium, cold tolerant chitinases could be isolated that would further reduce energy costs of chitin hydrolysis [194]. Efforts to further optimize the enzymes for direct GlcNAc production are being made to increase the output and reduce production steps. Chern *et al.*, identified a bacterium, *Chitinibacter tainanensis*, in Taiwan that seems to be able to synthesize GlcNAc into its environment when fed with chitin [195, 196]. A benefit of the new methods and processes that have been evolving over the past years is that via the enzymatic lysis by-products (proteins, lipids etc.) can be used as animal feed or for biogas-production as alternative energy source. The highly hazardous chitinous waste generated in millions of tons every year by the fishing industry could thereby be completely salvaged and prevent the otherwise ecological risks generated by disposal in landfills.

Extensive overfishing and the immense demand for fish led to a dramatic decline of natural fish stock in the past century. Therefore, commercial production of fish and crustaceans in large scale marine farms were established. Crustaceans (mainly shrimps) accounted for the highest percentage (57%) of farmed marine animals for food production in 2010. These farms are mainly built on land (in ponds) or sea near the coast and pose a high threat to coastal areas since the effluents from these farms contain vast amounts of organic matter, nitrogen and phosphate that harm and pollute the marine ecosystem [197]. The high density of crustaceans in the farms further raises the possibility that illnesses spread in the colony and therefore antibiotics are frequently administered raising the probability for antibiotic resistances. On the other hand, when antibiotics are omitted species such as *Vibrio* spp., the most common bacterial disease in shrimp farms, could also be transmitted to other inhabitants of the coast [197]. Thus, the environmental concerns caused by the high amounts of shrimp and crab production require other sources to be considered. Harvesting chitin from crustacean shells is extremely energy intensive since the vast amounts of pigments and residual proteins in and around the chitin shell need to be extracted first, and so far an effective enzymatic extraction (protease mixtures with maximum 20 % extraction efficiency [198]) for these contaminants has not been explored extensively. Therefore other sources of chitin with very low ash content are interesting targets for biotechnological exploitation. Moreover, obtaining chitin from crustacean shells for medical purposes is also challenging. This is due to the high amounts of proteins and pigments associated with chitin since residual solvent from the extraction process or proteins/pigments may create allergic reactions or intolerances [5]. Therefore, a current approach is to find and exploit new more ecologically save sources, which may yield easier extractable, more pure chitin. In an ongoing project insect farming[2] is tested where yellow mealworm larvae (*Tenebrio molitor)* are bred with controlled aliments. An advantage of insects is there easy and cheap breeding and their separation from the natural environment in a self-contained system. Trials conducted by the E.U. initiative PROteINSECT[3] have found that at least 150 tons of insect protein could be produced on one hectare of land per year [199]. Therefore, the insects would be bred in the first place not for chitin production, but to generate the high amounts of proteins and lipids currently needed as animal feed. The chitin shell of insect larvae further contains lower amounts of pigments - i.e. the percentage of ash in chitin is lower than in adults - which decreases the amount of chemicals needed to purify chitin [200, 201]. Thus chitin would only be a by-product of protein and lipid production but could more easily be extracted with less contaminant which would dramatically decrease costs and efforts for production [199].

Mushrooms, as source for chitin, represent another alternative to crustacean shells. Global edible fungi cultivation was estimated to 32 million tons in 2011 with *Agaricus bisporus*, leading the list of preferred fungi [8, 202]. Studies for chemical extraction from basidio- and ascomycetous wastes showed that fungi are an excellent source for chitin and chitosan. *A. bisporus*, *A. niger* and *M. rouxii* wastes contain 19, 12 and 20% harvestable chitin/chitosan in dry mycelia with lower deacetylation grades than shrimp shell chitin [203, 204]. Especially low DA grade chitosan could be directly harvested from *M. rouxii,* which harbors mainly chitosan with very low DA grades [205]. Pigmentation can be avoided when using white fungi such as *A. bisporus* or when only white parts of fungi, i.e. stalks or mycelium that is devoid of pigments, are processed so that chemical extraction of pigments could be bypassed

[1] ChiBio project: www.chibiofp7.eu; funded by European Union's Seventh Framework Programme (FP7) under the grant agreement n° 289284.

[2] YNSECT: Genopole – Campus 3, 1 rue Pierre Fontaine Bâtiment 2, 91058 Evry CEDEX, France, www.ynsect.com/

[3] PROteINSECT project: http://www.proteinsect.eu/; co-financed by the European Commission (EC) under the seventh framework program (FP7) n° 312084

or strongly reduced. Furthermore, in comparison to chitin from animal sources, chitin from edible fungi does not pose medical risks, since allergic reactions and transmission of diseases are considered unlikely, facilitating certification for pharmacological and medicinal purposes. The large scale enzymatic fermentation processes accumulate vast quantities of fungal biomass which often pose problems as unwanted byproducts. Extraction of chitin from these industrial sources would as well be a cheap alternative to crustacean chitin.

5.2 New products

Production of chitin, chitosan and derivatives thereof is now entering the third generation. When production of chitinous materials was established the so called 'first generation' of chitin and chitosans sufficed only for the use as biomaterial for e.g. waste water treatment or as livestock food additives. The poorly defined polymer mixtures of varying purity and composition rendered chitin applications and production at industrial scale unappealing. A 'second generation' with more reliable production standards after intense efforts to understand the structure and function of chitin and derivatives lead to well-defined new products in terms of degrees of polymerization and acetylation. They were more suitable for the development of reliable high value products due to known molecular structure-function relationships, which made it possible to increase affectivity for example in plant protection from 40kg/ha to 4g/ha. The challenge for future studies will be the exploitation and generation of new design products to enter the 'third generation' of chitin and chitosan based products. Chitosans with non-random patterns of acetylation and chitin and chitooligomers with clearly defined biological activities and cellular modes of action will be generated. The last section of this review therefore discusses new products that are already being designed or are envisaged for future applications.

Chitosan and chitooligomers of differing acetylation grades can be used in medical applications. They are highly antibacterial but can be degraded by endogenous human chitinases and therefore assist regeneration of human tissue after injury. Their current applications comprise wound dressings, separation membranes, antibacterial coatings for stents and tissue engineering scaffolds. With decreasing deacetylation grade chitosan also becomes soluble in mild acidic solutions producing hydrogels [206, 207] and sponges [208, 209], that can be used for drug delivery and scaffolds for mucosal tissue. Chitosan is the only known natural polymer with a positive charge owing to the free amino-groups generated by the partial deacetylation. The positive charge raises the possibility to crosslink chitosan to other negatively charged polymers or ions. Chitosan cross-linked to alginate or gelatin improves stability, cyto-biocompatibility and provides a better environment for cell attachment and proliferation [210-212]. The covalent combination of glycine and chitosan results in *N*-carboxymethyl chitosan. The amphiphilic behavior of the glycine moiety enhances the solubility over a continuous and extended pH range and the polymer has been demonstrated to serve as antioxidant in cellular processes [8]. Electrospinning is further applied now to generate higher molecular weight fibers from chitin solutions by pulling micron and nano-sized fibers from this polymer solution in an electric field. The higher molecular weight fibers can also serve as scaffolds in tissue engineering [213]. The project Nano3bio[4] aims at identifying chitinases and chitin deacetylases to obtain chitosans with known and defined, non-random patterns of deacetylation. Chitin deacetylases are capable of producing distinct patterns of DA and therefore will be used to generate chito-oligosaccharides with defined DA grades. Hamer *et al.* have produced specific chitosan oligomers, which are deacetylated with a novel and defined pattern by use of two chitin deacetylases [214]. By increasing the number of different deacetylases (also from fungal sources), novel chitosan oligomers with a fully defined architecture will be produced in the near future.
GlcNAc itself was discovered to be a valuable pharmacological agent in the treatment of a wide variety of maladies in the past decade. GlcNAc has been found effective to treat joint damage including arthritic diseases, cartilage- or joint injury and degenerative joint diseases when delivered to sites of damage. Furthermore, provident GlcNAc administration can prevent joint damage. GlcNAc also inhibits elastase activity and superoxide release from human polymorphonuclear leukocytes and is tested as potential candidate to treat inflammatory bowel disease [215-219]. Another field of application is cosmetics, hyaluronic acid is widely used to treat skin and mucosal damages despite inefficient absorption. GlcNAc, however, absorbs effectively and has been shown to induce a dose-dependent increase in the production of HA in cultured keratinocytes [220, 221].

N-acetylglucosamine and Glucosamine can serve as starting material (or platform chemical) to synthesize new polymers or N-containing compounds, such as isocyanates and polyamides which have so far not been produced from

[4] www.nano3bio.eu/start/ funded by European Union's Seventh Framework Programme (FP7) under the grant agreement n° 613931

renewable raw material. GlcNAc and GlcN can be harnessed as carbon and energy source for specialized yeast cells to produce functionalized fatty acids and amino-carboxylic acids as starting point for chemical synthesis processes. Both can be fed into the polymer production process. In a multi-enzymatic process currently developed[5] also heterocycles can be produced from GlcN. A process already developed is *N*-acetylneuraminic acid (Neu5Ac) production. Neu5Ac is one of the most common sialic acids, used to produce neuraminidase inhibitors to treat influenza infections [222]. For enzymatic synthesis of Neu5Ac *N*-acetylmannosamine (ManNAc) and pyruvate are used as substrates for recombinant Neu5Ac aldolase. ManNAc in turn can be produced inexpensively from GlcNAc, via epimerization with the GlcNAc 2-epimerase. Thus from 27 kg of GlcNAc 29 kg of Neu5Ac could be obtained using recombinant GlcNAc 2-epimerase and Neu5Ac lyase as catalysts [223].

6. Outlook

Over the past decades major efforts have been made to increase our knowledge about the structure and function of chitin/chitosan and its degradation products and how they are functionalized in chitin metabolism. With the use of new sources, that are more reliable, contain less pigments and contaminants and are thus medically safer, chitin and chitooligomers will be established as bio-based, renewable compounds and will serve as substrate for (bio-) chemical design processes, such as heterocycle chemistry and functionalized fatty acids. Moreover, a better understanding of the function and action of chitin metabolizing enzymes and the interplay with auxiliary proteins will help to increase their productivity and specificity via directed genetic engineering. Importantly, regulators for chitin metabolism need to be identified so that we are able to modulate activity of the enzymes and alter the time course of their action. With the identified regulators and the knowledge about all enzymes involved in anabolism and catabolism of chitin it will soon be possible to stop reactions at any given point and modify the produced intermediate with the introduction of heterologous enzymes to create new substrates for designed chemicals on a sustainable basis.

LIST OF ABBREVIATIONS

AAs	auxiliary activities
CBM	carbohydrate-binding module
CHS	chitin synthase
CP	cerato-platanin
CPP	cerato-platanin protein
CURLI	curled pili
DA-grade	deacetylation grade
ENGase	endo-β-*N*-acetylglucosaminidases
ER	endoplasmatic reticulum
ERAD	endoplasmatic reticulum associated degradation
ERAD	endoplasmic reticulum-associated protein degradation
GH	glycoside hydrolase
GlcN	*N*-glucosamine
GlcNAc	*N*-acetylglucosamine
$(GlcNAc)_2$	chitobiose
GPI-anchor	glycosylphosphatidylinositol anchor
HA	Hyaluronic acid
LPMOs	lytic polysaccharide monooxygenases
LysM	lysin motif
ManNAc	*N*-acetylmannosamine
MFS	major facilitator symporters
NAGase	*N*-acetylglucosaminidase
NAGases	*N*-acetylglucosaminidases
Neu5Ac	*N*-acetylneuraminic acid
UDP	uridine-5′-diphosphate

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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REFERENCES

[1] Dumitriu S, Dekker M. Polysaccharides: Structural Diversity and Functional Versatility. 2nd ed. New York: CRC Press 1998.

[2] Minke R, Blackwell J. The structure of alpha-chitin. J Mol Biol 1978; 120: 167-81.

[3] Jang MK, Kong BG, Jeong YI, Lee CH, Nah JW. Physicochemical characterization of α-chitin, β-chitin, and γ-chitin separated from natural resources. J Polym Sci Part A: Polym Chem 2004; 42: 3423-32.

[4] Gardner KH, Blackwell J. Refinement of the structure of beta-chitin. Biopolymers 1975; 14: 1581-95.

[5] Younes I, Hajji S, Rinaudo M, *et al*. Optimization of proteins and minerals removal from shrimp shells to produce highly acetylated chitin. Int J Biol Macromol 2015.

[5] ChiBio project: www.chibiofp7.eu; funded by European Union's Seventh Framework Programme (FP7) under the grant agreement n° 289284.

[6] Atkins E. Conformations in polysaccharides and complex carbohydrates. J Biosciences 1985; 8: 375-87.

[7] Kuhlmann K, Czupala A, Haunhorst J*, et al.* Preparation and characterization of chitosan from Mucorales. In: Peter, M. Domard, A. Muzzarelli, RAA, Eds. 3rd, European Chitin Society; Advances in chitin science. 1999; Potsdam, Germany 2000. pp. 7-14.

[8] Muzzarelli RAA, Boudrant J, Meyer D*, et al.* Current views on fungal chitin/chitosan, human chitinases, food preservation, glucans, pectins and inulin: A tribute to Henri Braconnot, precursor of the carbohydrate polymers science, on the chitin bicentennial. Carbohyd Polym 2012; 87: 995-1012.

[9] Muzzarelli RA. Native, industrial and fossil chitins. EXS 1999; 87: 1-6.

[10] Tharanathan RN, Kittur FS. Chitin-the undisputed biomolecule of great potential. Crit Rev Food Sci Nutr 2003; 43: 61-87.

[11] Duursma EK, Dawson R. Marine Organic Chemistry. In: Elsevier oceanography series; 31,: Elsevier Science Ltd 2011.

[12] Gal YL, Ulber R, Antranikian G. Marine Biotechnology. vol 1. In: Scheper T, Eds. Advances in biochnemical engineering/biotechnology: Springer US 2005.

[13] The State of World Fisheries and Aquaculture. FAO - Food and Agriculture Organization of the United Nations. Rome: FAO; 2014

[14] Wang SL, Chang WT. Purification and characterization of two bifunctional chitinases/lysozymes extracellularly produced by Pseudomonas aeruginosa K-187 in a shrimp and crab shell powder medium. Appl Environ Microbiol 1997; 63: 380-6.

[15] Subasinghe S. The development of crustacean and mollusk industries for chitin and chitosan resources. In: Zakaria MB, Muda WMW, Abdullah MP, Eds. Chitin and chitosan the versatile environmentally friendly modern materials: Universiti Kebangsaan Malaysia 1995.

[16] Cosgrove J. Chitin & Chitosan: A Global Strategic Business Report. 2010.

[17] Archer DB, Connerton IF, MacKenzie DA. Filamentous fungi for production of food additives and processing aids. Adv Biochem Eng Biotechnol 2008; 111: 99-147.

[18] Cheng JJ, Timilsina GR. Status and barriers of advanced biofuel technologies: A review. Renew Energy 2011; 36: 3541-9.

[19] Seiboth B, Ivanova C, Seidl-Seiboth V. *Trichoderma reesei:* A fungal enzyme producer for cellulosic biofuels. In: dos Santos Bernardes MA, Eds. Biofuel production-Recent developments and prospects. In Tech 2011. pp. 309-40.

[20] Roncero C. The genetic complexity of chitin synthesis in fungi. Curr Genet 2002; 41: 367-78.

[21] Baxter A, Dillon M, Taylor KD, Roberts GA. Improved method for i.r. determination of the degree of *N*-acetylation of chitosan. Int J Biol Macromol 1992; 14: 166-9.

[22] Gilbert NM, Baker LG, Specht CA, Lodge JK. A glycosylphosphatidylinositol anchor is required for membrane localization but dispensable for cell wall association of chitin deacetylase 2 in *Cryptococcus neoformans*. MBio 2012; 3.

[23] Arcidiacono S, Kaplan DL. Molecular weight distribution of chitosan isolated from *Mucor rouxii* under different culture and processing conditions. Biotechnol Bioeng 1992; 39: 281-6.

[24] Pochanavanich P, Suntornsuk W. Fungal chitosan production and its characterization. Lett Appl Microbiol 2002; 35: 17-21.

[25] Banks IR, Specht CA, Donlin MJ*, et al.* A chitin synthase and its regulator protein are critical for chitosan production and growth of the fungal pathogen *Cryptococcus neoformans*. Eukaryot Cell 2005; 4: 1902-12.

[26] Briza P, Ellinger A, Winkler G, Breitenbach M. Chemical composition of the yeast ascospore wall. The second outer layer consists of chitosan. J Biol Chem 1988; 263: 11569-74.

[27] Lenardon MD, Munro CA, Gow NA. Chitin synthesis and fungal pathogenesis. Curr Opin Microbiol 2010; 13: 416-23.

[28] Seidl-Seiboth V, Ihrmark K, Druzhinina IS, Karlsson M. Molecular evoluation of *Trichoderma* chitinases. In: Gupta, V.K. Schmoll, M. Herrera-Estrella, A. Upadhyay, R.S. Druzhinina, I. S. Tuohy, M.G, Eds. Biotechnology and Biology of *Trichoderma*. Oxford, UK: Elsevier 2014. pp. 67-78.

[29] Ruiz-Herrera J. Fungal cell wall: structure, synthesis and assembly. Boca Raton: CRC Press 1991.

[30] Chaffin WL, Lopez-Ribot JL, Casanova M, Gozalbo D, Martinez JP. Cell wall and secreted proteins of *Candida albicans*: identification, function, and expression. Microbiol Mol Biol Rev 1998; 62: 130-80.

[31] Davies G, Henrissat B. Structures and mechanisms of glycosyl hydrolases. Structure 1995; 3: 853-9.

[32] Henrissat B, Bairoch A. Updating the sequence-based classification of glycosyl hydrolases. Biochem J 1996; 316 (Pt 2): 695-6.

[33] Park JK, Morita K, Fukumoto I*, et al.* Purification and Characterization of the Chitinase (ChiA) from Enterobacter sp. G-l. Biosci Biotechnol Biochem 1997; 61: 684-9.

[34] Haran S, Schickler H, Chet I. Molecular mechanisms of lytic enzymes involved in the biocontrol activity of *Trichoderma harzianum*. Microbiology-sgm 1996; 142: 2321-31.

[35] Graham LS, Sticklen MB. Plant chitinases. Can J of Bot 1994; 72: 1057-83.

[36] Patil RS, Ghormade VV, Deshpande MV. Chitinolytic enzymes: an exploration. Enzyme Microb Technol 2000; 26: 473-83.

[37] Merzendorfer H, Zimoch L. Chitin metabolism in insects: structure, function and regulation of chitin synthases and chitinases. J Exp Biol 2003; 206: 4393-412.

[38] van Eijk M, van Roomen CP, Renkema GH, *et al.* Characterization of human phagocyte-derived chitotriosidase, a component of innate immunity. Int Immunol 2005; 17: 1505-12.

[39] Seidl V. Chitinases of filamentous fungi: a large group of diverse proteins with multiple physiological functions. Fungal Biol Rev 2008; 22: 36-42.

[40] Gao Q, Jin K, Ying SH, *et al.* Genome sequencing and comparative transcriptomics of the model entomopathogenic fungi *Metarhizium anisopliae* and *M. acridum*. PLoS Genet 2011; 7: e1001264.

[41] Kubicek CP, Herrera-Estrella A, Seidl-Seiboth V, *et al.* Comparative genome sequence analysis underscores mycoparasitism as the ancestral life style of *Trichoderma*. Genome Biol 2011; 12: R40.

[42] Horn SJ, Sorbotten A, Synstad B, *et al.* Endo/exo mechanism and processivity of family 18 chitinases produced by *Serratia marcescens*. FEBS J 2006; 273: 491-503.

[43] Seidl V, Huemer B, Seiboth B, Kubicek CP. A complete survey of *Trichoderma* chitinases reveals three distinct subgroups of family 18 chitinases. FEBS J 2005; 272: 5923-39.

[44] Karlsson M, Stenlid J. Evolution of family 18 glycoside hydrolases: diversity, domain structures and phylogenetic relationships. J Mol Microbiol Biotechnol 2009; 16: 208-23.

[45] van Aalten DM, Komander D, Synstad B, *et al.* Structural insights into the catalytic mechanism of a family 18 exo-chitinase. Proc Natl Acad Sci USA 2001; 98: 8979-84.

[46] Sikorski P, Sorbotten A, Horn SJ, Eijsink VG, Varum KM. *Serratia marcescens* chitinases with tunnel-shaped substrate-binding grooves show endo activity and different degrees of processivity during enzymatic hydrolysis of chitosan. Biochemistry 2006; 45: 9566-74.

[47] Hurtado-Guerrero R, van Aalten DM. Structure of *Saccharomyces cerevisiae* chitinase 1 and screening-based discovery of potent inhibitors. Chem Biol 2007; 14: 589-99.

[48] Hartl L, Zach S, Seidl-Seiboth V. Fungal chitinases: diversity, mechanistic properties and biotechnological potential. Appl Microbiol Biotechnol 2012; 93: 533-43.

[49] Dubey MK, Ubhayasekera W, Sandgren M, Jensen DF, Karlsson M. Disruption of the *Eng18B* ENGase gene in the fungal biocontrol agent *Trichoderma atroviride* affects growth, conidiation and antagonistic ability. PLoS One 2012; 7: e36152.

[50] Stals I, Samyn B, Sergeant K, *et al.* Identification of a gene coding for a deglycosylating enzyme in *Hypocrea jecorina*. FEMS Microbiol Lett 2010; 303: 9-17.

[51] Romisch K. Endoplasmic reticulum-associated degradation. Annu Rev Cell Dev Biol 2005; 21: 435-56.

[52] van Münster JM, van der Kaaij RM, Dijkhuizen L, van der Maarel MJ. Biochemical characterization of *Aspergillus niger* CfcI, a glycoside hydrolase family 18 chitinase that releases monomers during substrate hydrolysis. Microbiology 2012; 158: 2168-79.

[53] Cheng CY, Li YK. An *Aspergillus* chitosanase with potential for large-scale preparation of chitosan oligosaccharides. Biotechnol Appl Biochem 2000; 32 (Pt 3): 197-203.

[54] Eom TK, Lee KM. Characteristics of chitosanases from *Aspergillus fumigatus* KB-1. Arch Pharm Res 2003; 26: 1036-41.

[55] Zhang XY, Dai AL, Zhang XK, *et al.* Purification and characterization of chitosanase and Exo-beta-D-glucosaminidase from a Koji mold, *Aspergillus oryzae* IAM2660. Biosci Biotechnol Biochem 2000; 64: 1896-902.

[56] Zhang J, Cao H, Li S, *et al.* Characterization of a new family 75 chitosanase from *Aspergillus* sp. W-2. Int J Biol Macromol 2015; 81: 362-9.

[57] Hedges A, Wolfe RS. Extracellular enzyme from Myxobacter AL-1 that exhibits both beta-1,4-glucanase and chitosanase activites. J Bacteriol 1974; 120: 844-53.

[58] Kurakake M, Yo-u S, Nakagawa K, Sugihara M, Komaki T. Properties of chitosanase from *Bacillus cereus* S1. Curr Microbiol 2000; 40: 6-9.

[59] Jo YY, Jo KJ, Jin YL, *et al.* Characterization and kinetics of 45 kDa chitosanase from *Bacillus* sp. P16. Biosci Biotechnol Biochem 2003; 67: 1875-82.

[60] Yamasaki Y, Fukumoto I, Kumagai N, *et al.* Continuous chitosan hydrolyzate production by immobilized chitosanolytic enzyme from Enterobacter sp. G-1. Biosci Biotechnol Biochem 1992; 56: 1546-51.

[61] Osswald WF, Shapiro JP, Doostdar H, *et al.* Identification and characterization of acidic hydrolases with chitinase and chitosanase activities from sweet orange callus tissue. Plant Cell Physiol 1994; 35: 811-20.

[62] Sun L, Adams B, Gurnon JR, Ye Y, Van Etten JL. Characterization of two chitinase genes and one chitosanase gene encoded by *Chlorella* virus PBCV-1. Virology 1999; 263: 376-87.

[63] Cheng CY, Chang CH, Wu YJ, Li YK. Exploration of glycosyl hydrolase family 75, a chitosanase from *Aspergillus fumigatus*. J Biol Chem 2006; 281: 3137-44.

[64] Li S, Chen L, Wang C, Xia W. Expression, purification and characterization of endo-type chitosanase of *Aspergillus* sp. CJ22-326 from *Escherichia coli*. Carbohydr Res 2008; 343: 3001-4.

[65] Zhang XY, Dai AL, Kuroiwa K, *et al.* Cloning and characterization of a chitosanase gene from the koji mold *Aspergillus oryzae* strain IAM 2660. Biosci Biotechnol Biochem 2001; 65: 977-81.

[66] Rodriguez-Martin A, Acosta R, Liddell S, *et al.* Characterization of the novel antifungal chitosanase PgChP and the encoding gene from *Penicillium chrysogenum*. Appl Microbiol Biotechnol 2010; 88: 519-28.

[67] Kim HJ, Chen F, Wang X, Rajapakse NC. Effect of chitosan on the biological properties of sweet basil (Ocimum basilicum L.). J Agric Food Chem 2005; 53: 3696-701.

[68] Fukamizo T, Ohkawa T, Ikeda Y, Goto S. Specificity of chitosanase from *Bacillus pumilus*. Biochim Biophys Acta 1994; 1205: 183-8.

[69] Fukamizo T, Brzezinski R. Chitosanase from *Streptomyces* sp. strain N174: a comparative review of its structure and function. Biochem Cell Biol 1997; 75: 687-96.

[70] Gruber S, Seidl-Seiboth V. Self versus non-self: fungal cell wall degradation in *Trichoderma*. Microbiology 2012; 158: 26-34.

[71] Selvaggini S, Munro CA, Paschoud S, Sanglard D, Gow NAR. Independent regulation of chitin synthase and chitinase activity in *Candida albicans* and *Saccharomyces cerevisiae*. Microbiology 2004; 150: 921-8.

[72] Mach RL, Peterbauer CK, Payer K, *et al.* Expression of two major chitinase genes of *Trichoderma atroviride* (*T. harzianum* P1) is triggered by different regulatory signals. Appl Environ Microbiol 1999; 65: 1858-63.

[73] Carsolio C, Gutierrez A, Jimenez B, Van Montagu M, Herrera-Estrella A. Characterization of *ech-42*, a *Trichoderma harzianum* endochitinase gene expressed during mycoparasitism. Proc Natl Acad Sci USA 1994; 91: 10903-7.

[74] Zeilinger S, Galhaup C, Payer K, *et al.* Chitinase gene expression during mycoparasitic interaction of *Trichoderma harzianum* with its host. Fungal Genet Biol 1999; 26: 131-40.

[75] Garcia I, Lora JM, de la Cruz J, *et al.* Cloning and characterization of a chitinase (*chit42*) cDNA from the mycoparasitic fungus *Trichoderma harzianum*. Curr Genet 1994; 27: 83-9.

[76] Guigon-Lopez C, Vargas-Albores F, Guerrero-Prieto V, Ruocco M, Lorito M. Changes in *Trichoderma asperellum* enzyme expression during parasitism of the cotton root rot pathogen *Phymatotrichopsis omnivora*. Fungal Biol 2015; 119: 264-73.

[77] Shin KS, Kwon NJ, Kim YH, *et al.* Differential roles of the ChiB chitinase in autolysis and cell death of *Aspergillus nidulans*. Eukaryotic cell 2009.

[78] Yamazaki H, Yamazaki D, Takaya N, *et al.* A chitinase gene, *chiB*, involved in the autolytic process of *Aspergillus nidulans*. Curr Genet 2007; 51: 89-98.

[79] Jaques AK, Fukamizo T, Hall D, *et al.* Disruption of the gene encoding the ChiB1 chitinase of *Aspergillus fumigatus* and characterization of a recombinant gene product. Microbiology 2003; 149: 2931-9.

[80] Gruber S, Vaaje-Kolstad G, Matarese F, *et al.* Analysis of subgroup C of fungal chitinases containing chitin-binding and LysM modules in the mycoparasite *Trichoderma atroviride*. Glycobiology 2011a; 21: 122-33.

[81] Gruber S, Kubicek CP, Seidl-Seiboth V. Differential regulation of orthologous chitinase genes in mycoparasitic *Trichoderma* species. Appl Environ Microbiol 2011b; 77: 7217-26.

[82] Tzelepis GD, Melin P, Stenlid J, Jensen DF, Karlsson M. Functional analysis of the C-II subgroup killer toxin-like chitinases in the filamentous ascomycete *Aspergillus nidulans*. Fungal Genet Biol 2014; 64: 58-66.

[83] Karlsson M, Stenlid J. Comparative Evolutionary Histories of the Fungal Chitinase Gene Family Reveal Non-Random Size Expansions and Contractions due to Adaptive Natural Selection. Evol Bioinform Online 2008; 4: 47-60.

[84] Choquer M, Becker HF, Vidal-Cros A. Identification of two group A chitinase genes in *Botrytis cinerea* which are differentially induced by exogenous chitin. Mycol Res 2007; 111: 615-25.

[85] Adams DJ. Fungal cell wall chitinases and glucanases. Microbiology 2004; 150: 2029-35.

[86] Takaya N, Yamazaki D, Horiuchi H, Ohta A, Takagi M. Cloning and characterization of a chitinase-encoding gene (*chiA*) from *Aspergillus nidulans*, disruption of which decreases germination frequency and hyphal growth. Biosci Biotechnol Biochem 1998; 62: 60-5.

[87] Maddi A, Bowman SM, Free SJ. Trifluoromethanesulfonic acid-based proteomic analysis of cell wall and secreted proteins of the ascomycetous fungi *Neurospora crassa* and *Candida albicans*. Fungal Genet Biol 2009; 46: 768-81.

[88] Yamazaki H, Tanaka A, Kaneko J, Ohta A, Horiuchi H. *Aspergillus nidulans* ChiA is a glycosylphosphatidylinositol (GPI)-anchored chitinase specifically localized at polarized growth sites. Fungal Genet Biol 2008; 45: 963-72.

[89] Horsch M, Mayer C, Sennhauser U, Rast DM. β-*N*-acetylhexosaminidase: a target for the design of antifungal agents. Pharmacol Ther 1997; 76: 187-218.

[90] Tzelepis GD, Melin P, Jensen DF, Stenlid J, Karlsson M. Functional analysis of glycoside hydrolase family 18 and 20 genes in *Neurospora crassa*. Fungal Genet Biol 2012; 49: 717-30.

[91] Pusztahelyi T, Molnár Z, Emri T, *et al.* Comparative studies of differential expression of chitinolytic enzymes encoded by *chiA*, *chiB*, *chiC* and *nagA* genes in *Aspergillus nidulans*. Folia Microbiol (Praha) 2006; 51: 547-54.

[92] Cannon RD, Niimi K, Jenkinson HF, Shepherd MG. Molecular cloning and expression of the *Candida albicans* beta-*N*-acetylglucosaminidase (*HEX1*) gene. J Bacteriol 1994; 176: 2640-7.

[93] Lopez-Mondejar R, Catalano V, Kubicek CP, Seidl V. The beta-*N*-acetylglucosaminidases NAG1 and NAG2 are essential for growth of *Trichoderma atroviride* on chitin. FEBS J 2009; 276: 5137-48.

[94] Peterbauer CK, Lorito M, Hayes CK, Harman GE, Kubicek CP. Molecular cloning and expression of the *nag1* gene (*N*-acetyl-β-D- glucosaminidase-encoding gene) from *Trichoderma harzianum* P1. Curr Genet 1996; 30: 325-31.

[95] Konopka JB. *N*-acetylglucosamine (GlcNAc) functions in cell signaling. Scientifica (Cairo) 2012.

[96] Eijsink VG, Vaaje-Kolstad G, Varum KM, Horn SJ. Towards new enzymes for biofuels: lessons from chitinase research. Trends Biotechnol 2008; 26: 228-35.

[97] Guillen D, Sanchez S, Rodriguez-Sanoja R. Carbohydrate-binding domains: multiplicity of biological roles. Appl Microbiol Biotechnol 2010; 85: 1241-9.

[98] Limon MC, Margolles-Clark E, Benitez T, Penttilä M. Addition of substrate-binding domains increases substrate-binding capacity and specific activity of a chitinase from *Trichoderma harzianum*. FEMS Microbiol Lett 2001; 198: 57-63.

[99] Akcapinar GB, Kappel L, Sezerman OU, Seidl-Seiboth V. Molecular diversity of LysM carbohydrate-binding motifs in fungi. Curr Genet 2015; 61: 103-13.

[100] Bolton MD, van Esse HP, Vossen JH, *et al.* The novel *Cladosporium fulvum* lysin motif effector Ecp6 is a virulence factor with orthologues in other fungal species. Mol Microbiol 2008; 69: 119-36.

[101] de Jonge R, van Esse HP, Kombrink A, *et al.* Conserved fungal LysM effector Ecp6 prevents chitin-triggered immunity in plants. Science 2010; 329: 953-5.

[102] Marshall R, Kombrink A, Motteram J, *et al.* Analysis of two in planta expressed LysM effector homologs from the fungus *Mycosphaerella graminicola* reveals novel functional properties and varying contributions to virulence on wheat. Plant Physiol 2011; 156: 756-69.

[103] Mentlak TA, Kombrink A, Shinya T, *et al.* Effector-mediated suppression of chitin-triggered immunity by *Magnaporthe oryzae* is necessary for rice blast disease. Plant Cell 2012; 24: 322-35.

[104] Seidl-Seiboth V, Zach S, Frischmann A, *et al.* Spore germination of *Trichoderma atroviride* is inhibited by its LysM protein TAL6. FEBS J 2013: 1226-36.

[105] Baccelli I, Comparini C, Bettini PP, *et al.* The expression of the cerato-platanin gene is related to hyphal growth and chlamydospores formation in *Ceratocystis platani*. FEMS Microbiol Lett 2012; 327: 155-63.

[106] de Oliveira AL, Gallo M, Pazzagli L, *et al.* The structure of the elicitor Cerato-platanin (CP), the first member of the CP fungal protein family, reveals a double psibeta-barrel fold and carbohydrate binding. J Biol Chem 2011; 286: 17560-8.

[107] Frischmann A, Neudl S, Gaderer R, *et al.* Self-assembly at air/water interfaces and carbohydrate binding properties of the small secreted protein EPL1 from the fungus *Trichoderma atroviride*. J Biol Chem 2013; 288: 4278-87.

[108] Gaderer R, Bonazza K, Seidl-Seiboth V. Cerato-platanins: a fungal protein family with intriguing properties and application potential. Appl Microbiol Biotechnol 2014; 98: 4795-803.

[109] Pazzagli L, Seidl-Seiboth V, Barsottini M, *et al.* Cerato-platanins: elicitors and effectors. Plant Sci 2014; 228: 79-87.

[110] de O. Barsottini MR, de Oliveira JF, Adamoski D, *et al.* Functional diversification of cerato-platanins in *Moniliophthora perniciosa* as seen by differential expression and protein function specialization. Mol Plant Microbe Interact 2013; 26: 1281-93.

[111] Baccelli I, Luti S, Bernardi R, Scala A, Pazzagli L. Cerato-platanin shows expansin-like activity on cellulosic materials. Appl Microbiol Biotechnol 2014; 98: 175-84.

[112] Gaderer R, Lamdan NL, Frischmann A, *et al.* Sm2, a paralog of the *Trichoderma* cerato-platanin elicitor Sm1, is also highly important for plant protection conferred by the fungal-root interaction of *Trichoderma* with maize. BMC Microbiol 2015; 15: 2.

[113] Chen JK, Shen CR, Liu CL. *N*-acetylglucosamine: production and applications. Mar Drugs 2010; 8: 2493-516.

[114] Vaaje-Kolstad G, Westereng B, Horn SJ, *et al.* An oxidative enzyme boosting the enzymatic conversion of recalcitrant polysaccharides. Science 2010; 330: 219-22.

[115] Quinlan RJ, Sweeney MD, Lo Leggio L, *et al.* Insights into the oxidative degradation of cellulose by a copper metalloenzyme that exploits biomass components. Proc Natl Acad Sci USA 2011; 108: 15079-84.

[116] Forsberg Z, Vaaje-Kolstad G, Westereng B, *et al.* Cleavage of cellulose by a CBM33 protein. Protein Sci 2011; 20: 1479-83.

[117] Aachmann FL, Sorlie M, Skjak-Braek G, Eijsink VG, Vaaje-Kolstad G. NMR structure of a lytic polysaccharide monooxygenase provides insight into copper binding, protein dynamics, and substrate interactions. Proc Natl Acad Sci USA 2012; 109: 18779-84.

[118] Beeson WT, Phillips CM, Cate JH, Marletta MA. Oxidative cleavage of cellulose by fungal copper-dependent polysaccharide monooxygenases. J Am Chem Soc 2012; 134: 890-2.

[119] Hemsworth GR, Davies GJ, Walton PH. Recent insights into copper-containing lytic polysaccharide mono-oxygenases. Curr Opin Struct Biol 2013; 23: 660-8.

[120] Hemsworth GR, Henrissat B, Davies GJ, Walton PH. Discovery and characterization of a new family of lytic polysaccharide monooxygenases. Nat Chem Biol 2014; 10: 122-6.

[121] Roux D, Cywes-Bentley C, Zhang YF, *et al.* Identification of Poly-*N*-acetylglucosamine as a Major Polysaccharide Component of the *Bacillus subtilis* Biofilm Matrix. J Biol Chem 2015; 290: 19261-72.

[122] Itoh Y, Rice JD, Goller C, *et al.* Roles of pgaABCD genes in synthesis, modification, and export of the *Escherichia coli* biofilm adhesin poly-beta-1,6-*N*-acetyl-D-glucosamine. J Bacteriol 2008; 190: 3670-80.

[123] Simonetti N, Strippoli V, Cassone A. Yeast-mycelial conversion induced by *N*-acetyl-D-glucosamine in *Candida albicans*. Nature 1974; 250: 344-6.

[124] Perez-Campo FM, Dominguez A. Factors affecting the morphogenetic switch in *Yarrowia lipolytica*. Curr Microbiol 2001; 43: 429-33.

[125] Barnhart MM, Chapman MR. Curli biogenesis and function. Annu Rev Microbiol 2006; 60: 131-47.

[126] Ashry ESHE, Aly MRE. Synthesis and Biological Relevance of *N*-Acetylglucosamine-containing Oligosaccharides. Pure Appl Chem 2007; 12: 2229-42.

[127] Miller JB, Bull S, Miller J, McVeagh P. The oligosaccharide composition of human milk: temporal and individual variations in monosaccharide components. J Pediatr Gastroenterol Nutr 1994; 19: 371-6.

[128] Kobata A, Ginsburg V, Tsuda M. Oligosaccharides of human milk. I. Isolation and characterization. Arch Biochem Biophys 1969; 130: 509-13.

[129] Naseem S, Gunasekera A, Araya E, Konopka JB. *N*-acetylglucosamine (GlcNAc) induction of hyphal morphogenesis and transcriptional responses in *Candida albicans* are not dependent on its metabolism. J Biol Chem 2011; 286: 28671-80.

[130] Naderer T, Heng J, McConville MJ. Evidence that intracellular stages of Leishmania major utilize amino sugars as a major carbon source. PLoS Pathog 2010; 6: e1001245.

[131] Peri KG, Goldie H, Waygood EB. Cloning and characterization of the *N*-acetylglucosamine operon of *Escherichia coli*. Biochem Cell Biol 1990; 68: 123-37.

[132] Plumbridge JA. Repression and induction of the *nag* regulon of *Escherichia coli* K-12: the roles of *nagC* and *nagA* in maintenance of the uninduced state. Mol Microbiol 1991; 5: 2053-62.

[133] Naseem S, Araya E, Konopka JB. Hyphal growth in *Candida albicans* does not require induction of hyphal-specific gene expression. Mol Biol Cell 2015; 26: 1174-87.

[134] Wendland J, Schaub Y, Walther A. *N*-acetylglucosamine utilization by *Saccharomyces cerevisiae* based on expression of *Candida albicans* NAG genes. Appl Environ Microbiol 2009; 75: 5840-5.

[135] Ishijima SA, Hayama K, Takahashi M, *et al. N*-acetylglucosamine increases symptoms and fungal burden in a murine model of oral candidiasis. Med Mycol 2012; 50: 252-8.

[136] Rao KH, Ghosh S, Natarajan K, Datta A. *N*-Acetylglucosamine Kinase, *HXK1* Is Involved in Morphogenetic Transition and Metabolic Gene Expression in *Candida albicans*. PLoS One 2013; 8: e53638.

[137] Reedy JL, Floyd AM, Heitman J. Mechanistic plasticity of sexual reproduction and meiosis in the *Candida* pathogenic species complex. Curr Biol 2009; 19: 891-9.

[138] Gilmore SA, Naseem S, Konopka JB, Sil A. *N*-acetylglucosamine (GlcNAc) triggers a rapid, temperature-responsive morphogenetic program in thermally dimorphic fungi. PLoS Genet 2013; 9: e1003799.

[139] Alvarez FJ, Konopka JB. Identification of an *N*-acetylglucosamine transporter that mediates hyphal induction in *Candida albicans*. Mol Biol Cell 2007; 18: 965-75.

[140] Kumar MJ, Jamaluddin MS, Natarajan K, Kaur D, Datta A. The inducible *N*-acetylglucosamine catabolic pathway gene cluster in *Candida albicans*: discrete *N*-acetylglucosamine-inducible factors interact at the promoter of *NAG1*. Proc Natl Acad Sci U S A 2000; 97: 14218-23.

[141] Kappel L, Gaderer R, Flipphi M, V. S-S. The *N*-acetylglucosamine catabolic gene cluster in *Trichoderma reesei* is controlled by the Ndt80-like transcription factor RON1. Molecular Microbiology 2015: in press.

[142] Cheng Q, Li H, Merdek K, Park JT. Molecular characterization of the beta-*N*-acetylglucosaminidase of *Escherichia coli* and its role in cell wall recycling. J Bacteriol 2000; 182: 4836-40.

[143] Li H, Morimoto K, Katagiri N, *et al.* A novel beta-*N*-acetylglucosaminidase of *Clostridium paraputrificum* M-21 with high activity on chitobiose. Appl Microbiol Biotechnol 2002; 60: 420-7.

[144] Mayer C, Vocadlo DJ, Mah M, *et al.* Characterization of a beta-*N*-acetylhexosaminidase and a beta-*N*-acetylglucosaminidase/beta-glucosidase from *Cellulomonas fimi*. FEBS J 2006; 273: 2929-41.

[145] Tsujibo H, Fujimoto K, Tanno H, *et al.* Gene sequence, purification and characterization of *N*-acetyl-beta-glucosaminidase from a marine bacterium, *Alteromonas* sp. strain O-7. Gene 1994; 146: 111-5.

[146] Litzinger S, Fischer S, Polzer P, *et al.* Structural and kinetic analysis of *Bacillus subtilis N*-acetylglucosaminidase reveals a unique Asp-His dyad mechanism. J Biol Chem 2010; 285: 35675-84.

[147] Macdonald SS, Blaukopf M, Withers SG. *N*-acetylglucosaminidases from CAZy family GH3 are really glycoside phosphorylases, thereby explaining their use of histidine as an acid/base catalyst in place of glutamic acid. J Biol Chem 2015; 290: 4887-95.

[148] Chu S, Herskowitz I. Gametogenesis in yeast is regulated by a transcriptional cascade dependent on Ndt80. Mol Cell 1998; 1: 685-96.

[149] Fingerman IM, Sutphen K, Montano SP, Georgiadis MM, Vershon AK. Characterization of critical interactions between Ndt80 and MSE DNA defining a novel family of Ig-fold transcription factors. Nucleic Acids Res 2004; 32: 2947-56.

[150] Lamoureux JS, Glover JN. Principles of protein-DNA recognition revealed in the structural analysis of Ndt80-MSE DNA complexes. Structure 2006; 14: 555-65.

[151] Xu L, Ajimura M, Padmore R, Klein C, Kleckner N. *NDT80*, a meiosis-specific gene required for exit from pachytene in *Saccharomyces cerevisiae*. Mol Cell Biol 1995; 15: 6572-81.

[152] Chen CG, Yang YL, Tseng KY, *et al.* Rep1p negatively regulating MDR1 efflux pump involved in drug resistance in *Candida albicans*. Fungal Genet Biol 2009; 46(9): 714-20.

[153] Katz ME, Gray KA, Cheetham BF. The *Aspergillus nidulans xprG* (*phoG*) gene encodes a putative transcriptional activator involved in the response to nutrient limitation. Fungal Genet Biol 2006; 43: 190-9.

[154] Dementhon K, Iyer G, Glass NL. VIB-1 is required for expression of genes necessary for programmed cell death in *Neurospora crassa*. Eukaryot Cell 2006; 5: 2161-73.

[155] Gabriel I, Olchowy J, Stanislawska-Sachadyn A, *et al.* Phosphorylation of glucosamine-6-phosphate synthase is important but not essential for germination and mycelial growth of *Candida albicans*. FEMS Microbiol Lett 2004; 235: 73-80.

[156] Mio T, Yabe T, Arisawa M, Yamada-Okabe H. The eukaryotic UDP-*N*-acetylglucosamine pyrophosphorylases. Gene cloning, protein expression, and catalytic mechanism. J Biol Chem 1998; 273: 14392-7.

[157] Mio T, Yamada-Okabe T, Arisawa M, Yamada-Okabe H. *Saccharomyces cerevisiae GNA1*, an essential gene encoding a novel acetyltransferase involved in UDP-*N*-acetylglucosamine synthesis. J Biol Chem 1999; 274: 424-9.

[158] Milewski S, Gabriel I, Olchowy J. Enzymes of UDP-GlcNAc biosynthesis in yeast. Yeast 2006; 23: 1-14.

[159] Bartnicki-Garcia S. Cell wall chemistry, morphogenesis, and taxonomy of fungi. Ann Rev Microbiol 1968; 22: 87-108.

[160] Spiro RG. Protein glycosylation: nature, distribution, enzymatic formation, and disease implications of glycopeptide bonds. Glycobiology 2002; 12: 43-56.

[161] Ferguson MAJ, Kinoshita T, Hart GW. Glycosylphosphatidylinositol Anchors. In: Varki A, Cummings RD, Esko JD, *et al*., Eds. Essentials of Glycobiology. 2nd ed: Cold Spring Harbor Laboratory Press, Cold Spring Harbor (NY) 2009.

[162] Mio T, Kokado M, Arisawa M, Yamada-Okabe H. Reduced virulence of *Candida albicans* mutants lacking the *GNA1* gene encoding glucosamine-6-phosphate acetyltransferase. Microbiology 2000; 146 (Pt 7): 1753-8.

[163] Watzele G, Tanner W. Cloning of the glutamine:fructose-6-phosphate amidotransferase gene from yeast. Pheromonal regulation of its transcription. J Biol Chem 1989; 264: 8753-8.

[164] Boorsma A, de Nobel H, ter Riet B, *et al.* Characterization of the transcriptional response to cell wall stress in *Saccharomyces cerevisiae*. Yeast 2004; 21: 413-27.

[165] Bulik DA, Olczak M, Lucero HA, *et al.* Chitin synthesis in *Saccharomyces cerevisiae* in response to supplementation of growth medium with glucosamine and cell wall stress. Eukaryot Cell 2003; 2: 886-900.

[166] Lagorce A, Le Berre-Anton V, Aguilar-Uscanga B, *et al.* Involvement of GFA1, which encodes glutamine-fructose-6-phosphate amidotransferase, in the activation of the chitin synthesis pathway in response to cell-wall defects in *Saccharomyces cerevisiae*. Eur J Biochem 2002; 269: 1697-707.

[167] Ram AFJ, Arentshorst M, Damveld RA, *et al.* The cell wall stress response in *Aspergillus niger* involves increased expression of the glutamine: fructose-6-phosphate amidotransferase-encoding gene (*gfaA*) and increased deposition of chitin in the cell wall. Microbiology 2004; 150: 3315-26.

[168] Smith RJ, Milewski S, Brown AJ, Gooday GW. Isolation and characterization of the *GFA1* gene encoding the glutamine: fructose-6-phosphate amidotransferase of *Candida albicans*. J Bacteriol 1996; 178: 2320-7.

[169] Milewski S, Kuszczak D, Jedrzejczak R, *et al.* Oligomeric structure and regulation of *Candida albicans* glucosamine-6-phosphate synthase. J Biol Chem 1999; 274: 4000-8.

[170] Milewski S, Smith RJ, Brown AJP, Gooday GW. Purification and characterization of glucosamine-6-P synthase from *Saccharomyces cerevisiae*. In: Domard A, Jeuniaux C, Muzzarelli R, Roberts G, Eds. Advances in Chitin science. vol 1 Jacques Andre: Lyon 1996.

[171] Hofmann M, Boles E, Zimmermann FK. Characterization of the essential yeast gene encoding *N*-acetylglucosamine-phosphate mutase. Eu J Biochem 1994; 221: 741-7.

[172] Yamamoto K, Moriguchi M, Kawai H, Tochikura T. Inhibition of UDP-*N*-acetylglucosamine pyrophosphorylase by uridine. Biochim Biophys Acta 1980; 614: 367-72.

[173] Bowman SM, Free SJ. The structure and synthesis of the fungal cell wall. Bioessays 2006; 28: 799-808.

[174] Merzendorfer H. The cellular basis of chitin synthesis in fungi and insects: common principles and differences. Eur J Cell Biol 2011; 90: 759-69.

[175] Beth-Din A, Yarden O. The *Neurospora crassa chs3* gene encodes an essential class I chitin synthase. Mycologia 2000; 92: 65-73.

[176] Yarden O, Yanofsky C. Chitin synthase 1 plays a major role in cell wall biogenesis in *Neurospora crassa*. Genes Dev 1991; 5: 2420-30.

[177] Fukuda K, Yamada K, Deoka K, *et al.* Class III chitin synthase ChsB of *Aspergillus nidulans* localizes at the sites of polarized cell wall synthesis and is required for conidial development. Eukaryot Cell 2009; 8: 945-56.

[178] Aufauvre-Brown A, Mellado E, Gow NA, Holden DW. *Aspergillus fumigatus chsE*: a gene related to *CHS3* of *Saccharomyces cerevisiae* and important for hyphal growth and conidiophore development but not pathogenicity. Fungal Genet Biol 1997; 21: 141-52.

[179] Imai T, Watanabe T, Yui T, Sugiyama J. The directionality of chitin biosynthesis: a revisit. Biochem J 2003; 374: 755-60.

[180] Morgan JLW, Strumillo J, Zimmer J. Crystallographic snapshot of cellulose synthesis and membrane translocation. Nature 2013; 493: 181-6.

[181] Lenardon MD, Whitton RK, Munro CA, Marshall D, Gow NA. Individual chitin synthase enzymes synthesize microfibrils of differing structure at specific locations in the *Candida albicans* cell wall. Mol Microbiol 2007; 66: 1164-73.

[182] Chiou CC, Groll AH, Walsh TJ. New drugs and novel targets for treatment of invasive fungal infections in patients with cancer. Oncologist 2000; 5: 120-35.

[183] Valdivieso MH, Ferrario L, Vai M, Duran A, Popolo L. Chitin synthesis in a *gas1* mutant of *Saccharomyces cerevisiae*. J Bacteriol 2000; 182: 4752-7.

[184] Appeltauer U, Achstetter T. Hormone-induced expression of the *CHS1* gene from *Saccharomyces cerevisiae*. Eur J Biochem 1989; 181: 243-7.

[185] Fajardo-Somera RA, Johnk B, Bayram O, *et al.* Dissecting the function of the different chitin synthases in vegetative growth and sexual development in *Neurospora crassa*. Fungal Genet Biol 2015; 75: 30-45.

[186] Chen-Wu JL, Zwicker J, Bowen AR, Robbins PW. Expression of chitin synthase genes during yeast and hyphal growth phases of *Candida albicans*. Mol Microbiol 1992; 6: 497-502.

[187] Munro CA, Schofield DA, Gooday GW, Gow NA. Regulation of chitin synthesis during dimorphic growth of *Candida albicans*. Microbiology 1998; 144 (Pt 2): 391-401.

[188] Chuang JS, Schekman RW. Differential trafficking and timed localization of two chitin synthase proteins, Chs2p and Chs3p. J Cell Biol 1996; 135: 597-610.

[189] Choi WJ, Cabib E. The use of divalent cations and pH for the determination of specific yeast chitin synthetases. Anal Biochem 1994; 219: 368-72.

[190] DeMarini DJ, Adams AE, Fares H, *et al.* A septin-based hierarchy of proteins required for localized deposition of chitin in the *Saccharomyces cerevisiae* cell wall. J Cell Biol 1997; 139: 75-93.

[191] Ono N, Yabe T, Sudoh M, *et al.* The yeast Chs4 protein stimulates the trypsin-sensitive activity of chitin synthase 3 through an apparent protein-protein interaction. Microbiology 2000; 146 (Pt 2): 385-91.

[192] Sudoh M, Tatsuno K, Ono N, *et al.* The *Candida albicans CHS4* gene complements a *Saccharomyces cerevisiae* skt5/chs4 mutation and is involved in chitin biosynthesis. Microbiology 1999; 145 (Pt 7): 1613-22.

[193] Bajaj M, Freiberg A, Winter J, Xu Y, Gallert C. Pilot-scale chitin extraction from shrimp shell waste by deproteination and decalcification with bacterial enrichment cultures. Appl Microbiol Biotechnol 2015; 99: 9835-46.

[194] Barghini P, Moscatelli D, Garzillo AM, Crognale S, Fenice M. High production of cold-tolerant chitinases on shrimp wastes in bench-top bioreactor by the Antarctic fungus *Lecanicillium muscarium* CCFEE 5003: bioprocess optimization and characterization of two main enzymes. Enzyme Microb Technol 2013; 53: 331-8.

[195] Chern LL, Stackebrandt E, Lee SF, *et al. Chitinibacter tainanensis* gen. nov., sp. nov., a chitin-degrading aerobe from soil in Taiwan. Int J Syst Evol Microbiol 2004; 54: 1387-91.

[196] Chen JKb, Shen CR, Yeh CH, *et al. N*-acetyl glucosamine obtained from chitin by chitin degrading factors in *Chitinbacter tainanesis*. Int J Mol Sci 2011; 12: 1187-95.

[197] Barraza-Guardado RH, Arreola-Lizárraga JA, López-Torres MA, *et al.* Effluents of Shrimp Farms and Its Influence on the Coastal Ecosystems of Bahía de Kino, Mexico. ScientificWorldJournal 2013: 8.

[198] Babu CM, Chakrabarti R, Surya Sambasivarao KR. Enzymatic isolation of carotenoid-protein complex from shrimp

head waste and its use as a source of carotenoids. Lebenson Wiss Technol 2008; 41: 227-35.

[199] Zanolli L. Insect Farming Is Taking Shape as Demand for Animal Feed Rises. MIT Technology Review; 2014. Available from: http://www.technologyreview.com/news/529756/insect-farming-is-taking-shape-as-demand-for-animal-feed-rises/ [cited: 16th Oct 2015].

[200] Sajomsang W, Gonil P. Preparation and characterization of α-chitin from cicada sloughs. Mater Sci Eng 2010; 30: 357-63.

[201] Liu T, Liu Z, Song C, *et al.* Chitin-induced dimerization activates a plant immune receptor. Science 2012; 336: 1160-4.

[202] Zhang Y, Geng W, Shen Y, Wang Y, Dai Y-C. Edible Mushroom Cultivation for Food Security and Rural Development in China: Bio-Innovation, Technological Dissemination and Marketing. Sustainability 2014; 6: 2961.

[203] Wu T. Production and Characterization of Fungal Chitin and Chitosan. Master Thesis: University of Tennessee, Knoxville; 2004.

[204] Wu T, Zivanovic S, Draughon FA, Sams CE. Chitin and chitosan-value-added products from mushroom waste. J Agric Food Chem 2004; 52: 7905-10.

[205] Bartnicki-Garcia S, Nickerson WJ. Isolation, composition, and structure of cell walls of filamentous and yeast-like forms of *Mucor rouxii*. Biochim Biophys Acta 1962; 58: 102-19.

[206] Bhattarai N, Gunn J, Zhang M. Chitosan-based hydrogels for controlled, localized drug delivery. Adv Drug Deliv Rev 2010; 62: 83-99.

[207] Crompton KE, Goud JD, Bellamkonda RV, *et al.* Polylysine-functionalised thermoresponsive chitosan hydrogel for neural tissue engineering. Biomaterials 2007; 28: 441-9.

[208] Noel SP, Courtney HS, Bumgardner JD, Haggard WO. Chitosan sponges to locally deliver amikacin and vancomycin: a pilot *in vitro* evaluation. Clin Orthop Relat Res 2010; 468: 2074-80.

[209] Seol YJ, Lee JY, Park YJ, *et al.* Chitosan sponges as tissue engineering scaffolds for bone formation. Biotechnol Lett 2004; 26: 1037-41.

[210] Khong TT, Aarstad OA, Skjak-Braek G, Draget KI, Varum KM. Gelling concept combining chitosan and alginate proof of principle. Biomacromolecules 2013; 14: 2765-71.

[211] Baysal K, Aroguz AZ, Adiguzel Z, Baysal BM. Chitosan/alginate crosslinked hydrogels: preparation, characterization and application for cell growth purposes. Int J Biol Macromol 2013; 59: 342-8.

[212] Zhao P, Deng C, Xu H, *et al.* Fabrication of photo-crosslinked chitosan- gelatin scaffold in sodium alginate hydrogel for chondrocyte culture. Biomed Mater Eng 2014; 24: 633-41.

[213] Jayakumar R, Nair SV, Furuike T, Tamura H. Perspectives of Chitin and Chitosan Nanofibrous Scaffolds in Tissue Engineering. In : Daniel Eberli, Ed. Tissue Engineering 2010.

[214] Hamer SN, Cord-Landwehr S, Biarnés X, *et al.* Enzymatic production of defined chitosan oligomers with a specific pattern of acetylation using a combination of chitin oligosaccharide deacetylases. Sci Rep 2015; 5: 8716.

[215] Karzel K, Domenjoz R. Effects of hexosamine derivatives and uronic acid derivatives on glycosaminoglycane metabolism of fibroblast cultures. Pharmacology 1971; 5: 337-45.

[216] Burton AF, Freeman HJ. *N*-acetyl glucosamine as gastroprotective agent. WO 1993023055 A1. Google Patents; 1993.

[217] Mönnikes H, Schmidtmann M, van der Voort IR. [Drug therapy for irritable bowel syndrome. What works, what doesn't work and for whom? Internist (Berl) 2006; 47: 1073-6, 8-83.

[219] Burton AF. *N*-acetyl glucosamine as a cytoprotective agent. EP 0372730 A2. Google Patents; 1990.

[220] Sayo T, Sakai S, Inoue S. Synergistic effect of *N*-acetylglucosamine and retinoids on hyaluronan production in human keratinocytes. Skin Pharmacol Physiol 2004; 17: 77-83.

[221] Chen RH, Hsu CN, Chung MY, Tsai WL, Liu CH. Effect of Different Concentrations of Collagen, Ceramides, *N*-Acetyl Glucosamine, or their Mixture on Enhancing the Proliferation of Keratinocytes, Fibroblasts and the Secretion of Collagen and/or the Expression of mRNA of Type I Collagen. J Food Drug Anal 2008; 16: 66-74.

[222] Vimr E, Lichtensteiger C. To sialylate, or not to sialylate: that is the question. Trends Microbiol 2002; 10: 254-7.

[223] Maru I, Ohnishi J, Ohta Y, Tsukada Y. Simple and large-scale production of *N*-acetylneuraminic acid from *N*-acetyl-D-glucosamine and pyruvate using *N*-acyl-D-glucosamine 2-epimerase and *N*-acetylneuraminate lyase. Carbohydr Res 1998; 306: 575-8.

Chapter 1: GlcNAc-catabolism in filamentous fungi

Chapter 1.2

The *N*-acetylglucosamine catabolic gene cluster in *Trichoderma reesei* is controlled by the Ndt80-like transcription factor RON1.

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The *N*-acetylglucosamine catabolic gene cluster in *Trichoderma reesei* is controlled by the Ndt80-like transcription factor RON1

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Summary

Chitin is an important structural constituent of fungal cell walls composed of *N*-acetylglucosamine (GlcNAc) monosaccharides, but catabolism of GlcNAc has not been studied in filamentous fungi so far. In the yeast *Candida albicans*, the genes encoding the three enzymes responsible for stepwise conversion of GlcNAc to fructose-6-phosphate are clustered. In this work, we analysed GlcNAc catabolism in ascomycete filamentous fungi and found that the respective genes are also clustered in these fungi. In contrast to *C. albicans*, the cluster often contains a gene for an Ndt80-like transcription factor, which we named RON1 (regulator of *N*-acetylglucosamine catabolism 1). Further, a gene for a glycoside hydrolase 3 protein related to bacterial *N*-acetylglucosaminidases can be found in the GlcNAc gene cluster in filamentous fungi. Functional analysis in *Trichoderma reesei* showed that the transcription factor RON1 is a key activator of the GlcNAc gene cluster and essential for GlcNAc catabolism. Furthermore, we present an evolutionary analysis of Ndt80-like proteins in Ascomycota. All GlcNAc cluster genes, as well as the GlcNAc transporter gene *ngt1*, and an additional transcriptional regulator gene, *csp2*, encoding the homolog of *Neurospora crassa* CSP2/GRHL, were functionally characterised by gene expression analysis and phenotypic characterisation of knockout strains in *T. reesei*.

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Introduction

Chitin is a linear polysaccharide composed of β-(1,4) linked *N*-acetylglucosamine units (GlcNAc; 2-acetamido-2-deoxy-D-glucopyranose). In filamentous fungi, chitin is located in the inner layers of the cell wall, close to the plasma membrane (Ruiz-Herrera, 1991) and forms, together with β-(1,3–1,6) glucan, the structural scaffold of the fungal cell wall (Latgé, 2007). In the biosphere, chitin is not only found in fungal cell walls, but also in the exoskeletons of protists and arthropods, e.g. insects and shrimps. Chitin is the second most abundant biopolymer after cellulose with a natural turnover of at least 10^9 tons per year (Muzzarelli, 1999). Nonetheless, chitin does not visibly accumulate in the biosphere, which is indicative for its efficient natural recycling by microbes. Analysis of fungal genomes revealed that filamentous fungi feature a large repertoire of extracellular chitinolytic enzymes. They have typically between 10 and 35 different chitinases belonging to glycoside hydrolase (GH) family 18 and two extracellular GH 20 β-*N*-acetylglucosaminidases (chitobiases) that contribute to the degradation of chitin to GlcNAc monomers (Seidl, 2008). The genomes of fungi that parasitise other fungi (mycoparasites) or insects (entomopathogens), e.g. *Trichoderma* species and several entomopathogens, including *Metarhizium anisopliae*, *Beauveria bassiana* and *Cordyceps militaris*, are particularly enriched in chitinase genes (Seidl *et al*., 2005; Gao *et al*., 2011; Kubicek *et al*., 2011; Zheng *et al*., 2011; Xiao *et al*., 2012; Agrawal *et al*., 2015). Chitinases are not only involved in the breakdown of extracellular chitin, but also in self-digestive processes such as cell wall remodeling during fungal growth and asexual development, as well as in cell wall degradation during autolysis and apoptosis. The roles of chitinases in these processes, and their gene expression, have been studied in several fungal species, e.g. *Aspergillus nidulans* (Pusztahelyi *et al*., 2006; Emri *et al*., 2008; Pócsi *et al*., 2009; Shin *et al*., 2009), *Neurospora crassa* (Tzelepis *et al*., 2012), *Trichoderma atroviride* and *T. virens* (Gruber and Seidl-Seiboth, 2012), and *Penicillium chrysogenum* (Sámi *et al*., 2001; Kamerewerd *et al*., 2011; Pusztahelyi and Pócsi, 2014). The data suggest that several chitinases

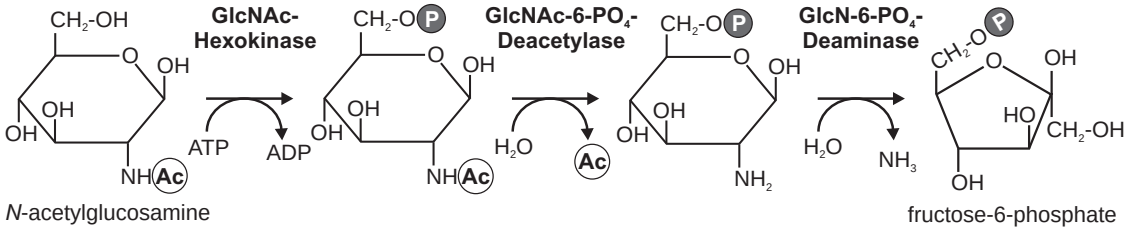


Fig. 1. Schematic representation of the GlcNAc catabolism pathway. GlcNAc is phosphorylated by GlcNAc hexokinase, then GlcNAc-6-phosphate is deacetylated by GlcNAc-6-phosphate deacetylase, and subsequently, GlcN-6-phosphate deaminase converts GlcN-6-phosphate into fructose-6-phosphate. Acetate and ammonium are the other end products of GlcNAc catabolism.

have dual roles and are involved in the degradation of extracellular chitin as well as remodeling and recycling of cell wall chitin during different growth stages (Gruber and Seidl-Seiboth, 2012). From this broad spectrum of different chitinases, the pathway of chitin degradation narrows down to one or two *N*-acetylglucosaminidases (Seidl, 2008). In *T. atroviride*, it was shown that the presence of either one of these two *N*-acetylglucosaminidases is essential for extracellular conversion of chitobiose to GlcNAc and for growth on chitin (López-Mondéjar *et al*., 2009). Uptake and intracellular catabolism of GlcNAc have scarcely been studied in fungi to date, with the notable exception of the human pathogenic diploid yeast *Candida albicans* (Kumar *et al*., 2000; Alvarez and Konopka, 2007; Biswas *et al*., 2007; Naseem *et al*., 2011; 2015; Konopka, 2012; Rao *et al*., 2013). *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* lack the genes needed to catabolise GlcNAc.

In *C. albicans*, GlcNAc is a potent inducer of hyphal growth related to the virulence of this dimorphic human pathogen (Naseem *et al*., 2011; Ishijima *et al*., 2012; Rao *et al*., 2013). GlcNAc stimulates the switch from budding to hyphal growth and induces the expression of virulence genes, but it also induces the expression of the genes needed to catabolise GlcNAc. To induce signaling, GlcNAc must enter *C. albicans* cells, although stimulation of hyphal growth does not require GlcNAc catabolism (Naseem *et al*., 2011; 2015). Transport of GlcNAc in *C. albicans* is mediated by the plasma membrane transporter Ngt1, which belongs to the large Major Facilitator Superfamily (Alvarez and Konopka, 2007). The three genes encoding the enzymes necessary for GlcNAc catabolism are organised in a gene cluster that consists of adjacent genes encoding a sugar kinase (Nag5/Hxk1) (EC 2.7.1.59) that phosphorylates GlcNAc to create GlcNAc-6-phosphate, a deacetylase (Nag2/Dac1) (EC 3.5.1.33) that splits it into acetate and glucosamine-6-phosphate, and a deaminase (Nag1) (EC 3.5.99.6) that subsequently converts glucosamine-6-phosphate to ammonium and fructose-6-phosphate, an intermediate of glycolysis (Kumar *et al*., 2000; Naseem *et al*., 2011) (Fig. 1). Deletion mutants of *HXK1*, *NAG1* and *DAC1* failed to utilise GlcNAc as carbon source, and surprisingly, GlcNAc inhibited the growth of *NAG1* and *DAC1* deletion mutants on other sugars (D-glucose, D-fructose, D-galactose), suggesting that excess GlcNAc-6-phosphate is deleterious (Naseem *et al*., 2011). However, the transcriptional regulator of the *C. albicans* GlcNAc catabolic pathway has not been identified so far.

In this study, we analysed the genomic organisation of the GlcNAc catabolic gene cluster in ascomycete filamentous fungi. We found that it often contains a gene for a transcription factor with an Ndt80-like DNA-binding domain. Expression of this transcription factor and the four structural GlcNAc catabolism genes was analysed in two species of *Trichoderma*, and the genes were functionally characterised upon generation of knockout strains in *T. reesei*.

Results

Organisation of the GlcNAc catabolic gene cluster in filamentous fungi

Fungal genomes were screened for the presence of homologs of the GlcNAc catabolic cluster genes and the GlcNAc transporter from *C. albicans*. The results showed that the respective genes are also clustered in ascomycete filamentous fungi and that a homolog of the *C. albicans* transporter gene *ngt1* can also be found in these genomes (Fig. 2A). Considering extant gene names in *Trichoderma* and other filamentous fungi (e.g. hexokinases *hxk1* and *hxk2*, β-*N*-acetylglucosaminidases *nag1* and *nag2*), the structural genes of the GlcNAc catabolic cluster were designated *hxk3* (GlcNAc-hexokinase), *dac1* (GlcNAc-6-phosphate deacetylase), *dam1* (glucosamine-6-phosphate deaminase) and *ngt1* (GlcNAc:H^+ symporter) respectively.

In filamentous fungi, besides these genes encoding the enzymes necessary for the stepwise conversion of GlcNAc into fructose-6-phosphate (see Fig. 1) and its transporter, a gene encoding a GH family 3 protein can often be found in

the cluster, which we have annotated as *nag3*. NAG3 exhibits similarity to bacterial GH3 β-*N*-acetylhexosaminidases (Tsujibo *et al*., 1994; Cheng *et al*., 2000; Li *et al*., 2002; Mayer *et al*., 2006; Litzinger *et al*., 2010) and http://www.cazy.org/GH3.html). In fungi, *N*-acetylglucosaminidases have so far only been described from GH family 20 (Seidl, 2008; López-Mondéjar *et al*., 2009). Recently, it was reported for Nag3 from *Cellulomonas fimi* that the GH3 enzyme is actually a GlcNAc-phosphorylase using phosphate rather than water as nucleophile (MacDonald *et al*., 2015).

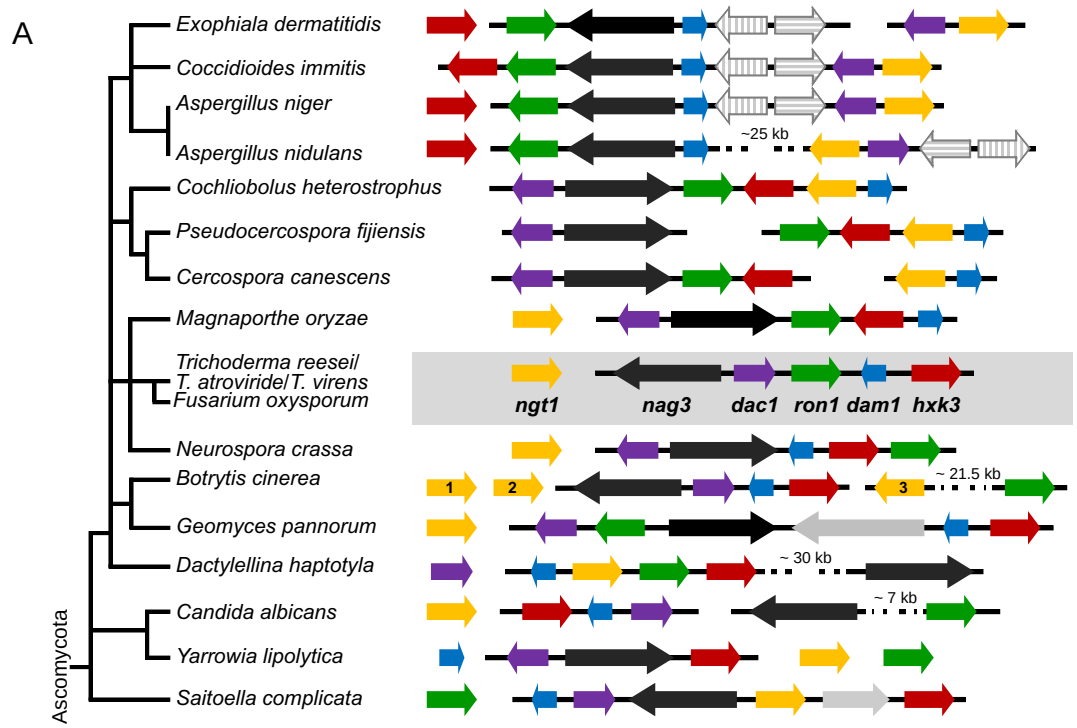
Interestingly, our analysis showed that the GlcNAc cluster in filamentous fungi in many cases also contains a gene for a transcription factor with an Ndt80-like DNA-binding domain (PFAM family PF05224). The transcription factor was designated RON1 (**r**egulator **o**f ***N***-acetylglucosamine catabolism 1). RON1 belongs to a rare family of exclusively fungal transcription factors, epitomised by *S. cerevisiae* Ndt80 (Xu *et al*., 1995; Chu and Herskowitz, 1998; Fingerman *et al*., 2004; Lamoureux and Glover, 2006). The genome of the filamentous model fungus *N. crassa* contains three genes encoding proteins with an Ndt80-like DNA-binding domain (Hutchison and Glass, 2010). While in *S. cerevisiae* Ndt80 is primarily involved in the regulation of meiosis, the Ndt80-like proteins in *N. crassa* (VIB-1, FSD-1) have other functions. Mutations in *fsd-1* affected the timing and development of female reproductive structures and ascospore maturation (Hutchison and Glass, 2010), and the *A. nidulans* homolog, *ndtA*, is apparently required for sexual reproduction (Katz *et al*., 2013). Further, VIB-1 is involved in the regulation of vegetative incompatibility and programmed cell death. In addition, VIB-1 is a major regulator of responses to nitrogen and carbon starvation, involved in protoperithecial development, and was recently reported to be essential for plant cell wall degradation by repressing glucose signalling and carbon catabolite repression (Dementhon *et al*., 2006; Hutchison and Glass, 2010; Xiong *et al*., 2014). For the third transcription factor in *N. crassa* belonging to the Ndt80-family (encoded by the gene at locus NCU04729), no phenotype or overlap in function with either *fsd-1* or *vib-1* has been reported (Hutchison and Glass, 2010). We have now found that the gene at locus NCU04729 is part of the GlcNAc catabolic cluster of *N. crassa* (Fig. 2A). In *A. nidulans*, classical loss-of-function mutations in the Ndt80 transcription factor gene *xprG*/*phoG* (initially called *pacG*) resulted in the loss of an acid phosphatase (Caddick and Arst, 1986) and in decreased extracellular protease production in response to carbon and nitrogen starvation (Katz *et al*., 2006). In *A. nidul*ans, the *xprG* gene is closely linked to three of the structural GlcNAc catabolic genes and neighbours the *nag3* ortholog (Fig. 2A), but the cluster is effectively split in two as a consequence of a chromosomal inversion.

In order to elucidate the evolutionary relationships among Ndt80-like transcription factors in filamentous fungi (Pezizomycotina), a phylogenetic analysis was performed, including some 500 proteins from over 200 species of Ascomycota. Figure 2B shows a condensed phylogenetic tree summarising our findings. A more detailed species-annotated tree of the same analysis can be found in Fig. S1. The results indicated that Ndt80-like proteins can be assigned to two groups that likely existed in a common ancestor of all Ascomycota. One superbranch contains direct orthologs of *S. cerevisiae* Ndt80, and includes members from many species belonging to the two subphyla Pezizomycotina and Saccharomycotina. A characterised filamentous fungal member of this superbranch is *N. crassa* FSD-1. *N. crassa* VIB-1 and *A. nidulans* PhoG/XprG-like proteins cluster in the other superbranch that constitutes two separate clades. One clade (termed VIB-1 clade in Fig. 2B) appeared exclusively in the classes Sordariomycetes (including *Trichoderma* spp.) and Leotiomycetes, while the other clade (XprG/RON1 clade in Fig. 2B) featured orthologs from all but one class of Pezizomycotina. Thus, this analysis shows that RON1 and XprG are orthologs that can be found throughout many different classes of Ascomycota, while VIB-1-like proteins are RON1-paralogs unique to Sordariomycetes and Leotiomycetes.

In general, these two clades (VIB-1-clade and RON1/XprG-clade) appear to have evolved in Pezizomycotina after the divergence from Saccharomycotina, including the early divergent yeasts *Yarrowia lipolytica* and *Blastobotrys adeninivorans*, both of which feature a complete set of GlcNAc-cluster genes (data not shown). Five out of the six GlcNAc cluster genes (including the designate Ndt80-family regulator) are neither present in the 14 species of Saccharomycetaceae nor in *Pichia pastoris*, which suggests that the GlcNAc cluster has been lost independently from this yeast lineage.

Expression of the GlcNAc cluster genes is upregulated during growth on GlcNAc and chitin

Gene regulation of chitinolytic enzymes and the use of chitin and *N*-acetylglucosamine as carbon sources have already received considerable attention in *Trichoderma* spp. In order to complement the currently available knowledge with data about GlcNAc catabolism, we decided to analyse the gene expression of the six GlcNAc cluster genes in *T. reesei* and *T. atroviride. T. atroviride* is a mycoparasite and has been the subject of several detailed studies on chitinase and *N*-acetylglucosaminidase gene regulation. *T. reesei* is a saprotroph and is widely used for biotechnological applications due to its remarkable cellulolytic potential. It is more amenable to molecular genetic manipulations than *T. atroviride*, and a range of different



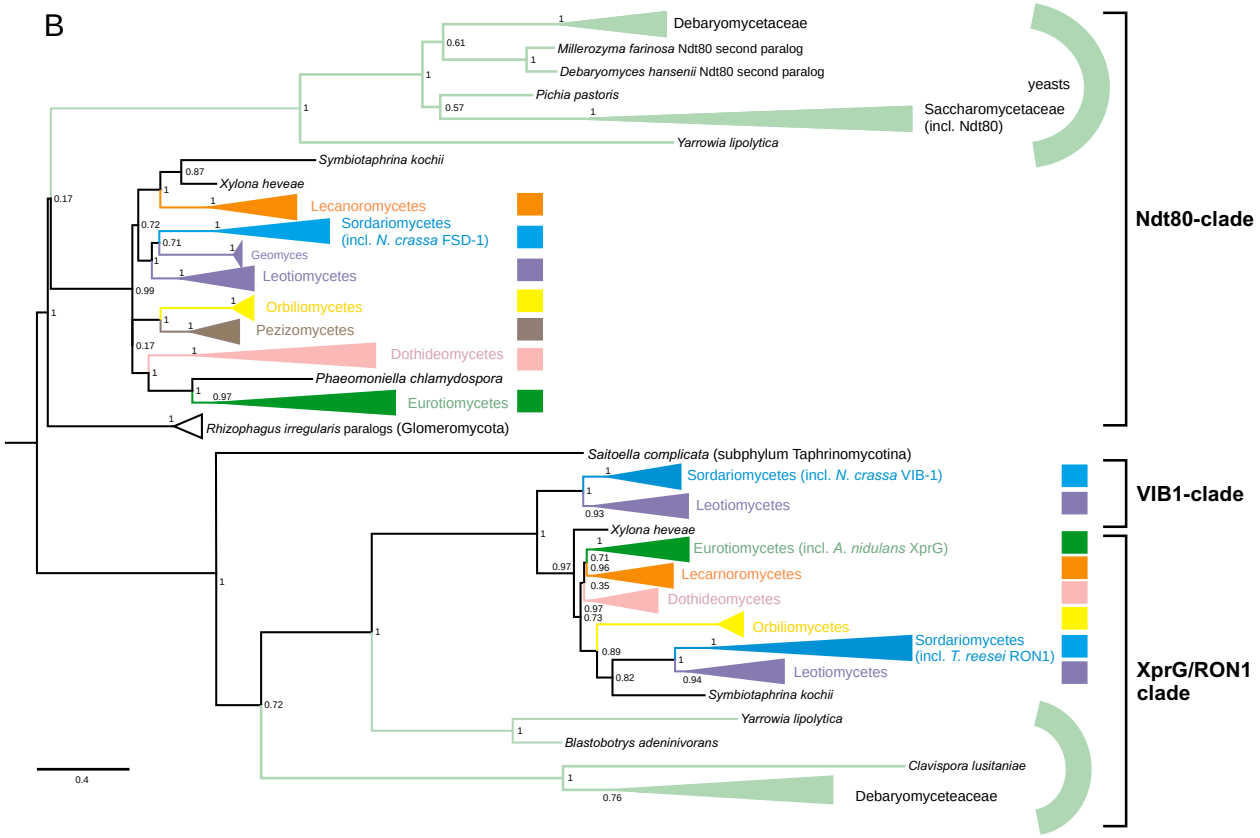


Fig. 2. (A) Genomic organisation of GlcNAc gene clusters in Ascomycota. GlcNAc cluster genes are depicted as coloured arrows to indicate their identity and orientation: *nag3*, black; *ngt1*, yellow; *hxk3*, red; *dac1*, purple; *dam1*, blue; *ron1*, green. Gene clustering is indicated by arrows superposed on an uninterrupted, thin horizontal black bar. Intergenic spaces are not drawn to scale. Unlinked genes involved in GlcNAc catabolism are always depicted with the arrowhead (3′ end) pointing to the right. Single genes unlikely to be involved in GlcNAc catabolism but nevertheless located between GlcNAc cluster genes in certain species are represented by grey arrows. Evolutionary relations among the 18 species are reflected by the schematic, flattened 'taxonomical tree' at the left. (B) Phylogenetic analysis of putative Ndt80 family proteins in Ascomycota. Data were condensed at the level of classes (Color code: Orbiliomycetes, yellow; Pezizomycetes, brown; Lecanoromycetes, orange; Leotiomycetes, purple; Sordariomycetes, blue; Eurotiomycetes, dark green; and Dothideomycetes, pink), while for Saccharomycotina, branches were collapsed at the level of families (essentially, Saccharomycetaceae – type species: *S. cerevisiae* – and Debaryomycetaceae – type species: *C. albicans*). To facilitate recognition, branches of yeast proteins are depicted in light green shades.

selection markers for gene deletion is readily available for *T. reesei* (Guangtao *et al*., 2009; Seiboth *et al*., 2011; Bischof and Seiboth, 2014). It has been established that GlcNAc is an efficiently consumable carbon source that promotes strong and fast growth of either species (Druzhinina *et al*., 2006; Seidl *et al*., 2006). Chitin, even when used in a pretreated, acid-hydrolysed form, allows only slow growth and weak biomass formation due to its recalcitrant polymer structure (López-Mondéjar *et al*., 2009; Gruber *et al*., 2011). For gene expression analysis of the GlcNAc cluster genes, biomass samples were taken from shake flask cultivations of *T. atroviride* and *T. reesei* in minimal medium containing 1% GlcNAc or glucose (control) as the carbon source at 16 h, 24 h and 38 h after inoculation. In both *T. reesei* and *T. atroviride*, *hxk3*, *dac1* and *dam1*, which encode the enzymes of GlcNAc catabolism, exhibited different basal expression levels on glucose, as well as a clear up-regulation during growth on GlcNAc for all three time points (Fig. 3A). Quantitative evaluation of the gene expression levels by qPCR in *T. reesei* (Fig. 3B) confirmed a particularly strong induction of the three genes *hxk3*, *dac1* and *dam1*, with 253, 104 and 60-fold, respectively, after 24 h on GlcNAc compared with the glucose control. In contrast, expression of *nag3*, encoding the GH 3 protein, was only increased 12-fold and that of the transcription factor *ron1*, 7-fold. Transcription of the putative GlcNAc-specific transporter-encoding gene *ngt1* was increased 40-fold after 24 h on GlcNAc, indicating involvement in GlcNAc uptake. These data show that the transcription of the genes encoding enzymatic and transport functions in the GlcNAc catabolism pathway is highly induced upon growth on this carbon source, which strongly suggests that the gene cluster is indeed involved in GlcNAc catabolism.

Next, we tested the gene expression of this cluster in *T. atroviride* and *T. reesei* during growth on chitin powder (from crab shells) and colloidal chitin, a better accessible, acid-pre-treated form of chitin. Liquid standing cultivations were used, which enable better growth of *Trichoderma* on chitin than shake flask cultivations (Gruber *et al*., 2011). Also under these growth conditions, increased expression of the GlcNAc catabolic gene cluster was detected, although it was less pronounced than on the monomer, GlcNAc (Fig. 3C). Again considerable induction of *hxk3*, *dac1* and *dam1* was observed, while *ron1* and *nag3* were less responsive. In *T. atroviride*, the expression profile of the GlcNAc cluster genes was similar on chitin and colloidal chitin at 48 h and 72 h, while *T. reesei* seemed to respond somewhat slower on untreated chitin and only at 72 h after inoculation, the highest levels of induction of the GlcNAc cluster genes were observed.

The transcription factor RON1 is essential for the induction of the GlcNAc catabolic gene cluster

Consequently, knockout strains of the GlcNAc catabolic cluster genes in *T. reesei* were generated and functionally analysed. First, single knockout strains were created for *nag3*, *hxk3*, *dac1*, *dam1*, *ron1* and *ngt1* (Table 1) in a Δ*tku70* background, which facilitates homologous integration of gene knockout cassettes due to the lesion in functional non-homologous end joining (see experimental procedures for details and Fig. S2 for genetic confirmation of the knockout strains). All strains used in this study are listed in Table 1. All subsequent analyses were performed with at least two independent knockout strains. In the figures involving gene deletion strains, the respective results from independent strains and biological replicates are included in the error bars or representative images (e.g. from plates) are presented.

All knockout strains exhibited wild-type growth rate and hyphal morphology as long as GlcNAc (or chitin) was not used as sole carbon source. Carbon sources that were tested are D-glucose, D-fructose, cellulose, chitin, glycerol and L-arabinose. Minimal medium from which a carbon source was omitted was also tested, but did not produce any marked difference in growth compared with the control strain. Figure 4A shows that growth of all deletions strains, except for Δ*ngt1* and Δ*hxk3*, was basically abolished on agar plates with minimal medium [Mandels-Andreotti (MA)] containing GlcNAc as the carbon source. Δ*hxk3* and Δ*ngt1* strains exhibited sparse outgrowth on agar plates with GlcNAc. Residual growth of Δ*ngt1* strains on agar plates could be due to expression of an alternative transporter that exhibits low affinity for GlcNAc and thereby enables some growth on the added carbon

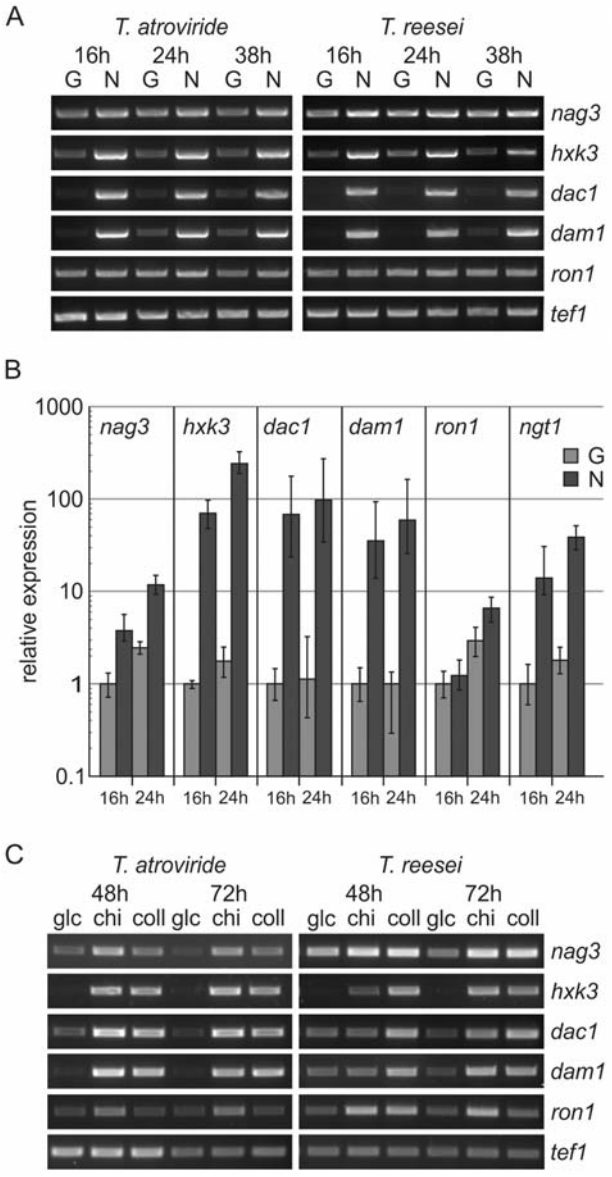


Fig. 3. Transcript levels of GlcNAc catabolic cluster genes are strongly increased during growth on GlcNAc.
A. Gene expression analysis (semi-quantitative RT-PCR) of GlcNAc cluster genes in *T. atroviride* and *T. reesei* after growth on 1% glucose (G) or 1% GlcNAc (N) for 16 h, 24 h and 38 h. As reference gene *tef1* was used.
B. Quantitative gene expression analysis (qRT-PCR) of the GlcNAc cluster genes in *T. reesei*. Samples were normalised to *tef1* expression levels and compared with the time point 'glucose, 16 h', which was set as 1 for the respective genes that were studied. The standard deviation of the mean expression values from at least two independent biological replicates is shown; *P* values are < 0.001 except for *hxk3* and *ngt1* ($P < 0.01$ for glucose, 24 h). *ron1* levels at 16 h induction with GlcNAc and *dac1* and *dam1* levels at 24 h on glucose are statistically not significantly different from glucose levels at 16 h.
C. Expression of GlcNAc cluster genes in *T. atroviride* and *T. reesei* after 48 h and 72 h of growth on the complex carbon sources chitin (1%, chi) and colloidal chitin (1%, coll) in comparison to growth on glucose (1%, glc). As reference gene, again, *tef1* was used.

source. In order to assess this possibility, agarose and Phytagel™ (a polymer gelling agent composed of glucuronic acid, rhamnose and glucose units) were also tested as solidifying reagents instead of agar. In each case, residual growth of the Δ*ngt1* strain was observed (data not shown).

Biomass formation of the knockout strains was also quantitatively determined from shake flask cultivations (Fig. 4B). Here, all knockout strains, including Δ*hxk3* and Δ*ngt1*, showed strongly reduced biomass formation on GlcNAc. Therefore, all GlcNAc catabolism cluster genes, including *nag3*, whose function in GlcNAc catabolism remains unclear, appear to be essential for growth on GlcNAc. The putative transporter NGT1 indeed seems to be of major importance for GlcNAc import into the cell. Importantly, Δ*ron1* knockout strains also could not grow on GlcNAc, suggesting that the putative transcription factor RON1 is essential for GlcNAc catabolism. In order to verify our results, complementation strains of *ron1* were generated, which contained an ectopically inserted, functional copy of *ron1* as well as the original deletion of the *ron1* locus (see experimental procedures for details and Fig. S2 for strain verification). Measurements from two independent *ron1*-complemented strains showed a complete restoration of wild-type biomass formation (Fig. S3A).

Upon exhaustion of medium nutrients, filamentous fungi undergo autolysis of ageing hyphae. At advanced autolytic growth stages, a decline of biomass can be observed in submerged cultivations. We tested whether a lack of GlcNAc catabolism causes alterations in autolysis due to impaired recycling of chitin from the cell wall. Biomass samples from shake flask cultivations with glucose as carbon source were taken at advanced time points, i.e., 120–216 h after inoculation (Fig. S4). Although onset of autolysis (i.e. the decrease of biomass) appeared delayed in Δ*ron1* compared with the control strain, this difference was not statistically significant. Similar results were obtained for the other knockout strains (data not shown), with the exception of Δ*hxk3* (Fig. S4) which exhibited a statistically significant stronger decrease in biomass than wild type.

The finding that *ron1* knockout strains could not grow on GlcNAc strongly suggested that RON1 is indeed an indispensable regulator of GlcNAc catabolism. To further confirm that RON1 is a transcriptional regulator of this pathway, transcript levels of the structural GlcNAc catabolic genes were assessed in Δ*ron1* strains. Mycelia were pre-grown on 1% glycerol and then replaced to either 1% glucose or 1% GlcNAc. Gene expression levels were determined at 4, 8 and 24 h after the replacement and were similar at all three tested time points. The results (Fig. 5) showed that high-level induction of the genes *hxk3*, *dac1* and *dam1* in the presence of the pathway's substrate, as

Table 1. Fungal strains used in this study.

Strain	Genotype	Reference
T. reesei QM9414	*mat1-2*	ATCC 26921
T. reesei QM9414 Δtku70	*Δtku70::pyr4 mat 1-2*	C. Ivanova and B. Seiboth; unpublished data (Ghassemi *et al*., 2015)
T. reesei Δnag3	*mat1-2 Δtku70 Δnag3::hph*	This study
T. reesei Δhxk3	*mat1-2 Δtku70 Δhxk3::hph*	This study
T. reesei Δdac1	*mat1-2 Δtku70 Δdac1::hph*	This study
T. reesei Δdam1	*mat1-2 Δtku70 Δdam1::hph*	This study
T. reesei Δron1	*mat1-2 Δtku70 Δron1::hph*	This study
T. reesei Δron1 ron1⁺	*mat1-2 Δtku70 Δron1::hph ron1:amdS*	This study
T. reesei Δngt1	*mat1-2 Δtku70 Δngt1::hph*	This study
T. reesei Δhxk3 G418	*mat1-2 Δtku70 Δhxk3::G418*	This study
T. reesei Δdam1 amdS	*mat1-2 Δtku70 Δdam1::amdS*	This study
T. reesei Δcsp2	*mat1-2 Δtku70 Δcsp2::hph*	This study
T. reesei Δdac1 Δhxk3	*mat1-2 Δtku70 Δdac1::hph Δhxk3::G418*	This study
T. reesei Δdam1 Δhxk3	*mat1-2 Δtku70 Δdam1::hph Δhxk3::G418*	This study
T. reesei Δdac1 Δdam1	*mat1-2 Δtku70 Δdac1::hph Δdam1::amdS*	This study
T. reesei Δhxk3 Δdam1	*mat1-2 Δtku70 Δhxk3::hph Δdam1::amdS*	This study
T. reesei Δhxk3 Δdac1 Δdam1	*mat1-2 Δtku70 Δhxk3::G418 Δdac1::hph Δdam1::amdS*	This study

well as that of *nag3* and *ngt1*, was virtually abolished in *ron1* deletion strains. Gene expression analysis of complemented Δ*ron1* strains (Fig. S3B) showed that an ectopic integration of *ron1* is sufficient to fully restore inducible expression of the other GlcNAc cluster genes in the presence of GlcNAc.

GlcNAc has also been reported to efficiently induce the expression of the GH20 *N*-acetylglucosaminidase genes *nag1* and *nag2* (López-Mondéjar *et al*., 2009). Therefore, we analysed whether the lack of *ron1* also influences the expression levels of these genes and found that the induction of *nag1* and *nag2* gene expression by GlcNAc is severely affected in Δ*ron1* strains (Fig. 5). Therefore, these data further substantiated that the transcription factor RON1 is indeed the key activator of the GlcNAc catabolic pathway in *T. reesei*.

Evaluation of growth conditions related to the GlcNAc catabolism based on observations in other fungi: starvation and regulation by the transcription factor CSP2

Our current study demonstrated for the first time the direct and essential involvement of a fungal Ndt80-like regulator in the turn-over of GlcNAc by mediating high-level induction of the GlcNAc-catabolic system. For *A. nidulans* XprG mutants, the ortholog of *T. reesei* RON1 (cf. Fig. 2A), a number of observations apparently unrelated to GlcNAc were published. An *xprG* gain-of-function mutant (*xprG1*) was described that overproduces extracellular proteases under starvation conditions (Katz *et al*., 2006; 2013). *xprG* loss-of-function mutants show reduced extracellular protease levels under both carbon and nitrogen starvation conditions. In addition, HxkC has been described as a non-catalytic hexokinase-like protein in *A. nidulans*, likewise modulating the production of extracellular protease (Bernardo *et al*., 2007). However, HxkC is the ortholog of *T. reesei* HXK3; thus, it is likely that HxkC is responsible for the phosphorylation of GlcNAc in *A. nidulans*. We tested growth of knockout strains of the *T. reesei* GlcNAc gene cluster on agar plates containing 1% skimmed milk, the growth condition under which increased protease levels were described for the *xprG* gain-of-function mutant. No growth differences (colony diameter or hyphal density) or variation in the diameter of the cleared halo around the growing colonies (indicative of the extracellular protease activity produced) could be detected on MA plates, regardless whether 1% (w/v) skimmed milk powder was used as the sole carbon- or as the sole nitrogen source (data not shown). Thus, the lack of *ron1* or *hxk3* or any other gene lesion in GlcNAc catabolism does not appear to affect extracellular protease production in *T. reesei* under these assay conditions.

A survey of the literature for transcription factors that might also be involved in the regulation of GlcNAc metabolism showed that in *N. crassa*, the transcriptional regulator termed GRHL (grainy head like protein) could be involved in the regulation of GlcNAc metabolism in aerial hyphae (Pare *et al*., 2012). On solid medium fungal hyphae grow on and in the substrate and some hyphae can extend into the air (aerial hyphae) and differentiate into specialised structures for the formation of asexual spores (conidia) upon certain stimuli, such as light or nutrient limitation. *N. crassa grhl* mutants are defective in conidial-spore dispersal in aerial hyphae due to an apparent inability to remodel the cell wall during conidiation. The

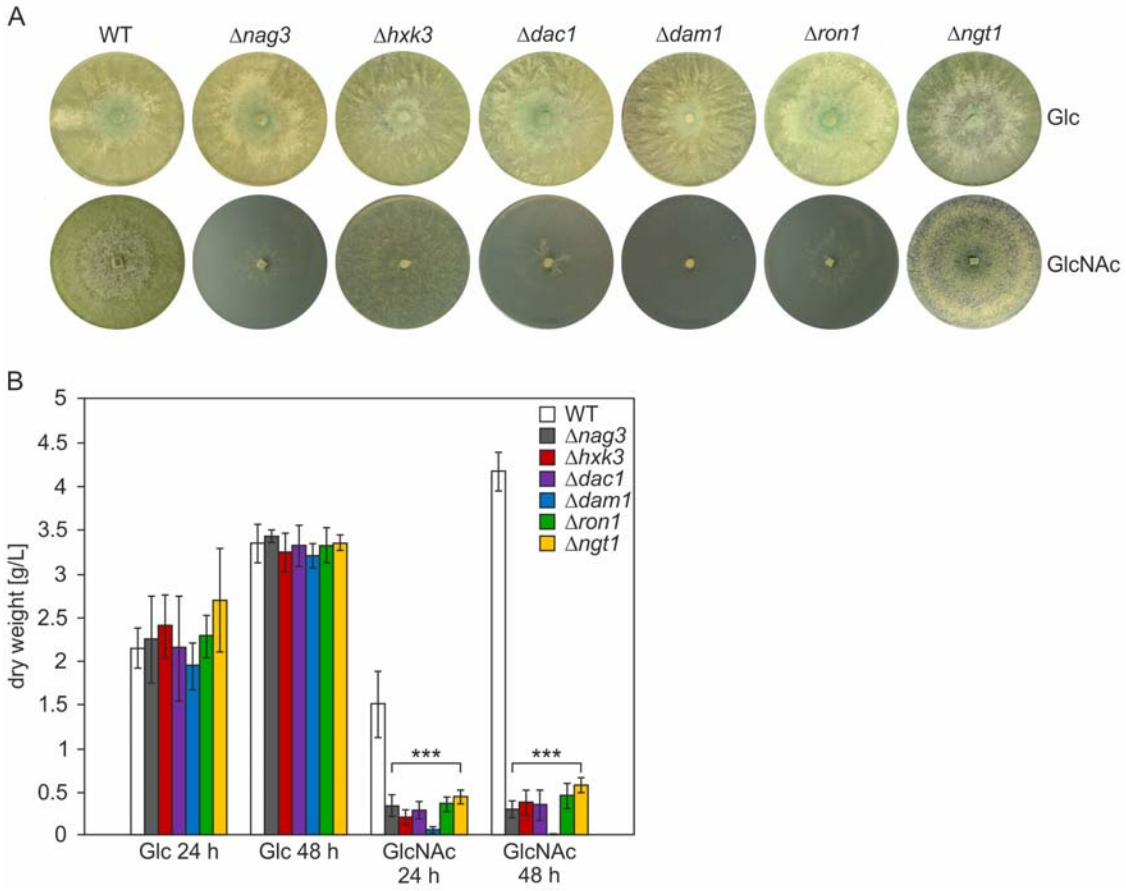


Fig. 4. Knockout strains of GlcNAc cluster genes exhibit strongly impaired growth on GlcNAc.
A. *T. reesei* strains WT (parental strain; QM9414 Δ*tku70*), Δ*nag3*, Δ*hxk3*, Δ*dac1*, Δ*dam1*, Δ*ron1* and Δ*ngt1* were grown for six days on MA agar plates (1.5% w/v agar) containing either 1% GlcNAc or 1% glucose (Glc) as carbon source.
B. For biomass measurements, strains were grown in liquid MA medium in shake flasks, containing either 1% GlcNAc or glucose (Glc) and dry weight was determined after 24 h and 48 h after inoculation. Dry weight of single knockout strains is in all cases statistically significantly different from WT when grown on GlcNAc as carbon source, with $p < 0.001$ for all knockout strains.

grhl gene is allelic to conidial separation-2 (*csp-2*) mutations. The genes of GlcNAc catabolism were among the set of down-regulated genes in aerial hyphae and conidia from a *N. crassa grhl* mutant (Pare *et al*., 2012).

GRHL proteins belong to the CP2 superfamily of transcription factors (PFAM family PF04516), and in the *T. reesei* genome database, only one predicted member can be found (NCBI accession number: XP_006965082), which we named CSP2. To address its function(s) and assess its relevance for GlcNAc catabolism, we generated knockout strains of *T. reesei csp2* (Fig. S2). *T. reesei* CSP2 and *N. crassa* CSP2 show a high degree of conservation (amino acid similarity: 69%; sequence coverage: 96%). We found that *T. reesei csp2* is expressed under various growth conditions, but we were not able to detect any morphological defects in our Δ*csp2* strains. Analyses included growth on agar plates with either complex medium (potato dextrose agar; PDA) (Fig. 6A) or MA medium with various carbon sources, but colony formation, radial growth rate and sporulation were not altered in Δ*csp2* strains. Further, no phenotype was evident for growth on GlcNAc and chitin (data not shown). Biomass formation in shake flask cultivations was normal for all tested growth substrates. Moreover, microscopical investigation of hyphae or sporulating cultures did not reveal any phenotype for *csp2* knockout strains (data not shown). These data show that, in contrast to *N. crassa*, the sole GRHL-type transcription factor in *T. reesei* does not seem to have critical functions in conidiation.

To assess potential connections between nutrient limitation and GlcNAc catabolism in more detail, we analysed whether the GlcNAc cluster genes in *T. reesei* are expressed during carbon starvation and whether this expression in the absence of externally supplied GlcNAc might be regulated by RON1 or CSP2. Deletion strains and a wild-type control strain were pre-grown for 16 h on 1% glucose and then replaced into medium without carbon source. After the medium shift, samples were

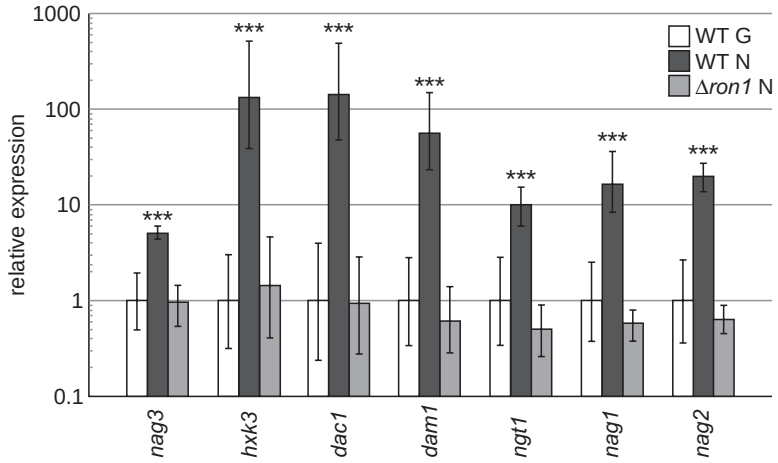


Fig. 5. The transcription factor RON1 is the key activator of the GlcNAc catabolic gene cluster. Fungi were grown on MA medium containing 1% glycerol as carbon source for 24 h and then shifted to minimal medium containing 1% GlcNAc (N) or 1% glucose (G) and cultivated for another 24 h. Gene expression levels of all GlcNAc cluster genes, *ngt1* as well as *nag1* and *nag2* were evaluated by qRT-PCR at 4, 8 and 24 h after the medium shift, normalised using *tef1* as reference gene, and compared with the respective gene expression levels of the parental strain on glucose (control) which was set as 1. The 4, 8 and 24 h values were combined since expression of all three time points was very similar. Statistically significant differences (*P* values < 0.001 were only detected for the parental strain (WT, QM9414 Δ*ku70*) for growth on GlcNAc vs glucose, but not for the Δ*ron1* strain, where gene expression levels on GlcNAc were with relative values around 1 rather similar to those on glucose.

taken at different time points (0 h (control), 5 h and 15 h), and expression analysis of the GlcNAc cluster genes was performed. The results showed that in the *T. reesei* parental strain, mainly *dac1* and *ngt1* transcript levels were increased during starvation and that this was dependent on *ron1*, but not on *csp2* (Fig. 6B). In Δ*csp2* strains, some transcript levels were increased transiently at 5 h after the medium shift, e.g. *hxk3* and *ron1*, but it was difficult to discern a trend or a biological consequence for GlcNAc metabolism from that. Interestingly, this experiment also suggested that RON1 antagonises apparent glucose repression of the *ngt1* and *hxk3* genes, i.e. the transporter and the dedicated GlcNAc kinase, directly after the transfer ($t = 0$ h), hence, in the absence of added GlcNAc or a starvation response.

Conidiation rates in GlcNAc cluster knockout strains

We also generated double and triple knockout strains of the GlcNAc cluster genes (Table 1, Fig. S2). Apart from the already observed phenotypes for single knockout strains on GlcNAc, no obvious phenotypic alterations were observed for the double and triple knockout strains except for Δ*hxk3*Δ*dam1* double knockout strains: PDA plates fully overgrown with sporulating colonies of the latter appeared to be 'less green', even after incubation for 12 days (data not shown). A more detailed analysis showed that Δ*hxk3*Δ*dam1* strains produced ca. 60% fewer spores/PDA plate than the parental strain (Fig. 7A), but pigmentation of the spores appeared to be normal (Fig. 7B). Spore counting of conidia from 12-days old PDA plates also revealed that Δ*ron1* produced 30% more conidia than the parental strain and all the other strains of our collection of deletion mutants (Fig. 7A). Microscopic analyses of conidia upon staining of chitin in the fungal cell wall with Calcofluor White did not reveal any differences, and germination rates of the produced conidia were not altered (data not shown). The phenotype of Δ*hxk3*Δ*dam1* strains (which was also verified with another combination of selection marker genes for deletion of *hxk3* and *dam1*, see Table 1, data not shown) was not observed in any of the other combinations of double knockout strains and also not in Δ*hxk3*Δ*dac1*Δ*dam1* triple knockouts. This suggested that the reduced number of conidia could be caused by aberrant *dac1* expression in Δ*hxk3*Δ*dam1* strains and/or a side activity of this enzyme. Gene expression analysis of *dac1* in conidia at different maturation stages showed indeed that this gene is up to 100-fold up-regulated in Δ*hxk3*Δ*dam1* double knockout strains compared with the parental strain or the respective single knockout strains (Fig. 7C). These data suggest that the roles of enzymes of the GlcNAc pathway and/or its intermediates could go beyond merely catabolic functions in mobilising reserve nutrients for energy production and nitrogen (re-)assimilation from cell wall polymers.

Discussion

The phenomenon of gene clustering has been a powerful aid in the identification, expression and characterisation of fungal secondary metabolism pathways, their intermediates and products (Brakhage, 2013; Wiemann and Keller,

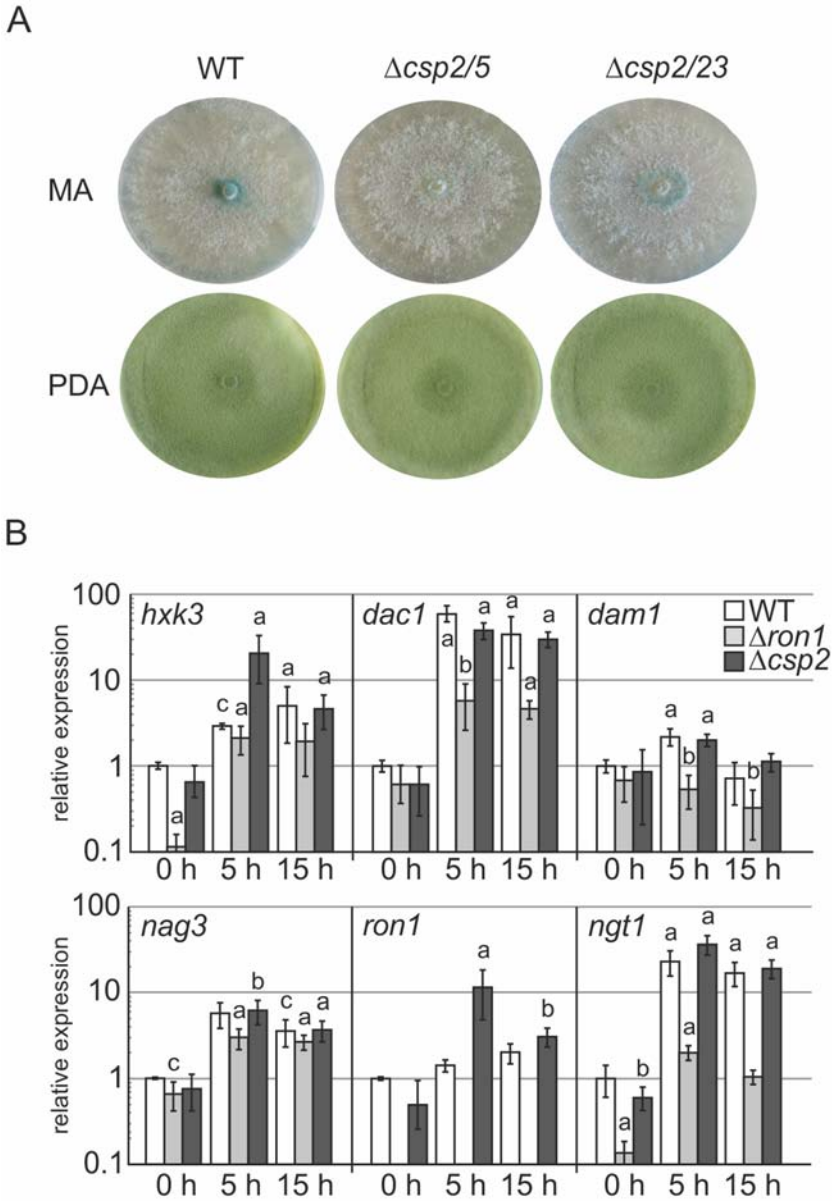


Fig. 6. CSP2 is not involved in the regulation of GlcNAc genes during vegetative growth or under carbon starvation conditions.
A. Growth of the *T. reesei* parental strain QM9414 Δ*tku70* (WT) and two *csp2* knockout strains (Δ*csp2/5* and Δ*csp2/23*) after 7 days on agar plates with MA medium with glucose and PDA.
B. qRT-PCR of the parental strain (WT), and two Δ*ron1* and Δ*csp2* strains. Mycelium was pre-cultivated for 16 h in MA medium containing 1% glucose, and biomass samples were taken at 0, 5 and 15 h after a shift to MA medium lacking added carbon sources. All measured values were normalised to *tef1* expression and compared with the time point [WT, 0 h], which was set at 1. Bars indicate the SEM (standard error of the mean). 'a', 'b' and 'c' indicate significance at $P < 0.001$, 0.01 and 0.05 respectively.

2014; Wisecaver and Rokas, 2015). Clustering of genes appears less frequently in primary metabolism, but both catabolic and anabolic gene clusters have already been described, e.g. for L-proline catabolism (Jones *et al*., 1981; Hull *et al*., 1989) and D-biotin biosynthesis (Magliano *et al*., 2011). Comparative genomics revealed that some clusters are quite ancient, while others, such as the ethanol utilisation cluster in certain *Aspergilli* (Flipphi *et al*., 2009), are more recent acquisitions. Clusters can contain structural genes for enzymes, pathway-related transport functions as well as pathway-specific regulatory genes, and their exact layout often varies among divergent lineages that share this higher level of organised, functionally related genetic information. In this work, we studied the genomic arrangement of the GlcNAc gene cluster in Ascomycota, generated transcriptional profiles of the respective genes in *T. reesei* and *T. atroviride* and finally characterised them functionally using knockout strains in *T. reesei*. We started by screening the position of the genes encoding homologs of the GlcNAc catabolism pathway in *C. albicans* in sequenced fungal genomes and established the occurrence of a GlcNAc catabolism cluster in all three subphyla of Ascomycota, right down to the most primitive ascomycete (Taphrinomycotina) sequenced to date, *Saitoella complicata* (Fig. 2A). The functional GlcNAc catabolism gene cluster contains two genes in addition to the catabolic enzymes. A gene encoding a GH3 protein (NAG3) is often associated with the four

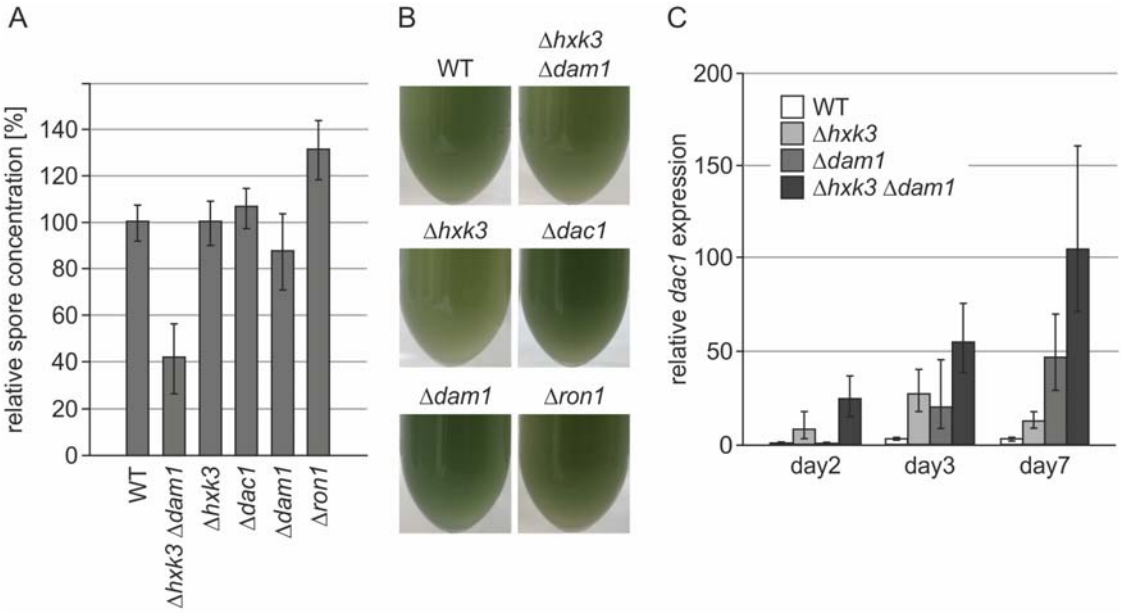


Fig. 7. Up-regulation of *dac1* expression in a Δ*hxk3*Δ*dam1* double knockout strain decreases the amount of produced conidia.
A. Percentage of produced conidia in gene knockout strains relative to the parental strain QM9414 Δ*tku70* (WT; 100%). Conidiospore numbers in the Δ*hxk3*Δ*dac1*, Δ*ron1* ($P < 0.001$) and Δ*dam1* ($P < 0.01$) strains are significantly different from the WT.
B. Conidiospores were harvested, counted (see Fig. 7A) and diluted to a final spore concentration of 1×10^7 spores ml^{-1}. Photographs were taken to visually document the color of the conidia.
C. *dac1* expression in spores of different age was assessed in the WT and selected knockout strains (Δ*hxk3*, Δ*dam1* single and Δ*hxk3* Δ*dam1* double knockout). Strains were grown on PDA plates, and spores were harvested after 2, 3 and 7 days after inoculation of the plate for qRT-PCR analysis, corresponding to nascent, pre-mature and mature spores of the control strain on PDA plates respectively. Expression levels of *dac1* were compared with WT levels at day 2 and normalised to *tef1* levels. Only the transcription levels of the knock out strains but not the WT levels were significantly different (*P* values < 0.001).

core cluster genes. The *nag3* gene is also present in Saccharomycetales that feature the GlcNAc cluster, as represented by *C. albicans* (not associated with the cluster; Locus CaO19.7516) and *Y. lipolytica* (Fig. 2A). It does not occur in yeasts that miss both the deacetylase and the deaminase, like budding yeast (*S. cerevisiae*) and fission yeast (*S. pombe*). Given the similarity to bacterial exo-*beta*-*N*-acetylhexosaminidases, e.g. *Escherichia coli* NagZ (Litzinger *et al*., 2010), a role in the mobilisation of GlcNAc from various poly and oligomeric precursors appears logical, although we cannot explain why the deletion of *nag3* in *T. reesei* results in a growth phenotype for the monomer (Fig. 4). We are currently studying this gene further to identify its function(s).

The other additional gene that we found in the cluster in comparison to *C. albicans* encodes the transcription factor with an Ndt80-like DNA-binding domain that we named RON1. Our *in silico* analysis showed that the GlcNAc cluster in ascomycete filamentous fungi (Pezizomycotina) frequently harbours a gene for this transcription factor. In *C. albicans*, the gene for the Ron1 structural ortholog was previously described as Rep1 and is linked to the GH3 ortholog gene (Fig. 2A). *REP1* is implicated in negative regulation of the multiple drug resistance efflux pump Mdr1: Overexpression in *S. cerevisiae* increased susceptibility to fluconazole, and deletion of *rep1* lead to increased efflux of azole antifungal drugs in *C. albicans* (Chen *et al*., 2009). The documented function of the Ndt80-like transcription factor Rep1 seems to be different from that of Ron1 in *T. reesei*. We have shown for the first time that an Ndt80-like transcription factor (RON1) is necessary for growth on GlcNAc as the sole carbon source (Fig. 4) and that it is essential for the high-level induction of the catabolic genes by GlcNAc (Fig. 5).

GlcNAc catabolism relates directly to nutrient starvation. Polymers of GlcNAc and N-glycosylated proteins – containing $GlcNAc_2$ as a basal anchor of the glycan to the protein – are mobilised to serve as transient carbon and nitrogen sources that allow the fungus to enter asexual reproduction and produce conidia to disseminate and to survive the prevalent unfavourable environmental conditions. Nevertheless, it can be expected that the intracellular GlcNAc pool not only undergoes catabolic recycling but also feeds into anabolism upon phosphorylation to GlcNAc-6P, followed by isomerisation into GlcNAc-1P with phospho-acetylglucosamine mutase (EC 5.4.2.3) and uridylation of the latter into UDP-GlcNAc with UDP-GlcNAc pyrophosphorylase (EC 2.7.7.23). The last two

enzymes are part of Leloir's UDP-GlcNAc biosynthetic pathway (for a review in fungi, see Milewski *et al*., 2006). This process provides building blocks for *de novo* chitin synthesis and glycosylation of secreted proteins bypassing the need to re-synthesise GlcNAc-6P from fructose-6P by means of the first two enzymes of UDP-GlcNAc biosynthesis, GlcN-6P synthase (EC 2.6.1.16) and GlcN-6P acetyltransferase (EC 2.3.1.4) (note: these enzymes catalyse the reverse reactions of DAM1 and DAC1 respectively). Indeed, diploid *C. albicans* strains, homozygote for GlcN-6P synthase deletion could not grow unless the growth medium contained GlcNAc (Gabriel *et al*., 2004).

In filamentous fungi, proteins belonging to the Ndt80-family have already received considerable attention. RON1 and XprG are orthologs, while VIB-1-like proteins are RON1 paralogs unique to Sordariomycetes and Leotiomycetes (Fig. 2B). We therefore speculate that XprG functions and their pleiotropic effects (as evident in *A. nidulans*) could be divided between the RON1- and VIB-1-like paralogs in species of the two filamentous fungal classes that feature both regulators, such as *T. reesei*, *N. crassa* and *Botrytis cinerea*. The involvement in multiple, seemingly unrelated processes has been reported for VIB-1, including the regulation of protoperithecial development, vegetative incompatibility and programmed cell death, regulation of responses to nitrogen and carbon starvation, as well as plant cell wall degradation by carbon catabolite repression (Dementhon *et al*., 2006; Hutchison and Glass, 2010; Xiong *et al*., 2014). In the endophytic plant-symbiont *Epichloë festucae* (Sordariomycetes), VIB-1 is involved in the production of antifungal compounds (Niones and Takemoto, 2015).

However, none of these studies have revealed the direct connection between Ndt80-like transcription factors and GlcNAc catabolism or studied GlcNAc as growth substrate although GlcNAc is an important, omnipresent constituent of the fungal cell wall. Additional functions of GlcNAc and its derivatives in signalling and other cellular processes, as reported in *C. albicans*, remain to be elucidated in filamentous fungi.

Our current work strongly suggests that HXK3, the ortholog of *A. nidulans* HxkC (Bernardo *et al*., 2007), is a sugar kinase dedicated to the phosphorylation of GlcNAc, whose induced expression is controlled by RON1, the ortholog of *A. nidulans* XprG (Katz *et al*., 2006). The increased induction levels of the structural GlcNAc catabolic genes in *T. reesei* in response to GlcNAc (Fig. 3) are substantially higher (> 100-fold) than for any gene mentioned by Katz *et al*. (2013) in their genome-wide transcriptome analysis of carbon starvation in *A. nidulans*. Nevertheless, one of the best induction ratios comparing xprG^{+} and xprG^{-} strains (6-fold) was reported for *hxkC*.

Very leaky (starvation) growth of *T. reesei hxk3* knockout strains was observed on GlcNAc agar plates, but not in shake flask cultivations (Fig. 4). This suggests that, although HXK3 is responsible for growth on the acetylated aminosugar, in surface cultures, some phosphorylation can take place in its absence. The enzyme responsible for this activity could be a glycolytic hexokinase; in *T. reesei*, the hexokinase HXK1 and the glucokinase GLK1 involved in the phosphorylation of D-fructose and D-glucose, respectively, have been described (Hartl and Seiboth, 2005), but in view of the strong growth defects on many other sugars exhibited by the knockout strains, we refrained from testing this trait further.

An interesting finding concerns simultaneous deletion of *hxk3* and *dam1* that leads to a 60% reduction in condiospore formation in this double knockout mutant and concomitantly, to an up to 100-fold increase in *dac1* expression in the absence of GlcNAc. In a recent publication, Häkkinen *et al*. found a strong dependence of *dac1* expression on PAC1, the principal mediator of the response to extracellular pH in *T. reesei* (known in *A. nidulans* as PacC: see Peñalva and Arst, 2004). The levels of *dac1* were strongly upregulated in a *pac1* deletion strain, which mimics acidic conditions (Häkkinen *et al*., 2015). A *pac1* deletion in *Trichoderma harzianum* was shown to result in decreased conidiation (Moreno-Mateos *et al*., 2007). Although we cannot directly explain the correlation between *dac1* up-regulation and conidiation with our current understanding of GlcNAc catabolism, a pleiotropic effect of elevated *dac1* levels on spore formation cannot be excluded.

In this study, we focussed on functional verification and characterisation of the GlcNAc catabolism pathway and the respective genes in *T. atroviride* and *T. reesei*. Further-reaching consequences of genetic alterations in this pathway and of its activator RON1 will be the subject of future studies. This could encompass potential feedback effects of the lack of GlcNAc catabolism on chitinase gene regulation as well as more general aspects related to, for example, carbon catabolite repression, as has been reported for *N. crassa* VIB-1 (Xiong *et al*., 2014). While *Trichoderma* chitinase genes have so far been reported to be inducible only by chito-oligosaccharides or chitin-containing polymers (e.g. chitin and fungal cell walls), the two *N*-acetylglucosaminidase-encoding genes *nag1* and *nag2* are strongly inducible by the monomer GlcNAc (Mach *et al*., 1999; Gruber and Seidl-Seiboth, 2012). Although the two proteins NAG1 and NAG2 exhibit different extracellular locations – NAG1 is predominantly secreted into the medium, while NAG2 remains attached to the fungal cell wall; the presence of one of these proteins is both necessary and sufficient to enable growth on chitin (López-Mondéjar *et al*., 2009). *T. atroviride* cannot grow on the dimer chitobiose when *nag1* and *nag2* genes are absent, which not only shows that there is no additional extracellular *N*-acetylglucosaminidase that would be able to compensate for NAG1 and NAG2, but also strongly suggests

that extracellular cleavage of the dimer into monomers is a prerequisite for uptake in *T. atroviride* (López-Mondéjar *et al*., 2009). In this study, we could now show that the expression of both *N*-acetylglucosaminidases is dependent on RON1 since expression of neither of them can be induced in the presence of GlcNAc in a *ron1* deletion mutant. This observation presents an interesting starting point for further investigations of the regulation of chitin metabolism.

As shown in this study, GlcNAc uptake proceeds mainly via the transporter NGT1 because growth of *ngt1* knockout strains was virtually abolished in liquid cultivations. On agar plates, some growth was evident, although the formed mycelium was less dense than in the control strains. The observed weak growth could be explained by presuming the existence of another hexose transporter that has a low affinity for GlcNAc and is expressed sufficiently due to the different type of colony morphology or the different environmental conditions on solid but not in liquid medium. Alternatively, it could be induced by (components of) the solidifying agent to support the observed residual growth on GlcNAc plates.

The GlcNAc gene cluster was not only inducible by GlcNAc, but also by chitin, although induction levels were lower. This is in agreement with the observed slow formation of biomass on chitin and suggests that extracellular depolymerisation is the bottleneck for growth on chitin. For proper understanding of the regulation of chitin degradation from external chitinous carbon (and nitrogen reserve) sources as well as for the process of cell wall remodelling and recycling, knowledge of the GlcNAc catabolic pathway and its possible regulatory and feed-back functions is pivotal. Chitin degradation narrows down from a multienzyme system at the level of polymeric chitin decomposition to a well-defined, singular pathway with unique genes for each step. It remains to be elucidated whether altering the flow through the GlcNAc catabolic pathway by manipulation of single genes leads to a better understanding of the regulation and functions of the whole chitin degradation machinery as well as of chitin biosynthesis.

In this study, we progressed towards a more complete insight in fungal chitin degradation by describing the GlcNAc catabolism pathway and its gene cluster in filamentous fungi and established the principle role of the Ndt80 family regulator RON1 as its transcriptional activator.

Experimental procedures

Strains and cultivation conditions

T. atroviride IMI206040 (ATCC 20476) and *T. reesei* QM9414 (ATCC 26921) were used in this study and maintained on potato dextrose agar (PDA, BD, Franklin Lakes, USA). Agar plates were kept in the dark at 28°C for *T. reesei* or in a 12 h light/12 h dark cycle at 28°C for *T. atroviride*. Stock cultures were kept in 50% glycerol at –80°C. For yeast transformation to generate deletion constructs, the shuttle vector pRS426 (Christianson *et al*., 1992) and the yeast strain WW-YH10 (ATCC 208405) were used. *E. coli* Stellar™ Competent Cells (Clontech Laboratories, Mountain View, CA) were used for propagation of all used and constructed plasmids.

For shake flask cultivations MA medium [pH 5; per liter: 1.4 g $(NH_4)_2SO_4$, 2.0 g KH_2PO_4, 0.3 g $MgSO_4*7H_2O$, 0.3 g $CaCl_2*2H_2O$, 5 mg $FeSO_4*7H_2O$, 1.6 mg $MnSO_4*H_2O$, 1.4 mg $ZnSO_4*7H_2O$ and 2 mg $CoCl_2*2H_2O$ for *T. reesei* (adapted from (Mandels and Andreotti, 1978)] and SM medium for *T. atroviride* (Seidl *et al*., 2004) with 0.05% peptone and 1% glucose (D-Glucose Monohydrate; Roth, Karlsruhe, Germany) or *N*-acetylglucosamine (GlcNAc; Sigma-Aldrich, St. Louis, MO) as carbon source was inoculated with 1×10^6 conidia ml^{-1} and cultivated at 28°C and 220 r.p.m. for the indicated time periods. For replacement of carbon sources, *T. reesei* strains were pre-grown in MA medium containing 0.05% peptone and 1% glycerol (for replacement with GlcNAc and glucose) or 1% glucose (for starvation) for 24 h and 16 h, respectively, harvested by filtration through sterile filter tissue (Miracloth; Calbiochem, Merck, Darmstadt, Germany), washed with sterile tap water and transferred to fresh MA medium, containing carbon sources as stated in the figure legends in the results section and again incubated at 28°C, 220 r.p.m. for the indicated time periods. Static liquid cultivations were performed in sterile Petri dishes (100 mm diameter, Greiner Bio-one, Kremsmünster, Austria) with MA medium containing 1% glucose, powdered chitin (Sigma-Aldrich) or colloidal chitin, inoculated with 1×10^6 conidia ml^{-1} and cultivated at 28°C. Colloidal chitin was prepared as described in Seidl *et al*. (2005). Mycelial samples from shake flask and static cultures were harvested by filtration through Miracloth (Calbiochem, Merck), washed with cold distilled water and immediately frozen in liquid nitrogen. Mycelial dry weight was determined by withdrawing 50 ml aliquots from shake flask cultivations, suction filtration through a glass wool filter, followed by extensive washing with tap water, and drying at 80°C to constant weight.

For preparation of conidia from different maturation stages, based on the appearance of the mycelium covered with conidia, ranging from white (2 days) to light green (3 days) and dark green conidia (7 days) strains were grown on PDA plates in the dark at 28°C. Conidia were harvested at the indicated time points by rinsing the sporulating mycelium with a 0.9% NaCl/0.05% Tween-80 solution, which was then transferred into a sterile 2 ml reaction tube, concentrated by centrifugation (2,500 *g*, 3 min), and the conidial pellet was immediately frozen in liquid nitrogen.

For spore counting strains were grown for 12 days on PDA. Conidiospores were harvested from plates in a defined volume of 0.9% NaCl/0.05% Tween-80 solution/plate and counted in a Thoma-cell counting chamber (Roth). The amount of total spores per ml was calculated using the formula given by the manufacturer. To visually determine the color of the mature spores, a dilution to 1×10^7 spores per ml was produced according to the spore concentration determined by cell counting. Statistical analysis for growth tests and spore generation was performed using the Student's *t*-test, assuming unequal variance of groups.

Isolation and purification of fungal DNA and RNA

For RNA isolation, the samples were ground with mortar and pestle to a fine powder under liquid nitrogen, and total RNA was isolated using the guanidinium thiocyanate method (Sambrook and Russell, 2001). Isolated RNAs were treated with DNAse I (Fermentas, St Leon-Roth, Germany), and cDNAs were subsequently generated with the Revert Aid H-minus cDNA synthesis kit (Fermentas). For verification of knockouts, genomic DNA was isolated using a rapid DNA purification protocol (Liu *et al*., 2000). Genomic DNA from *T. reesei* was isolated using the Wizard Genomic DNA Purification Kit (Promega).

Gene expression analysis (RT-PCR and quantitative RT-PCR)

RT–PCR (17-30 cycles depending on the probe, see Table S1) was performed using the gene-specific primers listed in Table S1 along with the database accession numbers of the genes analysed in this study. The *tef1* gene (JGI protein ID 83874 for *T. reesei* and JGI protein ID 300828 for *T. atroviride* in the JGI database; NCBI database: XP_006964056 and EHK42777 respectively) was used as the reference gene. qRT-PCR reactions were performed in a Bio-Rad (Hercules, CA, USA) iCycler IQ. The reaction mix contained 12.5 µl SYBR green Supermix (Bio-Rad), 8.5 µl pure water, 6.25 µM forward and 6.25 µM reverse primer, and 2 µl 1:50 diluted template cDNA. For cDNA synthesis, 5 µg of RNA/reaction were reverse-transcribed using the Revert Aid H-minus cDNA synthesis kit (Fermentas). Primer efficiency for qPCR was calculated using a dilution series from 1:5 to 1:5000 with the PCR baseline-subtracted mode. Amplification efficiency was then calculated from the given slopes in the IQ5 Optical system Software v2.0. The amplification protocol consisted of an initial denaturation step for 3 min at 95°C, followed by 40 cycles of denaturation (95°C for 15 s), annealing and elongation (60°C for 15 s). Oligonucleotides are listed in Table S1. The *tef1* gene was used as a reference. Relative gene expression ratios were calculated using REST software (Pfaffl *et al*., 2002). For analyses of the results, samples from at least two independent experiments with three technical replicates in each run were used. The significance of differences in gene expression between different knockout mutants and the parental strain as well as between cultivation conditions at a given time point was evaluated by the Student's *t*-test, assuming unequal variance of groups.

Generation of T. reesei *gene knockout strains*

Gene deletions were performed in a *T. reesei* QM9414 Δ*tku70* strain in which the *tku70* gene was replaced by the *pyr4* marker (Ghassemi *et al*., 2015; C. Ivanova and B. Seiboth, unpub. results). Effects of the deletion of *tku70* in *T. reesei* have been described in Guangtao *et al*. (2009). Gene knockout cassettes were generated using the yeast recombination system described in Schuster *et al*. (2012). For generation of the 5′- and 3′-flanking sequences consisting of around 1 kb of up- and downstream non-coding regions of the respective genes, primers were designed according to Schuster *et al*. (2012). Sequences were obtained from the *T. reesei* genome database (http://genome.jgi-psf.org/Trire2/Trire2.home.html). Primers for 5′- and 3′-flanking sequences for the knockout cassette construction are listed in Table S2. As selectable marker the hygromycin phosphotransferase gene *hph*B from *E. coli* was used to generate single knockout strains (Mach *et al*., 1994). For generation of double and triple knockout strains, the geneticin resistance gene (*npt2*, from *E. coli* (Bischof and Seiboth, 2014)) and acetamidase gene (*amd*S, from *Aspergillus nidulans*; described for application as selection marker in *Trichoderma* in Penttilä *et al*., 1987) were used as selectable markers (the primers for amplification of the marker genes are listed in Table S3). In the course of these experiments, also single knockout strains with *npt2* (*hxk3*) and *amdS* (*dam1*) markers were generated (Table 1) with which also the results from the *hph*B marker deletion strains could be verified. The amplified 5′ and 3′ flanking regions of the respective GlcNAc cluster genes and the marker genes were inserted in pRS426 by homolog recombination in yeast (Schuster *et al*., 2012). Transformation of *T. reesei* was performed essentially as described in Gruber *et al*. (1990). Correct integration of the gene deletion cassette was verified by PCR (primers are listed in Table S4) after two rounds of purification by single spore isolation on selective medium containing 0.1% Triton X-100. Selective media were PDA with 100 µg ml^{-1} Hygromycin B (Roth) or 120 µg ml^{-1} Geneticin (G418-Sulfat, Roth) for *hphB* and *npt2*, respectively, and 0.1 M acetamide (Merck) in MA medium lacking other nitrogen sources for *amdS*. A replacement strain of the Δ*ron1* strain was constructed by heterologously reintroducing the wild-type *ron1* gene coupled to an *amdS* cassette into the constructed Δ*ron1* strains. Therefore, *ron1* was amplified from genomic *T. reesei* DNA, assembled with the *amdS* cassette downstream of the gene in the pRS426 vector and transformed into the Δ*ron1* mutant strains as described above. Table 1 shows all generated knockout strains and the Δ*ron1 ron1*$^{+}$ replacement strain. Knockout strains and the Δ*ron1 ron1*$^{+}$ replacement strain were verified for correct integration of the cassette by PCR (Fig. S2) and by absence of the wild-type band of the respective target genes and gene expression (data not shown). At least two independent knockout strains for each of the analysed genes were used for characterisation.

Microscopic analyses

For detailed morphological characterisation of knockout strains, liquid cultures supplemented with the carbon sources indicated in the respective results sections were incubated for 24, 48 and 72 h; 200 µl of each culture was placed on a microscope chambered coverglass (Thermo Fisher Scientific) and imaged with an inverted Nikon T300 microscope (Nikon, Tokyo, Japan) with differential interference contrast optics and a DXM1200F digital camera (Nikon). For microscopical investigation of the chitin content of fungal cell walls, they were incubated with a solution containing Calcofluor White to a final concentration of 2.5 µM and imaged on a Nikon C1 confocal laser scanning unit containing a 405 nm laser mounted on a Nikon Eclipse TE2000 inverted microscope.

Bioinformatic analyses

For analysis of the GlcNAc catabolic gene cluster in filamentous fungi, genomic data of the 18 token species of Ascomycota depicted in Fig. 2A, accessible at the National Centre for Biotechnology Information (NCBI), were mined for orthologs of the six genes involved in GlcNAc catabolism that are described in this paper, i.e. *hxk3*, *dac1*, *dam1*, *ngt1*, *ron1* and *nag3*, by TBLASTN screening (Altschul *et al*., 1990). The query proteins used were the functional *C. albicans* structural proteins Nag1 (*T. reesei* DAM1), Nag2 (*T. reesei* DAC1), Nag5 (*T. reesei* HXK3) and Ngt1 (*T. reesei* NGT1) (all of these, encoded by intron-less genes), the predicted GH family 3 protein from *T. reesei* (NAG3, protein ID: 79669, NCBI accession number: XP_006966911) and *A. nidulans* (NCBI TPA accession number CBF84813; 923 aa) and the Ndt80-like regulator PhoG/XprG from *A. nidulans* (NCBI TPA accession number CBF84810: 588 aa), as well as its ortholog in *T. reesei* RON1 (protein ID: 79673, NCBI accession number: XP_006966820). Gene models were determined manually, and clustering and orientation were subsequently deduced for the closely linked genes. Intergenic spaces within (sub)clusters were checked for (additional) reading frames.

To study the evolutionary relations among ascomycete proteins harbouring an *NDT80*-like DNA-binding domain, we mined the NCBI Whole Genome Shotgun contigs database – including an ample range of species of Eurotiomycetes, Sordariomycetes and Dothideomycetes – for the encoding genes in Pezizomycotina and Taphrinomycotina and the NCBI Reference Genomic Sequences database for the encoding genes in Saccharomycetales. As queries for TBLASTN screening, we used the characterised regulatory proteins *Saccharomyces cerevisiae* Ndt80 (Xu *et al*., 1995), *Neurospora crassa* Vib1 (Xiang and Glass, 2002), *Aspergillus nidulans* PhoG/XprG (version TPA Accession CBF84810; (Wortman *et al*., 2009) and its *T. reesei* ortholog, RON1 (Kubicek *et al*., 2011). Gene models and products were deduced manually, in some cases with the aid of publicly available EST sequences, for instance, for the correct 586 residues-long Ndt80-ortholog in *Neurospora crassa*, GenBank accessions GE976053 and BG280050. PhoG/XprG- and Vib1-like proteins were assigned to two groups (tagged _1 and _2 respectively) based on the gene model (intron-exon structure) of the encoding genes, while Ndt80-like proteins (labelled _3) are clearly distinct. In the subphylum Taphrinomycotina, we only found a PhoG/XprG-like protein specified in the primitive species *Saitoella complicata*. Two paralog Ndt80-like proteins deduced from the genomes of seven strains of *Rhizophagus irregularis*, belonging to the phylum of Glomeromycota, were included to provide an evolutionary anchor that eventually appeared at the basis of the branch of the Ndt80-like (group 3) ascomycete proteins.

Approximately 500 resulting protein sequences from this search were aligned with MAFFT version 7 (Katoh and Toh, 2008) using the E-INS-i algorithm and a BLOSUM 45 similarity matrix. The alignment was curated with Block Mapping and Gathering using Entropy (Criscuolo and Gribaldo, 2010) using a BLOSUM 40 similarity matrix and a block size of 3, yielding 135 informative residues per protein. A maximum likelihood tree was then calculated with the PhyML program applying the WAG substitution model (Guindon *et al*., 2010) and drawn with FigTree (available at http://tree.bio.ed.ac.uk/software/figtree). The drawing software allows simplification of branches or clades to group phylogenetically related proteins with retention of species information in cartoons (Fig. S1) as well as collapsing branches or clades to the level of entire classes or entire families (Fig. 2B) (NB: all known Saccharomycotina belong to the order of Saccharomycetales). Hence, Fig. 2B shows an extremely compressed version while Fig. S1 is a more extensive, albeit still simplified, reflection of the same maximum likelihood tree. Approximate likelihood ratio tests (Anisimova and Gascuel, 2006) were calculated integrally by PhyML using Chi2-based parametric, and aLRT values (0–1) are given at the connecting nodes in the tree.

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References

Agrawal, Y., Khatri, I., Subramanian, S., and Shenoy, B.D. (2015) Genome sequence, comparative analysis and evolutionary insights into chitinases of entomopathogenic fungus *Hirsutella thompsonii*. *Genome Biol Evol* **7:** 916–930.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990) Basic local alignment search tool. *J Mol Biol* **215:** 403–410.

Alvarez, F.J., and Konopka, J.B. (2007) Identification of an *N*-acetylglucosamine transporter that mediates hyphal induction in *Candida albicans*. *Mol Biol Cell* **18:** 965–975.

Anisimova, M., and Gascuel, O. (2006) Approximate likelihood-ratio test for branches: A fast, accurate, and powerful alternative. *Syst Biol* **55:** 539–552.

Bernardo, S.M., Gray, K.A., Todd, R.B., Cheetham, B.F., and Katz, M.E. (2007) Characterization of regulatory non-catalytic hexokinases in *Aspergillus nidulans*. *Mol Genet Genomics* **277:** 519–532.

Bischof, R., and Seiboth, B. (2014) Molecular tools for strain improvement of *Trichoderma* spp. In *Biotechnology and Biology of Trichoderma*. Gupta, V.K., Schmoll, M., Herrera-Estrella, A., Upadhyay, R.S., Druzhinina, I.S., and Tuohy, M.G. (eds). Oxford, UK: Elsevier, pp. 179–192.

Biswas, S., Van Dijck, P., and Datta, A. (2007) Environmental sensing and signal transduction pathways regulating morphopathogenic determinants of *Candida albicans*. *Microbiol Mol Biol Rev* **71:** 348–376.

Brakhage, A.A. (2013) Regulation of fungal secondary metabolism. *Nat Rev Microbiol* **11:** 21–32.

Caddick, M.X., and Arst, H.N., Jr (1986) Structural genes for phosphatases in *Aspergillus nidulans*. *Genet Res* **47:** 83–91.

Chen, C.G., Yang, Y.L., Tseng, K.Y., Shih, H.I., Liou, C.H., Lin, C.C., and Lo, H.J. (2009) Rep1p negatively regulating MDR1 efflux pump involved in drug resistance in *Candida albicans*. *Fungal Genet Biol* **46:** 714–720.

Cheng, Q., Li, H., Merdek, K., and Park, J.T. (2000) Molecular

characterization of the beta-*N*-acetylglucosaminidase of *Escherichia coli* and its role in cell wall recycling. *J Bacteriol* **182:** 4836–4840.
Christianson, T.W., Sikorski, R.S., Dante, M., Shero, J.H., and Hieter, P. (1992) Multifunctional yeast high-copy-number shuttle vectors. *Gene* **110:** 119–122.
Chu, S., and Herskowitz, I. (1998) Gametogenesis in yeast is regulated by a transcriptional cascade dependent on Ndt80. *Mol Cell* **1:** 685–696.
Criscuolo, A., and Gribaldo, S. (2010) BMGE (Block Mapping and Gathering with Entropy): a new software for selection of phylogenetic informative regions from multiple sequence alignments. *BMC Evol Biol* **10:** 210.
Dementhon, K., Iyer, G., and Glass, N.L. (2006) VIB-1 is required for expression of genes necessary for programmed cell death in *Neurospora crassa*. *Eukaryot Cell* **5:** 2161–2173.
Druzhinina, I.S., Schmoll, M., Seiboth, B., and Kubicek, C.P. (2006) Global carbon utilization profiles of wild-type, mutant, and transformant strains of *Hypocrea jecorina*. *Appl Environ Microbiol* **72:** 2126–2133.
Emri, T., Molnár, Z., Szilágyi, M., and Pócsi, I. (2008) Regulation of autolysis in *Aspergillus nidulans*. *Appl Biochem Biotechnol* **151:** 211–220.
Fingerman, I.M., Sutphen, K., Montano, S.P., Georgiadis, M.M., and Vershon, A.K. (2004) Characterization of critical interactions between Ndt80 and MSE DNA defining a novel family of Ig-fold transcription factors. *Nucleic Acids Res* **32:** 2947–2956.
Flipphi, M., Sun, J., Robellet, X., Karaffa, L., Fekete, E., Zeng, A.P., and Kubicek, C.P. (2009) Biodiversity and evolution of primary carbon metabolism in *Aspergillus nidulans* and other *Aspergillus* spp. *Fungal Genet Biol* **46** (Suppl. 1): S19–S44.
Gabriel, I., Olchowy, J., Stanislawska-Sachadyn, A., Mio, T., Kur, J., and Milewski, S. (2004) Phosphorylation of glucosamine-6-phosphate synthase is important but not essential for germination and mycelial growth of *Candida albicans*. *FEMS Microbiol Lett* **235:** 73–80.
Gao, Q., Jin, K., Ying, S.H., Zhang, Y., Xiao, G., Shang, Y., *et al*. (2011) Genome sequencing and comparative transcriptomics of the model entomopathogenic fungi *Metarhizium anisopliae* and *M. acridum*. *PLoS Genet* **7:** e1001264.
Ghassemi, S., Lichius, A., Bidard, F., Lemoine, S., Rossignol, M.N., Herold, S., *et al*. (2015) The importin KAP8 (Pse1/Kap121) is required for nuclear import of the cellulase transcriptional regulator XYR1, asexual sporulation and stress resistance in *Trichoderma reesei*. *Mol Microbiol* **96:** 405–418.
Gruber, F., Visser, J., Kubicek, C.P., and de Graaf, L.H. (1990) Cloning of the *Trichoderma reesei pyrG*-gene and its use as a homologous marker for a high-frequency transformation system. *Curr Genet* **18:** 447–451.
Gruber, S., and Seidl-Seiboth, V. (2012) Self vs. non-self: Fungal cell wall degradation in *Trichoderma*. *Microbiology* **158:** 26–34.
Gruber, S., Kubicek, C.P., and Seidl-Seiboth, V. (2011) Differential regulation of orthologous chitinase genes in mycoparasitic *Trichoderma* species. *Appl Environ Microbiol* **77:** 7217–7226.
Guangtao, Z., Hartl, L., Schuster, A., Polak, S., Schmoll, M., Wang, T., *et al*. (2009) Gene targeting in a nonhomologous end joining deficient *Hypocrea jecorina*. *J Biotechnol* **139:** 146–151.
Guindon, S., Dufayard, J.F., Lefort, V., Anisimova, M., Hordijk, W., and Gascuel, O. (2010) New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Syst Biol* **59:** 307–321.
Hartl, L., and Seiboth, B. (2005) Sequential gene deletions in *Hypocrea jecorina* using a single blaster cassette. *Curr Genet* **48:** 204–211.
Häkkinen, M., Sivasiddarthan, D., Aro, N., Saloheimo, M., and Pakula, T.M. (2015) The effects of extracellular pH and of the transcriptional regulator PACI on the transcriptome of *Trichoderma reesei*. *Microb Cell Fact* **14:** 63.
Hull, E.P., Green, P.M., Arst, H.N., Jr, and Scazzocchio, C. (1989) Cloning and physical characterization of the L-proline catabolism gene cluster of *Aspergillus nidulans*. *Mol Microbiol* **3:** 553–559.
Hutchison, E.A., and Glass, N.L. (2010) Meiotic regulators Ndt80 and ime2 have different roles in *Saccharomyces* and *Neurospora*. *Genetics* **185:** 1271–1282.
Ishijima, S.A., Hayama, K., Takahashi, M., Holmes, A.R., Cannon, R.D., and Abe, S. (2012) *N*-acetylglucosamine increases symptoms and fungal burden in a murine model of oral candidiasis. *Med Mycol* **50:** 252–258.
Jones, S.A., Arst, H.N., Jr, and MacDonald, D.W. (1981) Gene roles in the *prn* cluster of *Aspergillus nidulans*. *Curr Genet* **3:** 49–56.
Kamerewerd, J., Zadra, I., Kurnsteiner, H., and Kück, U. (2011) PcchiB1, encoding a class V chitinase, is affected by PcVelA and PcLaeA, and is responsible for cell wall integrity in *Penicillium chrysogenum*. *Microbiology* **157:** 3036–3048.
Katoh, K., and Toh, H. (2008) Recent developments in the MAFFT multiple sequence alignment program. *Brief Bioinform* **9:** 286–298.
Katz, M.E., Gray, K.A., and Cheetham, B.F. (2006) The *Aspergillus nidulans xprG* (*phoG*) gene encodes a putative transcriptional activator involved in the response to nutrient limitation. *Fungal Genet Biol* **43:** 190–199.
Katz, M.E., Braunberger, K., Yi, G., Cooper, S., Nonhebel, H.M., and Gondro, C. (2013) A p53-like transcription factor similar to Ndt80 controls the response to nutrient stress in the filamentous fungus. *Aspergill Nidul. F1000Res* **2:** 72.
Konopka, J.B. (2012) *N*-acetylglucosamine (GlcNAc) functions in cell signaling. *Sci (Cairo)* **2012**. (In press).
Kubicek, C.P., Herrera-Estrella, A., Seidl-Seiboth, V., Martinez, D.A., Druzhinina, I.S., Thon, M., *et al*. (2011) Comparative genome sequence analysis underscores mycoparasitism as the ancestral life style of *Trichoderma*. *Genome Biol* **12:** R40.
Kumar, M.J., Jamaluddin, M.S., Natarajan, K., Kaur, D., and Datta, A. (2000) The inducible *N*-acetylglucosamine catabolic pathway gene cluster in *Candida albicans*: discrete *N*-acetylglucosamine-inducible factors interact at the promoter of *NAG1*. *Proc Natl Acad Sci USA* **97:** 14218–14223.
Lamoureux, J.S., and Glover, J.N. (2006) Principles of protein-DNA recognition revealed in the structural analysis of Ndt80-MSE DNA complexes. *Structure* **14:** 555–565.

Latgé, J.P. (2007) The cell wall: a carbohydrate armour for the fungal cell. *Mol Microbiol* **66:** 279–290.

Li, H., Morimoto, K., Katagiri, N., Kimura, T., Sakka, K., Lun, S., and Ohmiya, K. (2002) A novel beta-*N*-acetylglucosaminidase of *Clostridium paraputrificum* M-21 with high activity on chitobiose. *Appl Microbiol Biotechnol* **60:** 420–427.

Litzinger, S., Fischer, S., Polzer, P., Diederichs, K., Welte, W., and Mayer, C. (2010) Structural and kinetic analysis of *Bacillus subtilis N*-acetylglucosaminidase reveals a unique Asp-His dyad mechanism. *J Biol Chem* **285:** 35675–35684.

Liu, D., Coloe, S., Baird, R., and Pederson, J. (2000) Rapid mini-preparation of fungal DNA for PCR. *J Clin Microbiol* **38:** 471.

López-Mondéjar, R., Catalano, V., Kubicek, C.P., and Seidl, V. (2009) The beta-*N*-acetylglucosaminidases NAG1 and NAG2 are essential for growth of *Trichoderma atroviride* on chitin. *FEBS J* **276:** 5137–5148.

MacDonald, S.S., Blaukopf, M., and Withers, S.G. (2015) *N*-acetylglucosaminidases from CAZy family GH3 are really glycoside phosphorylases, thereby explaining their use of histidine as an acid/base catalyst in place of glutamic acid. *J Biol Chem* **290:** 4887–4895.

Mach, R.L., Schindler, M., and Kubicek, C.P. (1994) Transformation of *Trichoderma reesei* based on hygromycin B resistance using homologous expression signals. *Curr Genet* **25:** 567–750.

Mach, R.L., Peterbauer, C.K., Payer, K., Jaksits, S., Woo, S.L., Zeilinger, S., *et al*. (1999) Expression of two major chitinase genes of *Trichoderma atroviride* (*T. harzianum* P1) is triggered by different regulatory signals. *Appl Environ Microbiol* **65:** 1858–1863.

Magliano, P., Flipphi, M., Sanglard, D., and Poirier, Y. (2011) Characterization of the *Aspergillus nidulans* biotin biosynthetic gene cluster and use of the *bioDA* gene as a new transformation marker. *Fungal Genet Biol* **48:** 208–215.

Mandels, M.M., and Andreotti, R.E. (1978) The cellulose to cellulase fermentation. *Proc Biochem* **13:** 6–13.

Mayer, C., Vocadlo, D.J., Mah, M., Rupitz, K., Stoll, D., Warren, R.A., and Withers, S.G. (2006) Characterization of a beta-*N*-acetylhexosaminidase and a beta-*N*-acetylglucosaminidase/beta-glucosidase from *Cellulomonas fimi*. *FEBS J* **273:** 2929–2941.

Milewski, S., Gabriel, I., and Olchowy, J. (2006) Enzymes of UDP-GlcNAc biosynthesis in yeast. *Yeast* **23:** 1–14.

Moreno-Mateos, M.A., Delgado-Jarana, J., Codón, A.C., and Benítez, T. (2007) pH and Pac1 control development and antifungal activity in *Trichoderma harzianum*. *Fungal Genet Biol* **44:** 1355–1367.

Muzzarelli, R.A. (1999) Native, industrial and fossil chitins. *EXS* **87:** 1–6.

Naseem, S., Gunasekera, A., Araya, E., and Konopka, J.B. (2011) *N*-acetylglucosamine (GlcNAc) induction of hyphal morphogenesis and transcriptional responses in *Candida albicans* are not dependent on its metabolism. *J Biol Chem* **286:** 28671–28680.

Naseem, S., Araya, E., and Konopka, J.B. (2015) Hyphal growth in *Candida albicans* does not require induction of hyphal-specific gene expression. *Mol Biol Cell* **26:** 1174–1187.

Niones, J.T., and Takemoto, D. (2015) VibA, a homologue of a transcription factor for fungal heterokaryon incompatibility, is involved in antifungal compound production in the plant-symbiotic fungus *Epichloe festucae*. *Eukaryot Cell* **14:** 13–24.

Pare, A., Kim, M., Juarez, M.T., Brody, S., and McGinnis, W. (2012) The functions of grainy head-like proteins in animals and fungi and the evolution of apical extracellular barriers. *PLoS ONE* **7:** e36254.

Penttilä, M., Nevalainen, H., Ratto, M., Salminen, E., and Knowles, J. (1987) A versatile transformation system for the cellulolytic filamentous fungus *Trichoderma reesei*. *Gene* **61:** 155–164.

Peñalva, M.A., and Arst, H.N., Jr (2004) Recent advances in the characterization of ambient pH regulation of gene expression in filamentous fungi and yeasts. *Annu Rev Microbiol* **58:** 425–451.

Pfaffl, M.W., Horgan, G.W., and Dempfle, L. (2002) Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res* **30:** e36.

Pócsi, I., Leiter, E., Kwon, N.J., Shin, K.S., Kwon, G.S., Pusztahelyi, T., *et al*. (2009) Asexual sporulation signalling regulates autolysis of *Aspergillus nidulans* via modulating the chitinase ChiB production. *J Appl Microbiol* **107:** 514–523.

Pusztahelyi, T., and Pócsi, I. (2014) Chitinase but *N*-acetyl-beta-D-glucosaminidase production correlates to the biomass decline in *Penicillium* and *Aspergillus* species. *Acta Microbiol Immunol Hung* **61:** 131–143.

Pusztahelyi, T., Molnár, Z., Emri, T., Klement, E., Miskei, M., Kerékgyárto, J., *et al*. (2006) Comparative studies of differential expression of chitinolytic enzymes encoded by *chiA*, *chiB*, *chiC* and *nagA* genes in *Aspergillus nidulans*. *Folia Microbiol (Praha)* **51:** 547–554.

Rao, K.H., Ghosh, S., Natarajan, K., and Datta, A. (2013) *N*-Acetylglucosamine kinase, HXK1 is involved in morphogenetic transition and metabolic gene expression in *Candida albicans*. *PLoS ONE* **8:** e53638.

Ruiz-Herrera, J. (1991) *Fungal Cell Wall: Structure, Synthesis and Assembly*. Boca Raton: CRC Press.

Sámi, L., Pusztahelyi, T., Emri, T., Varecza, Z., Fekete, A., Grallert, A., *et al*. (2001) Autolysis and aging of *Penicillium chrysogenum* cultures under carbon starvation: Chitinase production and antifungal effect of allosamidin. *J Gen Appl Microbiol* **47:** 201–211.

Sambrook, J., and Russell, D.W. (2001) *Molecular Cloning: A Laboratory Manual*. Painview, NY: Cold Spring Harbor Lab. Press.

Schuster, A., Bruno, K.S., Collett, J.R., Baker, S.E., Seiboth, B., Kubicek, C.P., and Schmoll, M. (2012) A versatile toolkit for high throughput functional genomics with *Trichoderma reesei*. *Biotechnol Biofuels* **5:** 1.

Seiboth, B., Ivanova, C., and Seidl-Seiboth, V. (2011) Trichoderma reesei: A fungal enzyme producer for cellulosic biofuels. In *Biofuel Production-Recent Developments and Prospects*. dos Santos Bernardes, M.A. (ed.). Rijeka, Croatia: InTech. pp. 309–340. [WWW document]. URL http://www.intechopen.com/books/biofuel-production-recent-developments-and-prospects/trichoderma-reesei-a-fungal-enzyme-producer-for-cellulosic-biofuels:

Seidl, V. (2008) Chitinases of filamentous fungi: a large group of diverse proteins with multiple physiological functions. *Fungal Biol Rev* **22:** 36–42.

Seidl, V., Seiboth, B., Karaffa, L., and Kubicek, C.P. (2004) The fungal STRE-element-binding protein Seb1 is involved but not essential for glycerol dehydrogenase (*gld1*) gene expression and glycerol accumulation in *Trichoderma atroviride* during osmotic stress. *Fungal Genet Biol* **41:** 1132–1140.

Seidl, V., Huemer, B., Seiboth, B., and Kubicek, C.P. (2005) A complete survey of *Trichoderma* chitinases reveals three distinct subgroups of family 18 chitinases. *FEBS J* **272:** 5923–5939.

Seidl, V., Druzhinina, I.S., and Kubicek, C.P. (2006) A screening system for carbon sources enhancing β-*N*-acetylglucosaminidase formation in *Hypocrea atroviridis* (*Trichoderma atroviride*). *Microbiology* **152:** 2003–2012.

Shin, K.S., Kwon, N.J., Kim, Y.H., Park, H.S., Kwon, G.S., and Yu, J.H. (2009) Differential roles of the ChiB chitinase in autolysis and cell death of *Aspergillus nidulans*. *Eukaryot Cell* **8:** 738–746.

Tsujibo, H., Fujimoto, K., Tanno, H., Miyamoto, K., Imada, C., Okami, Y., and Inamori, Y. (1994) Gene sequence, purification and characterization of *N*-acetyl-beta-glucosaminidase from a marine bacterium, *Alteromonas* sp. strain O-7. *Gene* **146:** 111–115.

Tzelepis, G.D., Melin, P., Jensen, D.F., Stenlid, J., and Karlsson, M. (2012) Functional analysis of glycoside hydrolase family 18 and 20 genes in *Neurospora crassa*. *Fungal Genet Biol* **49:** 717–730.

Wiemann, P., and Keller, N.P. (2014) Strategies for mining fungal natural products. *J Ind Microbiol Biotechnol* **41:** 301–313.

Wisecaver, J.H., and Rokas, A. (2015) Fungal metabolic gene clusters-caravans traveling across genomes and environments. *Front Microbiol* **6:** 161.

Wortman, J.R., Gilsenan, J.M., Joardar, V., Deegan, J., Clutterbuck, J., Andersen, M.R., *et al*. (2009) The 2008 update of the *Aspergillus nidulans* genome annotation: a community effort. *Fungal Genet Biol* **46** (Suppl. 1): S2–S13.

Xiang, Q., and Glass, N.L. (2002) Identification of *vib-1*, a locus involved in vegetative incompatibility mediated by *het-c* in *Neurospora crassa*. *Genetics* **162:** 89–101.

Xiao, G., Ying, S.H., Zheng, P., Wang, Z.L., Zhang, S., Xie, X.Q., *et al*. (2012) Genomic perspectives on the evolution of fungal entomopathogenicity in *Beauveria bassiana*. *Scientific Reports* **2:** 483.

Xiong, Y., Sun, J., and Glass, N.L. (2014) VIB1, a link between glucose signaling and carbon catabolite repression, is essential for plant cell wall degradation by *Neurospora crassa*. *PLoS Genet* **10:** e1004500.

Xu, L., Ajimura, M., Padmore, R., Klein, C., and Kleckner, N. (1995) NDT80, a meiosis-specific gene required for exit from pachytene in *Saccharomyces cerevisiae*. *Mol Cell Biol* **15:** 6572–6581.

Zheng, P., Xia, Y., Xiao, G., Xiong, C., Hu, X., Zhang, S., *et al*. (2011) Genome sequence of the insect pathogenic fungus *Cordyceps militaris*, a valued traditional Chinese medicine. *Genome Biol* **12:** R116.

Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Chapter 1: GlcNAc-catabolism in filamentous fungi

Chapter 1.3

N-acetylglucosamine, the monomeric building block of chitin, inhibits growth of *Neurospora crassa*.

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N-acetylglucosamine, the monomeric building block of chitin, inhibits growth of *Neurospora crassa*

Romana Gaderer, Verena Seidl-Seiboth and Lisa Kappel

Abstract

N-acetylglucosamine (GlcNAc) is the monomer of the polysaccharide chitin and is in fungi an important structural component of the fungal cell wall. GlcNAc can also serve as nutrient source for filamentous fungi. We could recently show that the genes encoding the enzymes necessary for catabolism of GlcNAc are clustered in filamentous ascomycete fungi, including *Neurospora crassa*. But GlcNAc-catabolism has not been studied in *N. crassa* so far. In this study we investigated the GlcNAc-catabolism cluster genes in *N. crassa* with respect to their gene expression and analysed growth behaviour and biomass formation of *N. crassa* on GlcNAc as carbon source. The results showed that, in contrast to *Trichoderma* species, GlcNAc is surprisingly a very poor carbon source for *N. crassa*. These results were confirmed with four *N. crassa* wild-type isolates. GlcNAc turned out to be even growth-inhibiting in the presence of other carbon sources. Nonetheless we found that the GlcNAc-catabolism cluster genes were still expressed on GlcNAc. Since our findings, as well as data from other organisms reported in the literature, suggested that the enzymatic conversion from glucosamine-6-phosphate to fructose-6-phosphate performed by the glucosamine-6-phosphate deaminase could be a bottleneck in GlcNAc-catabolism, we attempted to overexpress the functional GlcN-6-phosphate deaminase gene (*dam1*) from *Trichoderma reesei* in a *N. crassa* wild-type and a *dam-1* deletion strain. Indeed, investigations of the *T. reesei dam1* overexpression strains exhibited improved GlcNAc utilisation, but the results showed that growth was still relatively sparse in comparison to D-glucose, which is in contrast to other fungi such as *Trichoderma* species.

Introduction

Chitin consists of β-(1,4) linked residues of *N*- acetylglucosamine units (GlcNAc, 2-acetamido-2-deoxy-D-glucopyranose) and is a major constituent of the shells of crustaceans and insects.

Chitin can also be found as a major component in the exoskeleton of arthropods and nematodes and in the cell walls of algae and fungi, and is overall considered to be the second most abundant polymer on earth [1-5]. In fungi, chitin is located in the inner layer of the cell wall close to the plasma membrane [6] and constitutes together with β-(1,3 1,6) glucan the structural scaffold of the cell wall [7]. Bacteria and fungi are responsible for the turnover of chitin in soil and marine environments. Genomic studies showed that filamentous fungi have a multitude of genes encoding chitinolytic enzymes [8, 9]. Enzymatic degradation of chitin involves chitinases (EC.3.2.1.14), which are in fungi members of the glycoside hydrolase (GH) 18 family and catalyze the hydrolysis of the β-1,4 linkages in chitin and chitooligomers, releasing short-chain chitooligosaccharide products with a minimum chain length of 2 GlcNAc molecules [10]. Filamentous fungi have typically between 10 and 30 different chitinases, whereas fungi with yeast and yeast-like forms exhibit low numbers of chitinases [8, 9]. $(GlcNAc)_2$ is converted into GlcNAc monomers by *N*-acetylglucosaminidases belonging to GH family 20 in fungi [8]. GlcNAc can subsequently be taken up into the cell and used as carbon and nitrogen source via three additional steps that lead to generation of fructose-6-phosphate, which subsequently can enter glycolysis. However, GlcNAc not only undergoes catabolic recycling, but also feeds into anabolism as UDP-GlcNAc, which is a building block for *de novo* chitin synthesis in the cell wall [11, 12]. We were recently able to show that the genes involved in the conversion of GlcNAc to fructose-6-phosphate are clustered in fungi. The GlcNAc-catabolism gene cluster was first described in the human pathogen *Candida albicans*. *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* lack the genes needed to catabolize GlcNAc [13-15]. In our recent study we found that this cluster is also conserved in filamentous fungi, including *Trichoderma* spp. and *Neurospora crassa* [16]. The three main enzymes that are responsible for the stepwise conversion of GlcNAc into fructose-6-phosphate are a GlcNAc-hexokinase (*Trichoderma*: HXK3; *C. albicans:* Nag5/Hxk1), a GlcNAc-6-phosphate deacetylase (*Trichoderma*: DAC1; *C. albicans*: Nag2/Dac1) and a glucosamine-6-phosphate deaminase (*Trichoderma*: DAM1; *C. albicans:* Nag1) [17, 18]. Furthermore, a gene belonging to GH3-family, namely *nag3*, occurs in the GlcNAc-catabolism cluster and the protein exhibits similarity to bacterial β-*N*-acetylhexosaminidases. A GlcNAc transporter, named NGT1 ($GlcNAc:H^+$ symporter), is located distantly from the cluster ([19-23] and http://www.cazy.org/GH3.html). In contrast to *C. albicans*, the GlcNAc-catabolism cluster of filamentous fungi often includes an Ndt80-

like transcription factor, called RON1 (regulator of *N*-acetylglucosamine catabolism 1), which was recently shown to operate as a key activator of the GlcNAc gene cluster and is essential for GlcNAc-catabolism [16]. The genome of *N. crassa* contains all six genes from the GlcNAc-catabolism, the catabolic genes are similar to the other filamentous fungi, highly conserved and clustered together with the Ndt80-like transcription factor. *N. crassa* has two other Ndt80-like transcription factors, VIB1 and FSD1, whose biological functions seem to be unrelated to GlcNAc-catabolism and therefore are different from that of RON1. FSD1 plays an important role in timing and development of female reproductive structures and ascospore maturation [24] and VIB1 controls the production of proteases upon nutrient starvation, is involved in the regulation of vegetative incompatibility and programmed cell death, as well as plant cell wall degradation by repressing glucose signalling and carbon catabolite repression (CCR) [24-26].

In this study, we investigated the growth of *N. crassa* on chitin and GlcNAc and the inhibitory growth effect of GlcNAc on other carbon sources. Moreover, we characterized the GlcNAc-catabolism cluster genes in *N. crassa* with respect to their gene expression profiles and their effect on GlcNAc utilization.

Material and Methods

Strains and cultivation conditions

The following fungal strains were used in this study: *Neurospora crassa* wild-type (WT) strains 74-OR23-1A(FGSC987), Chilton (FSGC1691), Lindegren25a (FSGC353), Fast growth (FSGC5729) and the *N. crassa* 74-OR23-1A knockout strains Δ*ron-1*(FGSC12181; NCU04729), Δ*dam-1* (FGSC16718; NCU04727), Δ*ngt-1* (FGSC18566; NCU09771), Δ*nag-3* (FGSC22042; NCU04726), Δ*hxk-1* (FGSC 16566; NCU04728). All strains were obtained from the Fungal Genetics Stock Center (FGSC, Kansas City, MO) [27], and maintained on Vogel`s minimal medium (VM) with 1% sucrose at 28°C [28].

Escherichia coli Stellar^{TM} Competent Cells (Clontech Laboratories, Mountain View, CA, USA) were used for propagation of all used and constructed plasmids.

For biomass measurements, samples for gene expression analysis on insoluble crab-shell chitin and growth tests liquid standing cultivations were performed. $1x10^5$ conidia/ ml were cultivated in VM with 1 % of the indicated carbon sources [glycerol (Roth, Karlsruhe, Germany), GlcNAc (Sigma-Aldrich, St.Louis, MO), chitin from crab shell (Sigma-Aldrich, St. Louis, MO), D-glucose (Roth, Karlsruhe, Germany), D-fructose (Sigma-Aldrich, St. Louis, MO) and cellulose (Serva, Heildelberg, Germany)] at 28°C with 12h/ 12h dark/ light cycles. For biomass measurements, cultivations were performed in sterile petri dishes with 5 cm diameter (Greiner Bio-one, Kremsmünster, Austria) containing a volume of 10 ml VM and 1 % carbon source. For gene expression analysis on chitin and growth assays on different carbon sources, cultivations were performed in sterile petri dishes with 9 cm diameter (Greiner Bio-one, Kremsmünster, Austria) containing a volume of 15 ml VM and 1 % carbon source. Mycelia for RNA synthesis were harvested by filtration through Miracloth (Calbiochem, Merck, Darmstadt, Germany), washed briefly with cold tap water and stored at −80°C, samples were taken after 16 h, 24 h and 48 h. Images of the plates from *N. crassa* WT and gene deletion strains were taken after seven days and from *N. crassa* WT and overexpression strains after 12 days.

For gene expression analysis via induction by GlcNAc liquid shaking cultivations were performed in flasks containing 200ml VM medium and 1% glycerol. They were inoculated with $1x10^5$ conidia/ ml and incubated for 24 h at 200 rpm and 28°C, and then the formed fungal biomass was replaced into VM medium with either 1 % glycerol (as control) or 1 % GlcNAc. Samples were taken after 0.5 h, 1 h and 2 h after shifting. Mycelia were harvested by filtration through Miracloth (Calbiochem, Merck, Darmstadt, Germany), washed briefly with cold tap water, immediately immersed in liquid nitrogen and stored at −80°C.

For viability assays on GlcNAc *N. crassa* WT conidia were incubated in 1 ml liquid standing VM medium containing 1 % GlcNAc in 2 ml tubes for 20 h. Thereafter the spores were washed twice with distilled water and shifted into 6 well plates (Greiner Bio-one, Kremsmünster, Austria) containing 3 ml VM medium each with either 1 % glycerol, 1 % D-glucose or 1 % GlcNAc (negative control). As positive control *N. crassa* conidia were pre-incubated in VM containing 1 % D-glucose or 1 % glycerol instead of GlcNAc.

Biomass measurements

The total protein concentration, corresponding to biomass formation, was measured for mycelial biomass after extraction with NaOH as described in [29]. 10 ml of each sample were harvested by centrifugation at 5000 x *g* for 10 min and mycelia were washed twice with distilled water. Then mycelia were incubated with 1 ml 1M NaOH for 3 h by shaking (1000 rpm) at room temperature, and centrifuged again. The protein content was determined using the Bradford protein assay (Bio-rad, Hercules, CA, USA) with BSA as standard. All extractions and measurements were performed at least in duplicates. Statistical analysis of the results was performed with student´s t-test, assuming unequal variance of groups.

Plasmid construction

For generation of the *dam1* overexpression construct, the respective coding region of *dam1*, the GlcNAc-deaminase of *Trichoderma reesei* (JGI Protein ID 49898, NCBI accession number: XP_006966912.1), was amplified from *T. reesei* cDNA with the primer pair FW_INFU_DAM_T.r. and RV_INFU_DAM_T.r (see Suppl. Tab.1). A suitable host vector, pAL6-lifeact (FGSC760, http://www.fgsc.net), which harbours a strong Pccg-1 promoter [30, 31] and a nourseothricin (*nat1*) [32] selection gene, was chosen and linearized via PCR using FW_pAL6_linear and RV_pAL6_linear primer (see Suppl. Tab.1). The reporter peptide (Lifeact) and the fluorescent protein (TagRFP-T) of the pAL6-lifeact plasmid were regarded disadvantageous for this experiment, and therefore replaced with *T. reesei dam1* gene. Plasmid construction was carried out using the *in vitro* cloning In-Fusion® PCR cloning system (Clontech, Takara Bio Europe, Saint-Germain-en-Laye, France). The construct was verified by sequencing and ectopically transformed into *N. crassa* WT 74-OR23-1A and the corresponding Δ*dam1* strain using a standard electroporation protocol for *N. crassa* [33]. Transformants were selected by recovery on VM medium with 10 % $CaCl_2$ and 10 % $MgSO_4$ and 400 µg/ ml nourseothricin and subsequently purified by single spore isolation, twice, and screened with PCR using the primers FW_Trafo_check_DAM and RV_Trafo_check_DAM (Suppl. Fig.2a). For verification of overexpression strains, genomic DNA was isolated using a rapid DNA purification protocol [34]. And the expression of *T. reesei* dam1 was checked by

RT-PCR using the primers Tr_DAM_RTs and Tr_DAM_RTa (Suppl. Fig.2b). All oligonucleotides are listed in Suppl. Tab.1.

RNA Isolation

For RNA isolation the mycelia from those samples that were grown on D-glucose, glycerol and GlcNAc, were ground to fine powder under liquid nitrogen and total RNA was isolated using the guanidinium thiocyanate method [35]. RNA from chitin samples was isolated with the RNeasy Plant Mini Kit (Qiagen). Isolated RNAs were treated with DNAse I (Fermentas, St Leon-Rot, Germany), and cDNAs were generated with the Revert Aid H-minus cDNA synthesis kit (Fermentas), using 5 µg RNA.

Gene expression analysis

qPCR reactions were performed in an Eppendorf Realplex thermal cycler. The reaction mix contained 12.5 µl SYBR green Supermix (Bio-Rad Laboratories, Hercules, CA, USA), 8.5 µl pure water, 6.25 µM forward and 6.25 µM reverse primer, and 2µl 1:50 diluted template cDNA. Reactions were performed in triplicates. Primer efficiency was calculated using a diluted series from 1:5 to 1:5000 with the PCR baseline-subtracted mode. The amplification protocol consisted of an initial denaturation step for 3 min 95°C followed by 40 cycles of denaturation (95°C for 15 s), annealing, and elongation (60°C for 15 s). Oligonucleotides and protein IDs in the *N. crassa* genome database are listed in Suppl. Tab1. The *act1* (*actin1*, NCU04173) gene was used as reference. Expression data were evaluated using REST software [36]. All samples were analysed in at least two independent experiments with three replicates in each run. Statistical analysis of the results was performed with student´s t-test, assuming unequal variance of groups.

Microscopic analyses

For morphological characterization, liquid VM medium supplemented with the carbon sources indicated in the respective results sections, were incubated for 8 h at 28°C. 200 µl of

each culture were placed on microscope chambered coverglass devices with eight wells (Thermo Fisher Scientific) and imaged with an inverted Nikon T300 microscope (Nikon, Tokyo, Japan) with differential interference contrast optics and a DXM1200F digital camera (Nikon).

Results

***1) GlcNAc inhibits growth of* N. crassa**

Several bacteria as well as various fungi have been reported to be able to utilize GlcNAc as sole carbon source, including filamentous fungi from the genera *Trichoderma* and *Apergillus* [37, 38]. In analogy to other filamentous fungi, the genome of *N. crassa* contains a gene cluster located on linkage group (chromosome) 6 encoding the enzymes involved in GlcNAc-catabolism and a transcription factor. Distant from the GlcNAc gene cluster, a gene encoding the GlcNAc transporter is located on linkage group (chromosome) 2. Following the nomenclature that was used for *T. reesei* [16], we named the respective genes in *N. crassa* *hxk-3* (NCU04728), *dac-1* (NCU04725), *dam-1* (NCU04727), *nag-3* (NCU04726), *ngt-1* (NCU09771) and *ron-1* (NCU04729), one of the three Ndt80-like transcription factors also described in Hutchison, *et al* [24]. Since *Trichoderma* species are readily able to utilize GlcNAc as carbon source and the GlcNAc-catabolic cluster is strongly conserved between *Trichoderma* spp. and *N. crassa,* we anticipated that *N. crassa* would grow equally well on GlcNAc as sole carbon source. However, when we compared the growth of *N. crassa* WT 74A on VM medium containing different carbon sources (glycerol, D-glucose, cellulose, D-fructose, chitin and GlcNAc), the results (Fig.1a) showed surprisingly that *N. crassa* was unable to grow on GlcNAc.

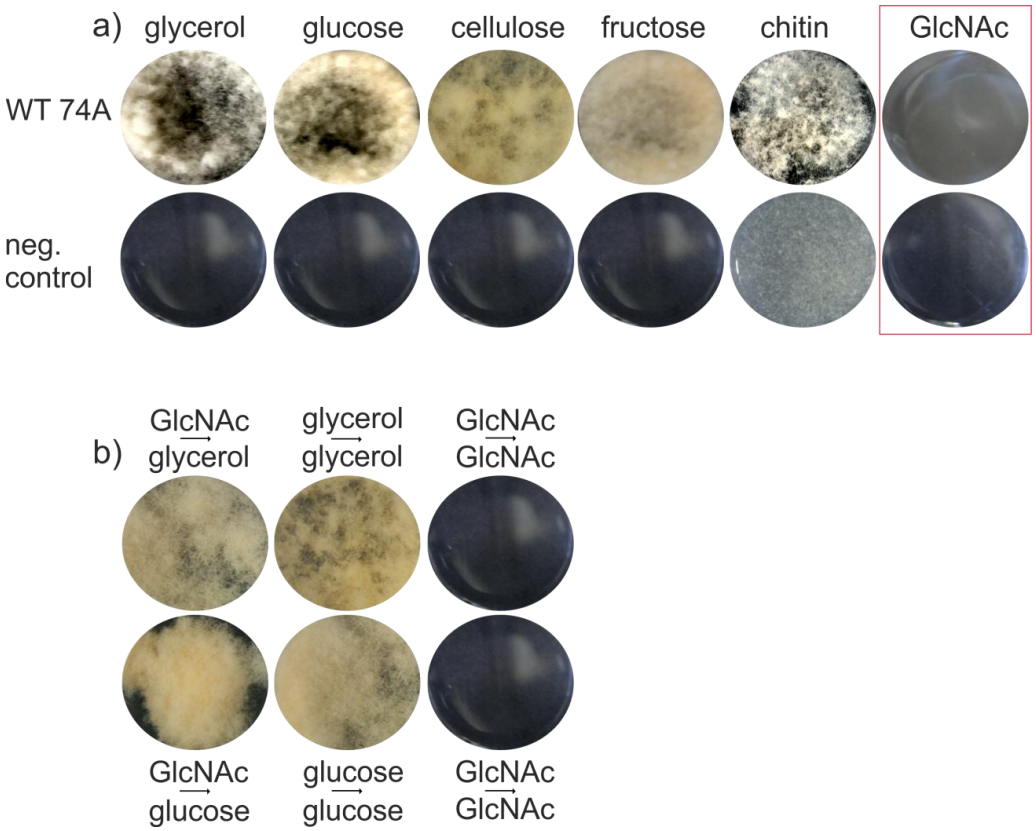


Fig.1: Growth of *N. crassa* WT 74A on different carbon sources. a) Growth of the WT on VM containing different carbon sources (1% glycerol, 1% glucose, 1% cellulose, 1% fructose, 1% chitin and 1% GlcNAc) after 7 days. Negative control was VM medium containing the appropriate carbon source without spores. **b)** WT on VM containing 1% glycerol or 1% glucose after incubation for 20 h on VM with 1% GlcNAc. Positive control was only incubated and replaced on VM containing 1% glycerol or 1% glucose and negative control was replaced again on VM containing 1% GlcNAc. Pictures were taken after 7 days.

Somewhat in contrast to that finding, *N. crassa* was able to grow on medium containing purified chitin powder from crab shells (Suppl. Fig.1) and colloidal chitin, a better accessible, acid-pre-treated, purified form of chitin. In order to verify that the observed growth defect was not specific for *N. crassa* WT strain 74A, we tested three additional WT strains (Chilton (FSGC1691), Lindegren25a (FSGC353), Fast growth (FSGC5729)). All tested *N. crassa* WT strains were unable to grow on GlcNAc (data not shown).

In order to analyse the growth defect on GlcNAc in more detail, the concentration of GlcNAc was varied between 0.1 % and 1 %. With all tested GlcNAc concentrations, growth of *N. crassa* was inhibited.

Next we tested if GlcNAc is simply not catabolized by the fungus or, beyond that, if it is even growth-inhibiting in the presence of other carbon sources. Therefore, growth assays with the *N. crassa* WT 74A on VM containing 1 % GlcNAc and 1 % glycerol, chitin, cellulose, D-glucose or D-fructose were performed. GlcNAc was clearly growth-inhibitory in the presence of glycerol (Fig.2a), and growth was strongly diminished. The growth-inhibitory effect on glycerol was less pronounced when the GlcNAc concentration was lowered. Combination of 1 % GlcNAc with 1 % of the polymeric carbon sources cellulose and chitin resulted also in strong growth inhibition (data not shown). Only upon supplementation with D-glucose and D-fructose, restoration of growth was observed (data not shown). This might be due to carbon catabolite repression that is caused by these carbon sources, in contrast to glycerol, which is generally regarded as a non-repressive carbon source. Biomass formation of the WT strain that was cultivated on VM containing 1% glycerol, 1% glycerol + 1 % GlcNAc and 1% GlcNAc was analysed after 16 h, 24 h and 48 h of growth. Since biomass formation was too low for mycelial dry weight measurements, biomass was carefully harvested (by centrifugation), lysed and quantified by total protein measurements (see Materials and Methods for details). As observed by visual inspection in the growth assays described above, quantification of the biomass showed that growth of *N. crassa* WT was very low on medium containing GlcNAc as sole carbon source. After 48 h of growth a value of 13 +/- 1 µg/ ml protein was measured and also on medium containing both, glycerol and GlcNAc, the value was with 55 +/- 1.5 µg/ ml still only 29 % of that on glycerol alone (192 +/- 33 µg/ ml) (Fig.2b).

Further, we investigated if incubation of *N. crassa* conidia in medium containing GlcNAc solely inhibits growth or if GlcNAc leads to cell death of the conidia or germlings. In order to test that, conidia were incubated in medium containing 1% GlcNAc for 20 h, thereafter washed twice with distilled water and then replaced into medium containing 1% glycerol or 1% D-glucose, respectively. Interestingly, on both carbon sources normal growth was restored upon medium shift (Fig.1b). Thus, these results showed that under our experimental conditions, i.e. incubation with GlcNAc for up to 20 h, *N. crassa* conidia were still viable. Germination was initiated on GlcNAc but hyphal growth appeared to proceed very slowly. In order to test whether GlcNAc exerted its growth-inhibitory effect mainly during the stages of spore germination and initial growth or if it is also growth inhibitory for mature hyphae, we also tested if *N. crassa* WT strains could grow on GlcNAc containing

medium when mycelium was used as starting material. The findings showed that also growth of the mycelium itself was affected by GlcNAc (data not shown).

In *C. albicans* a growth defect on GlcNAc-containing medium was described for the three single knockout strains *hxk1*, *nag1*, and *dac1* (and a triple mutant lacking all three catabolic genes (*hxk1*Δ*nag1*Δdac*1*Δ)) [18]. Deletion mutants of *nag1* and *dac1* were not even able to grow on medium containing GlcNAc with a supplementary carbon source such as D-glucose, D-fructose and D-galactose, suggesting that excess GlcNAc-6-phosphate is deleterious. However, growth of *hxk1* single knockout strain and the triple mutant (*hxk1*Δ*nag1*Δ*dac1*Δ) was recovered on medium containing GlcNAc in addition to galactose [14, 18]. In the *N. crassa* strain collection from FGSC, gene knockout strains for *hxk-3*, *dam-1*, *ron-1*, *nag-3* and *ngt-1* are available. We therefore investigated growth of these strains on GlcNAc (Suppl. Fig.1) in order to test whether a defect in the GlcNAc-catabolism pathway at certain steps would have an effect on growth on GlcNAc alone or in the presence of other carbon sources. However, the results were similar as those that we found for the WT and our findings again showed that growth was also inhibited in the presence of glycerol (Fig.2a), but not glucose (data not shown).

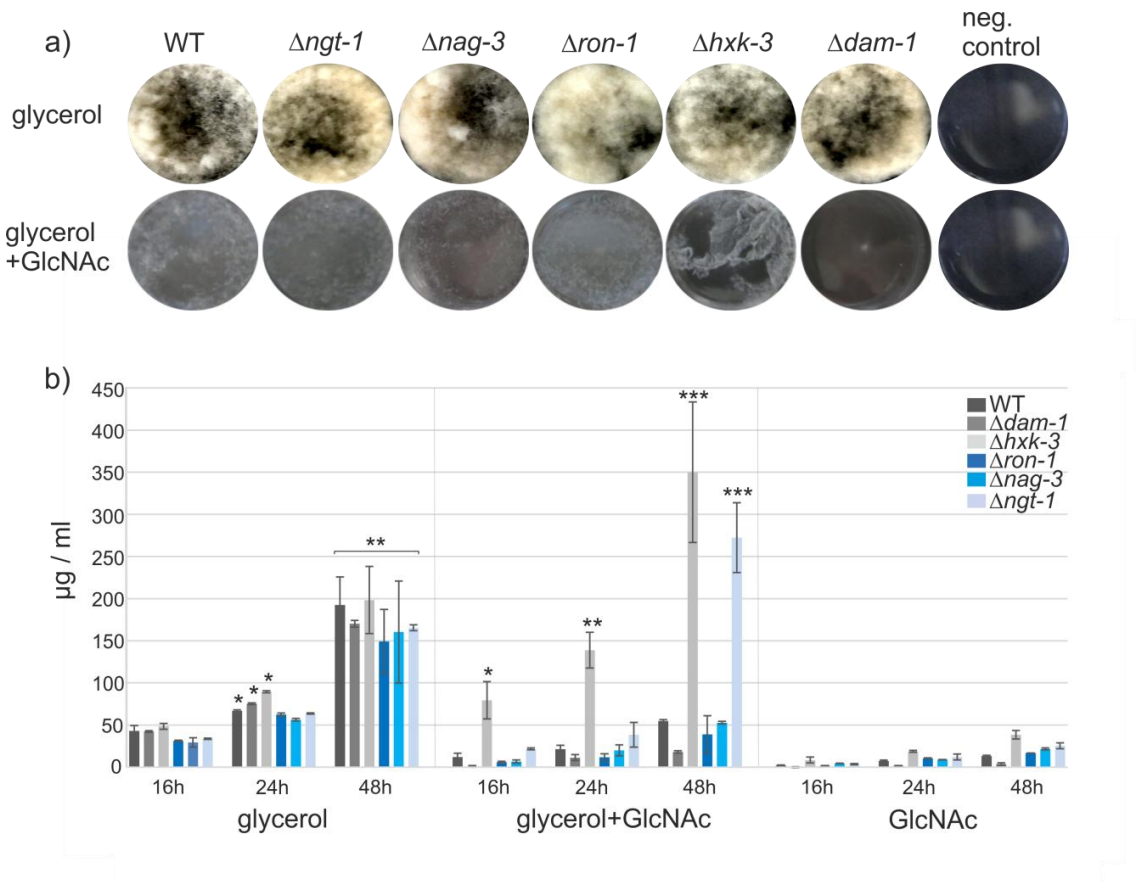


Fig.2: Growth tests and biomass formation of *N. crassa* WT 74A and GlcNAc catabolic gene knockout strains (Δ*ngt-1*, Δ*nag-3*, Δ*ron-1*, Δ*hxk-3*, Δ*dam-1*). a) WT and knockout strains were grown for seven days on VM containing 1% glycerol and VM containing 1% glycerol + 1% GlcNAc as carbon source. Negative control was VM with the corresponding carbon source without spores. **b)** For biomass measurements, strains were grown in liquid standing VM medium, containing 1% glycerol or 1% gylcerol+1% GlcNAc or 1% GlcNAc and total protein concentration was determined after 16 h, 24 h and 48 h after inoculation.

Quantification of biomass based on total protein measurements – biomass formation was too low for mycelial dry weight measurements – showed that *N. crassa* WT and all tested knockout strains exhibited no significant differences in biomass content on glycerol as carbon source, which was used as control. On medium containing only GlcNAc as carbon source, biomass formation of all tested strains was very low (between 4-25 µg/ ml after 48 h), similar to the results of the visual inspection of the growth tests, except for Δ*hxk-3*, with

slightly higher - but not statistically significant – values of 38 +/- 5 µg/ ml after 48 h of growth. Interestingly, on medium containing both, 1 % glycerol and 1 % GlcNAc, biomass formation of Δ*hxk-3* was significantly increased (350 +/- 83 µg/ ml after 48 h) and was considerably higher than that of the WT with 55 +/- 1.5 µg/ ml and even higher than Δ*hxk-3* on medium containing only glycerol with 198 +/- 39 µg/ ml (Fig. 2b). In Fig.2a the difference between Δ*hxk-3* and WT on medium containing glycerol and GlcNAc was not obviously discernible with the naked eye, but we observed that Δ*hxk-3* formed plenty of mycelium but did not sporulate well. Interestingly also the *ngt-1* knockout strain showed increased biomass formation (272 +/- 41 µg/ ml) after 48 h of growth compared to the WT.

2) *The GlcNAc-catabolism cluster genes are induced by GlcNAc but not during growth on chitin*

In order to get first insights into gene expression levels of the GlcNAc-catabolic cluster genes i.e. *hxk-3*, *dac-1*, *dam-1*, *ron-1*, *nag-3*, as well as the transporter-encoding gene *ngt-1*, we analysed their expression in *N. crassa* WT strain 74A on medium containing chitin or GlcNAc as carbon source. For analysis via induction by GlcNAc as carbon source a replacement strategy was followed. Shake flask cultivations with VM medium containing 1 % glycerol as non-repressive carbon source were inoculated with *N. crassa* WT conidia and incubated for 24 hours, and after that replaced into VM medium with 1 % glycerol (as control) or 1% GlcNAc. Samples were taken after 0.5h, 1h and 2h after replacing (for more detailed information see Materials and Methods section). Induction of gene expression by chitin was investigated in liquid standing cultivations with 1 % chitin after incubation for 16h, 24h and 48h and compared to cultivations with glucose as carbon source. Transcription levels of the *N. crassa* GlcNAc catabolic genes were investigated by RT-qPCR. As shown in Fig.3ab, gene expression patterns of all six GlcNAc cluster genes on medium containing chitin showed almost no difference compared to the control (D-glucose). Interestingly, none of the genes was significantly upregulated on chitin, except for *ngt-1*, for which a slight induction (12-fold after 48 h) could be detected. Gene expression of the transcription factor gene *ron-1* and GH3-encoding gene *nag-3* was even somewhat lower on chitin than on D-glucose.

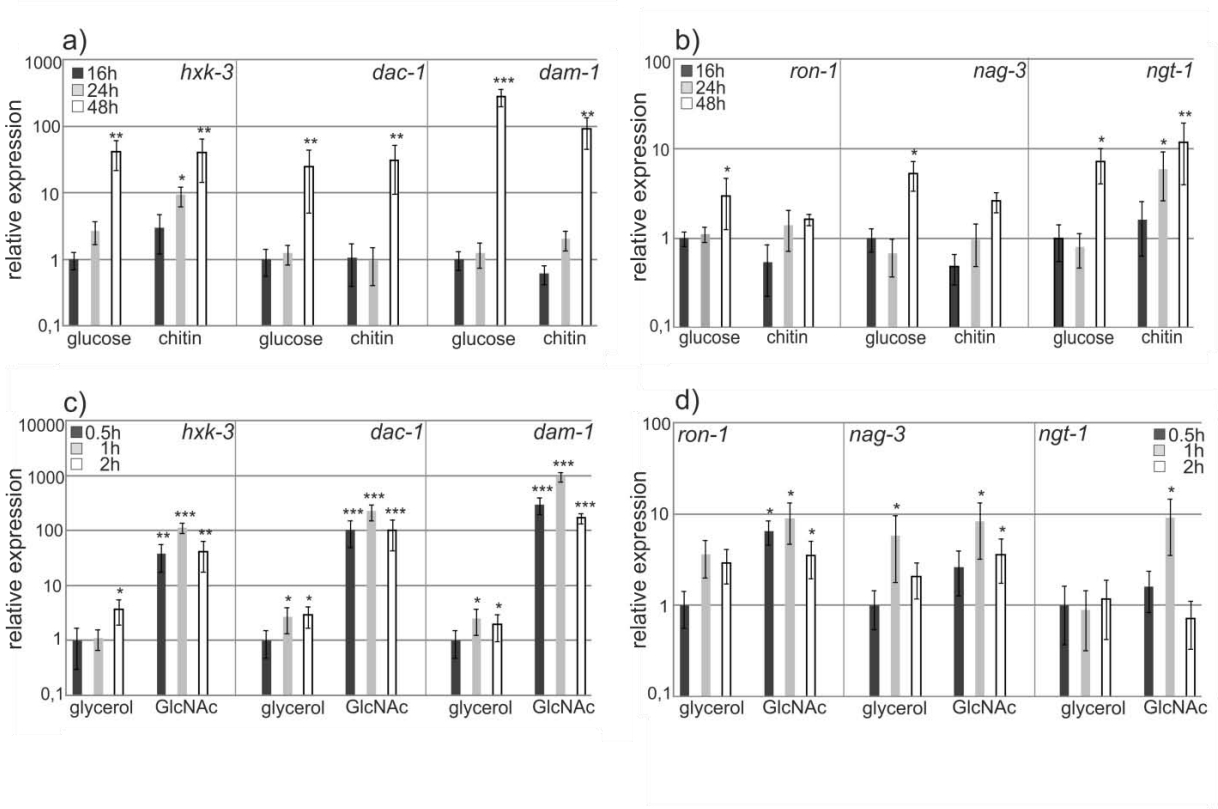


Fig.3: Gene expression (RT-qPCR) of GlcNAc-catabolism cluster genes in the WT 74A strain. a-b) Samples were taken after 16 h, 24 h and 48 h from liquid standing cultivations with glucose or chitin as carbon source. All samples were normalized to the 16 h glucose sample. **c-d)** Samples were taken after 0.5 h, 1 h and 2h after replacing from 1% glycerol into 1% glycerol (control) or 1% GlcNAc. All samples were normalized to the 0.5 h glycerol sample. *act1* was used as reference gene. The standard deviation of the mean expression values from at least two independent biological replicates is shown. Bars indicate the SEM (*, ** and *** indicate significance at $P < 0.05$, 0.01 and 0.001).

In contrast to these findings with chitin as carbon source, all three GlcNAc catabolic genes (*hxk-3*, *dac-1*, *dam-1*) were significantly stronger induced on GlcNAc than on the control glycerol, with 102, 224 and 956-fold, respectively, after 1 h, summarized in Fig.3cd. However, expression of the genes *nag-3*, *ron-1* and *ngt-1* was only moderately higher (9-fold after 1 h) on GlcNAc than on glycerol. After 2 hours, at this time point expression of all three genes is even decreasing on GlcNAc-containing medium, with 4, 4, and 1- fold, respectively. These results therefore showed that the genes of the GlcNAc-catabolism cluster were interestingly inducible by GlcNAc although *N. crassa* is not able to grow on this carbon source. With chitin as carbon source they did not appear to be strongly induced, which was

actually in agreement with the rather puzzling finding that *N. crassa* could grow rather well on chitin and that it did not seem to be growth inhibiting.

3) *Overexpression of the* T. reesei *glucosamine-6-phosphate-deaminase gene* dam1 *in* N. crassa *WT and a Δ*dam-1 *strain partially restores growth on GlcNAc*

The amino acid sequences of the enzymes involved in GlcNAc-catabolism in *N. crassa, T. reesei, C. albicans* and *E. coli* were compared in order to detect possible clues why growth of *N. crassa* is abolished when GlcNAc is added to the medium. *N. crassa* Hxk3 and Dac1 exhibit high similarities with their respective orthologs in *Trichoderma* spp.. A first investigation of Dam1 suggested an N-terminal truncation of this protein because the first 70 amino acids seemed to be missing in *N. crassa* in comparison to other fungi. However, a closer examination revealed solely a wrong annotation of the introns leading to a falsely predicted protein sequence. The correct open reading frame was confirmed using cDNA and specific primers that bind in exons and introns, respectively, in our corrected model (PCR data not shown). If the correct annotation were used, the resulting protein would exhibit a very similar amino acid sequence to the other Dam1 orthologs (Suppl. Fig.3). Furthermore, *in silico* analysis showed that the highly conserved catalytic residues described from *E. coli* are also present in *N. crassa* Dam1. Nonetheless, since we observed the strongest growth defect on GlcNAc in Δ*dam1* and the findings in recent studies corroborate the importance of Dam1 in GlcNAc-catabolism, we hypothesised that the glucosamine-6-phophate-deaminase gene could be a bottleneck in the GlcNAc utilisation process of *N. crassa.* Additional support for the hypothesis that Dam1 plays an important role in the GlcNAc-catabolism came from the fact that *C. albicans* deletion mutants of *nag1* (homologue of *N. crassa dam-1*) exhibit massive growth defects on medium containing GlcNAc as carbon source, even in the presence of additional carbon sources (D-glucose, D-fructose, D-galactose) [18]. Therefore we decided to overexpress *T. reesei dam1*, which has already been shown to be important and functional in *T. reesei* [16], in the *N. crassa* WT and *N. crassa* Δ*dam-1* strain using the strong *N. crassa ccg-1* promoter. For construction and verification of the overexpression strains see also Materials and Methods section and Suppl. Fig.2ab.

In order to test if the presence of *T. reesei dam1* improved growth of *N. crassa* on GlcNAc, we investigated the growth of the verified transformants on different carbon sources and pictures were taken after 12 days to ensure complete sporulation. As shown in Fig.4a the overexpression strains, WT::*ccg1*:*Trdam1* and Δ*dam-1*::*ccg1*:*Trdam1*, showed normal growth on medium containing glycerol. Additionally, both strains were also able to grow on medium containing both, glycerol and GlcNAc. However, although growth of the WT::*ccg1*:*Trdam1* and Δ*dam-1*::*ccg1*:*Trdam1* strains was clearly better than that of the Δ*dam-1* strain, it was only marginally improved compared to the WT. Growth on medium containing only GlcNAc as carbon source did not show a clear improvement.

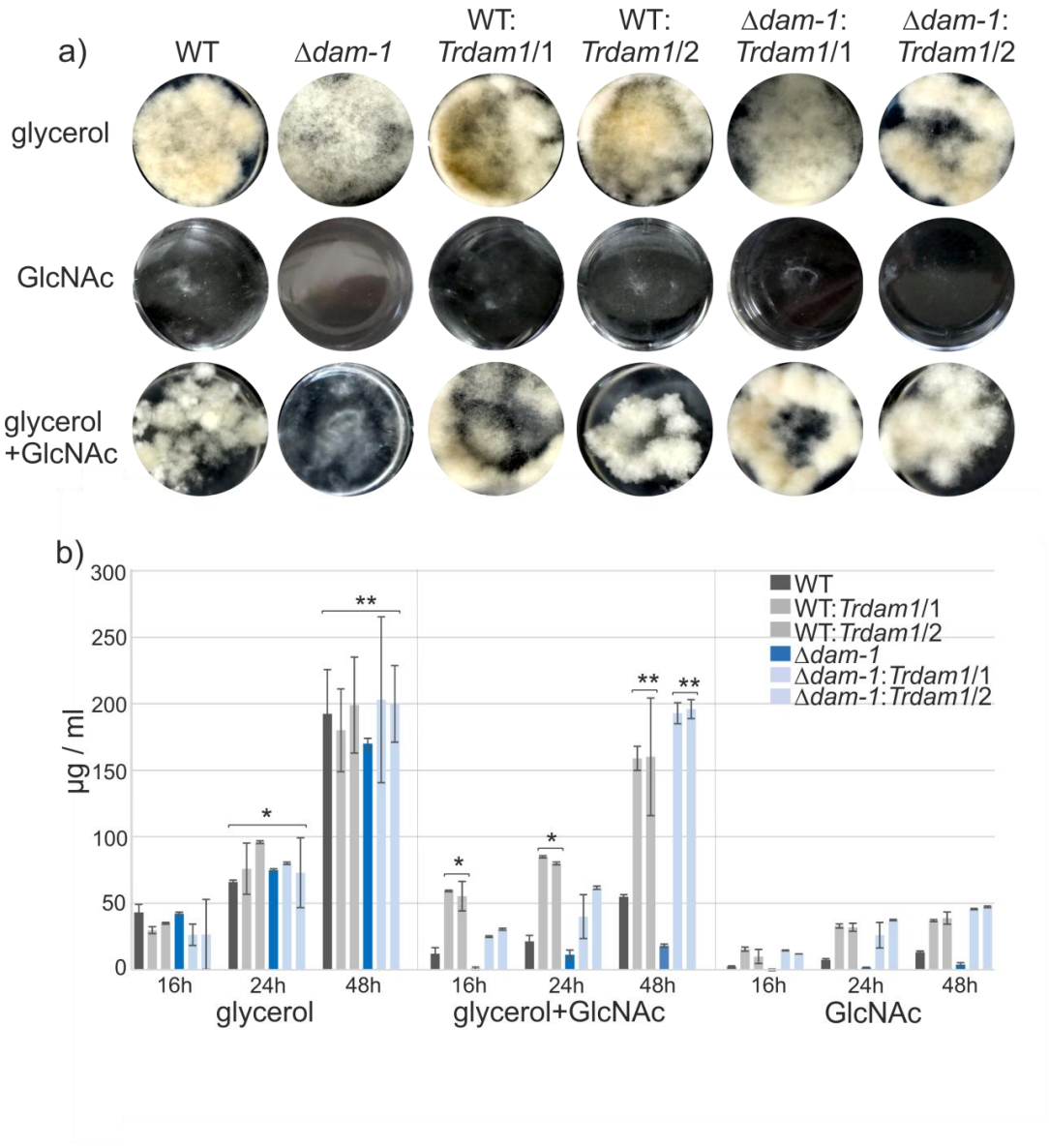


Fig.4: Growth tests and biomass formation of *N. crassa* WT 74A and overexpression strains (WT::*ccg1*:*Trdam1* and Δ*dam-1*::*ccg1*:*Trdam1*). a) WT and overexpression strains were grown for 12 days on VM containing 1% glycerol, 1% GlcNAc and 1% glycerol + 1% GlcNAc as

carbon source. Negative control was VM with the corresponding carbon source without spores. **b)** For biomass measurements, strains were grown in liquid standing VM medium, containing 1% glycerol or 1% gylcerol+1% GlcNAc or 1% GlcNAc and total protein concentration was determined after 16 h, 24 h and 48 h after inoculation.

Quantification of biomass formation of the corresponding strains on VM containing 1% glycerol, 1% glycerol+1% GlcNAc and 1% GlcNAc after 16 h, 24 h and 48 h of growth based on total protein measurements, is shown in Fig.4b. All tested strains exhibited similar values on the control glycerol. On medium containing GlcNAc as sole carbon source, biomass formation of the WT::*ccg1*:*Trdam1* and Δ*dam-1*::*ccg1*:*Trdam1* strains was slightly higher (37 +/- 0.7 and 39 +/- 4 µg/ ml, 46 +/- 0.5 and 47 +/- 0.6 µg/ ml, respectively, after 48 h), but not statistically significant different, but in general biomass formation was still very low, for the WT with 13 +/- 1 µg/ ml and Δ*dam-1* with 4 +/- 1.5 µg/ ml after 48 h, compared to other carbon sources. The results for the cultivation on medium containing both, glycerol and GlcNAc, showed that overexpression of *T. reesei dam1* successfully improved biomass formation of all tested strains. Biomass formation of the WT and Δ*dam-1* strain was much lower with 55 +/- 1.5 µg/ ml and 18 +/- 1.3 µg/ ml after 48 h than the results for the overexpression strains WT::*ccg1*:*Trdam1*/ Δ*dam-1*::*ccg1*:*Trdam1* with 159 +/- 9 µg/ ml and 160 +/- 44 µg/ ml, 193 +/- 8 µg/ ml and 195 +/- 7 µg/ ml, respectively.

Furthermore, we investigated morphological features of the WT, Δ*dam-1*, WT::*ccg1*:*Trdam1* and Δ*dam-1*::*ccg1*:*Trdam1* strains on VM containing 1% glycerol, 1% GlcNAc and 1% glycerol+1% GlcNAc. Macroscopically we could see that, although there was more biomass formation and mycelium of the overexpression strains was dense, the strains produced only small amounts of spores compared to the WT on glycerol (data not shown). Microscopic analysis revealed clear differences in hyphal development between WT/ Δ*dam-1* and WT::*ccg1*:*Trdam1*/ Δ*dam-1*::*ccg1*:*Trdam1* strains grown on medium containing glycerol+GlcNAc and GlcNAc as sole carbon source (Fig. 5ab). Especially the WT::*ccg1*:*Trdam1* strains showed more germinated spores and the germ tubes were even longer than that of the WT on glycerol (Fig. 5a).

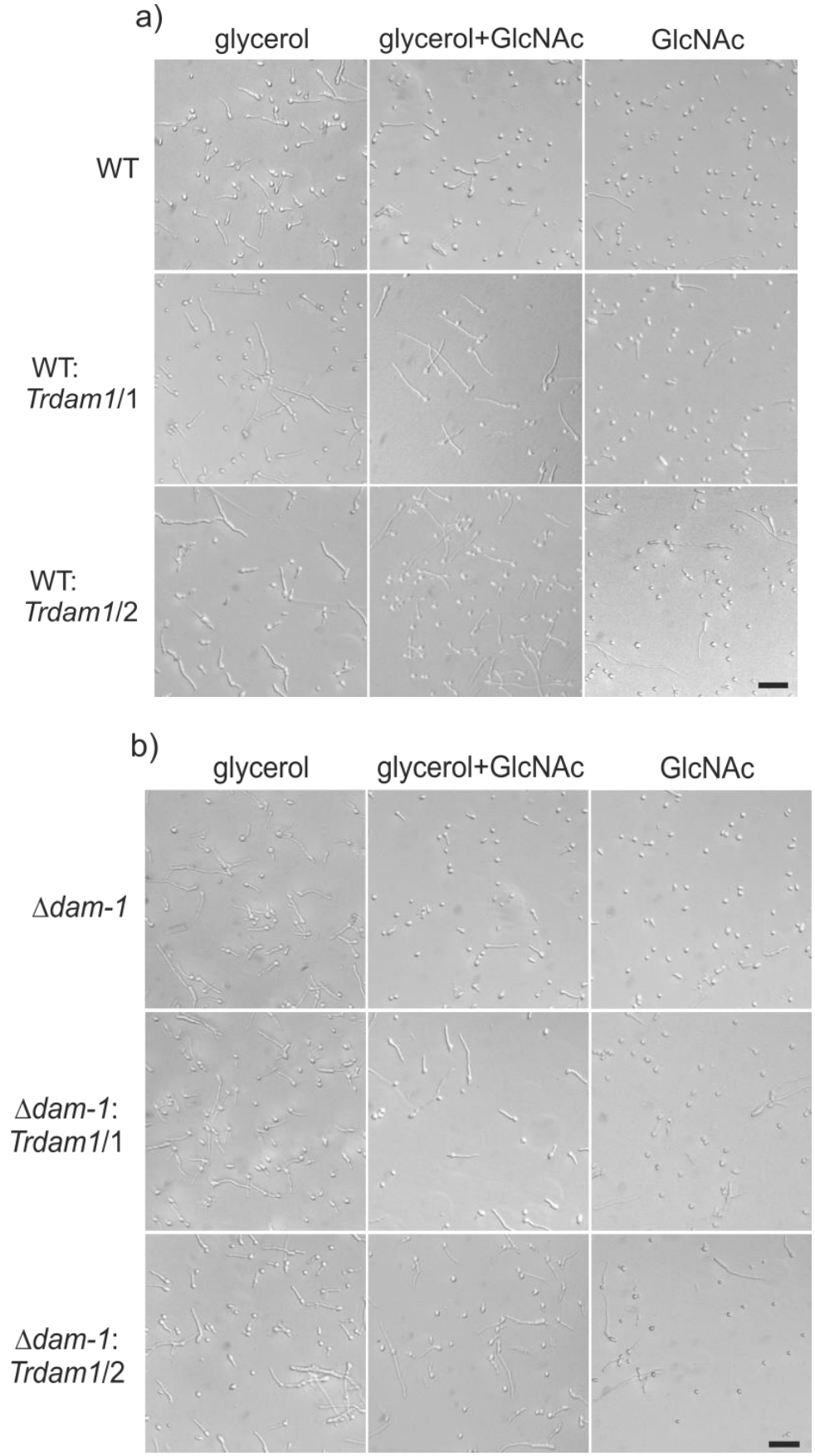


Fig.5: Microscopic analysis of the WT 74A, *dam1* deletion strain and overexpression strains (WT::*ccg1*:*Trdam1* and Δ*dam-1*::*ccg1*:*Trdam1*). a) WT, WT::*ccg1*:*Trdam1*/1 and

WT::*ccg1*:*Trdam1*/2 strains and **b)** Δ*dam,* Δ*dam-1*::*ccg1*:*Trdam1*/1 *and* Δ*dam-1*::*ccg1*:*Trdam1*/2 strains on liquid standing culture containing VM with 1% glycerol, 1% glycerol+ 1% GlcNAc and 1% GlcNAc after 8 h incubation are shown. Scale bars = 50 µm.

Discussion

In this study, we focussed on GlcNAc-catabolism in *N. crassa*. We started by testing the growth of four *N. crassa* WT isolates on different carbon sources, such as glycerol, D-glucose, D-fructose, cellulose, chitin and GlcNAc (Fig.1a). Although all tested WT strains (74-OR23-1A: FGSC987; Chilton: FSGC1691, Lindegren25a: FSGC353, Fast growth: FSGC5729) were able to grow on VM medium containing glycerol, D-glucose, D-fructose, cellulose and even chitin, no growth was observed on GlcNAc and it turned out to be even growth-inhibiting in the presence of a non-repressive carbon source such as glycerol. Only D-glucose and D-fructose were able to outcompete the growth-repressing effect of GlcNAc, which could be due to carbon catabolite repression resulting in repression of gene expression of the GlcNAc gene cluster. This points to the fact that an intermediate of the GlcNAc-catabolism pathway is responsible for the observed growth defect on GlcNAc. Despite the deleterious effects of GlcNAc on growth of *N. crassa*, it seemed to only cause a transient inhibition of growth but did not lead to immediate cell death. When *N. crassa* conidia were incubated in 1 % GlcNAc for 20 h, germination and growth could be completely restored after shifting them to medium without GlcNAc, containing glycerol or D-glucose. This finding is in agreement with observations in *C. albicans*, where addition of GlcNAc was also reported to have only an inhibitory but no lethal effect [18].

Interestingly, *N. crassa* was readily able to utilize the polymer chitin in powder form from crab shells and the better accessible and purified, acid-pre-treated form, colloidal chitin. One possible explanation for that could be that GlcNAc or one of its intermediates in the GlcNAc-catabolism pathway that are seemingly toxic for *N. crassa*, do not accumulate to sufficiently high levels to lead to growth inhibition due to the slow release of the monomer from the polymer chitin. However, in our assays even low amounts of the monomer GlcNAc were growth inhibitory and therefore this hypothesis can in our opinion be rejected. An alternative explanation could be that not chitinases but other, non-hydrolytic enzymes, e.g. lytic polysaccharide monooxygenases are mainly responsible for chitin degradation in *N.*

crassa, leading to chitin-degradation products other than GlcNAc, which would not be catabolised via the GlcNAc pathway and therefore not lead to accumulation of the growth-inhibitory intermediate. This is supported by our finding that the GlcNAc gene cluster was highly inducible by GlcNAc, similar to the findings in *T. reesei*, which very efficiently uses GlcNAc as carbon source [16], but none of the respective genes was found to be upregulated on chitin. There was almost no difference in gene expression to the control D-glucose. These findings are different from *T. reesei* which showed a clear upregulation for the GlcNAc catabolic cluster genes on GlcNAc and chitin containing-medium. Although *N. crassa* is able to utilize chitin as carbon source, the absence of the expression of the GlcNAc-cluster genes suggests that the GlcNAc-catabolism pathway is bypassed. We investigated gene expression at different time points (16 h, 24 h, 48 h) and biomass formation was clearly observed during these growth stages. As stated above, we speculate that lytic polysaccharide monooxygenases could be important for chitin degradation in *N. crassa*, due to their ability to cleave polysaccharides oxidatively [39-41] and leading to other chitin-degradation products than by GlcNAc-catabolism and therefore no toxic concentration of the growth inhibitory intermediate from the GlcNAc-catabolism pathway would be produced.

C. albicans is able to grow on GlcNAc containing medium, but Nassem *et al.* [18] described that single and triple deletion strains of the genes encoding the three main enzymes (*hxk1*, *dac1*, *nag1*) responsible for converting GlcNAc into fructose-6-phosphate failed to utilize GlcNAc. Even in the presence of an additional carbon source, such as galactose, the *dac1* and *nag1* deletion mutants were not able to grow, suggesting that an excess of GlcNAc-6-phosphate is toxic to cells [18]. Similar inhibitory growth effects of GlcNAc were observed for *E. coli* deletion strains lacking GlcNAc deacetylase or deaminase activity [42]. An exception in *C. albicans* were Δ*hxk1* and triple (*hxk1*Δ*dam1*Δ*dac1*Δ) knockout strains, which failed to phosphorylate GlcNAc and could recover on medium containing GlcNAc and a supplementary sugar (reviewed in Konopka *et al*, 2012 [14]). Based on these findings we tested the growth of all *N. crassa* GlcNAc-catabolism gene knockout strains (except for the not available Δ*dac-1*) on VM containing GlcNAc and glycerol. In agreement with the findings in *C. albicans*, the *hxk-3* deletion strain showed slightly better growth on medium containing GlcNAc and glycerol (Fig.2). Thus, a possible explanation is that in analogy to *C. albicans* due to the lack of the sugar kinase no toxic concentration of GlcNAc-6-phosphate will be

produced and therefore the organism is able to utilize the supplementary carbon source and is able grow again.

The *N. crassa dam-1* deletion strain seemed to be most severely affected by GlcNAc even in the presence of an alternative carbon source. The outcomes of this study confirmed the assumption that the conversion from glucosamine-6-phosphate to fructose-6-phosphate carried out by *dac-1* is a precarious step in the GlcNAc-catabolism pathway. Based on the findings in this study and previous data from *C. albicans* and *E. coli* [14, 42], where similar results are described, the functional *T. reesei* deaminase gene *dam1* was heterologously overexpressed in *N. crassa* WT and Δ*dam-1* background to see if high expression by another functional deaminase gene can alleviate the observed severe growth defects. Investigation of the overexpression strains showed that growth of the WT::*ccg1*:*Trdam1* and Δ*dam-1*::*ccg1*:*Trdam1* strains was partially restored on medium containing both, glycerol and GlcNAc. This suggests that overexpression of *T. reesei dam1* indeed seems to slightly restore *N. crassa* growth on GlcNAc in the presence of glycerol. On medium containing GlcNAc as sole carbon source the inhibitory effect of GlcNAc was still present (Fig.4). Thus, overexpression of *T. reesei dam1* in *N. crassa* restored growth on GlcNAc to a certain extent, but did not completely recover the growth defect, suggesting that more than one step in GlcNAc-catabolism is responsible for the growth inhibition. Due to the findings in *C. albicans* where deletion strains of *nag1* (*N. crassa*: *dam-1*) and *dac1* showed an inhibitory effect on GlcNAc containing medium, one possibility could be that the approach to solving this problem is the interaction between the two enzymes *dam-1* and *dac-1*.

Another reason could be a context to plasma membrane permeability as described for chitosan. Chitosan has been shown to inhibit growth of bacteria [43-45] and fungi, including *N. crassa* [46-50] and has been reported to damage the plasma membranes of both bacteria [44] and the yeast *Saccharomyces cerevisiae* [51]. Chitosan permeabilizes the plasma membrane of filamentous fungi and kills cells by an unknown energy-dependent mechanism that does not involve endocytosis [52]. The composition of the plasma membrane determine the sensitivity against chitosan, an important role plays the content of polyunsaturated fatty acids [53]. Although the fundamental difference between glucosamine and *N*-acetylglucosamine is the free amino group of the former sugar, it would be interesting to test if the deleterious effects of GlcNAc - or any of its metabolic intermediates, maybe even

unrelated to the GlcNAc catabolic pathway - are related to these processes. Further studies in this area will be necessary to elucidate the inhibitory effect of *N. crassa* by GlcNAc.

In this study we were able to show that GlcNAc can act as strong growth inhibitor under non-carbon catabolite repressive growth conditions in *N. crassa* and that the reason for that seems to be connected to the GlcNAc-catabolism pathway, which seems to be fully intact. Therefore, the observed growth defect seems rather to be connected to the toxic effects of an intermediate of this pathway but not to the inability of *N. crassa* to catabolize GlcNAc. Further, we found that this feature is interestingly not necessarily related to chitin utilization in *N. crassa*. Our growth assays and gene expression data suggest that the GlcNAc-catabolism pathway is efficiently bypassed during growth of *N. crassa* on chitin. Both of these key findings from our study have not been reported for any other fungus yet and highlight the variability of GlcNAc and chitin utilization strategies and features in the fungal kingdom.

References:

[1] Rudall KM, Kenchington W. THE CHITIN SYSTEM. Biological Reviews 1973; 48: 597-633.

[2] Muzzarelli RA. Native, industrial and fossil chitins. EXS 1999; 87: 1-6.

[3] Willis J. Cuticular Proteins in Insects and Crustaceans. American Zoologist 1999; 39: 600-9.

[4] Synowiecki J, Al-Khateeb NA. Production, properties, and some new applications of chitin and its derivatives. Crit Rev Food Sci Nutr 2003; 43: 145-71.

[5] Tharanathan RN, Kittur FS. Chitin-the undisputed biomolecule of great potential. Crit Rev Food Sci Nutr 2003; 43: 61-87.

[6] Ruiz-Herrera J. Fungal cell wall: structure, synthesis and assembly. Boca Raton: CRC Press 1991.

[7] Latgé JP. The cell wall: a carbohydrate armour for the fungal cell. Mol Microbiol 2007; 66: 279-90.

[8] Seidl V. Chitinases of filamentous fungi: a large group of diverse proteins with multiple physiological functions. Fungal Biol Rev 2008; 22: 36-42.

[9] Kubicek C, Herrera-Estrella A, Seidl-Seiboth V*, et al.* Comparative genome sequence analysis underscores mycoparasitism as the ancestral life style of *Trichoderma*. Genome Biol 2011; 12: r40.

[10] Horn SJ, Sorbotten A, Synstad B*, et al.* Endo/exo mechanism and processivity of family 18 chitinases produced by *Serratia marcescens*. FEBS J 2006; 273: 491-503.

[11] Mio T, Yabe T, Arisawa M, Yamada-Okabe H. The eukaryotic UDP-*N*-acetylglucosamine pyrophosphorylases. Gene cloning, protein expression, and catalytic mechanism. J Biol Chem 1998; 273: 14392-7.

[12] Milewski S, Gabriel I, Olchowy J. Enzymes of UDP-GlcNAc biosynthesis in yeast. Yeast 2006; 23: 1-14.

[13] Cannon RD, Niimi K, Jenkinson HF, Shepherd MG. Molecular cloning and expression of the *Candida albicans* beta-*N*-acetylglucosaminidase (*HEX1*) gene. J Bacteriol 1994; 176: 2640-7.

[14] Konopka JB. *N*-acetylglucosamine (GlcNAc) functions in cell signaling. Scientifica (Cairo) 2012.

[15] Naseem S, Araya E, Konopka JB. Hyphal growth in *Candida albicans* does not require induction of hyphal-specific gene expression. Mol Biol Cell 2015; 26: 1174-87.

[16] Kappel L, Gaderer R, Flipphi M, Seidl-Seiboth V. The *N*-acetylglucosamine catabolic gene cluster in *Trichoderma reesei* is controlled by the Ndt80-like transcription factor RON1. Mol Microbiol 2015.

[17] Kumar MJ, Jamaluddin MS, Natarajan K, Kaur D, Datta A. The inducible *N*-acetylglucosamine catabolic pathway gene cluster in *Candida albicans*: discrete *N*-acetylglucosamine-inducible factors interact at the promoter of *NAG1*. Proc Natl Acad Sci U S A 2000; 97: 14218-23.

[18] Naseem S, Gunasekera A, Araya E, Konopka JB. *N*-acetylglucosamine (GlcNAc) induction of hyphal morphogenesis and transcriptional responses in *Candida albicans* are not dependent on its metabolism. J Biol Chem 2011; 286: 28671-80.

[19] Cheng Q, Li H, Merdek K, Park JT. Molecular characterization of the beta-*N*-acetylglucosaminidase of *Escherichia coli* and its role in cell wall recycling. J Bacteriol 2000; 182: 4836-40.

[20] Li H, Morimoto K, Katagiri N*, et al.* A novel beta-*N*-acetylglucosaminidase of *Clostridium paraputrificum* M-21 with high activity on chitobiose. Appl Microbiol Biotechnol 2002; 60: 420-7.

[21] Mayer C, Vocadlo DJ, Mah M*, et al.* Characterization of a beta-*N*-acetylhexosaminidase and a beta-*N*-acetylglucosaminidase/beta-glucosidase from *Cellulomonas fimi*. FEBS J 2006; 273: 2929-41.

[22] Tsujibo H, Fujimoto K, Tanno H*, et al.* Gene sequence, purification and characterization of *N*-acetyl-beta-glucosaminidase from a marine bacterium, *Alteromonas* sp. strain O-7. Gene 1994; 146: 111-5.

[23] Litzinger S, Fischer S, Polzer P*, et al.* Structural and kinetic analysis of *Bacillus subtilis N*-acetylglucosaminidase reveals a unique Asp-His dyad mechanism. J Biol Chem 2010; 285: 35675-84.

[24] Hutchison EA, Glass NL. Meiotic regulators Ndt80 and ime2 have different roles in *Saccharomyces* and *Neurospora*. Genetics 2010; 185: 1271-82.

[25] Dementhon K, Iyer G, Glass NL. VIB-1 is required for expression of genes necessary for programmed cell death in *Neurospora crassa*. Eukaryot Cell 2006; 5: 2161-73.

[26] Xiong Y, Sun J, Glass NL. VIB1, a link between glucose signaling and carbon catabolite repression, is essential for plant cell wall degradation by *Neurospora crassa*. PLoS Genet 2014; 10: e1004500.

[27] McCluskey K, Wiest A, Plamann M. The Fungal Genetics Stock Center: a repository for 50 years of fungal genetics research. J Biosci 2010; 35: 119-26.

[28] Vogel HJ. A convenient growth medium for *Neurospora* (Medium N). Microbiol Genet Bull 1956; 13: 42-3.

[29] Bischof R, Fourtis L, Limbeck A*, et al.* Comparative analysis of the *Trichoderma reesei* transcriptome during growth on the cellulase inducing substrates wheat straw and lactose. Biotechnol Biofuels 2013; 6: 127.

[30] Freitag M, Hickey PC, Raju NB, Selker EU, Read ND. GFP as a tool to analyze the organization, dynamics and function of nuclei and microtubules in *Neurospora crassa*. Fungal Genet Biol 2004; 41: 897-910.

[31] McNally MT, Free SJ. Isolation and characterization of a *Neurospora* glucose-repressible gene. Curr Genet 1988; 14: 545-51.

[32] Kück U, Hoff B. Application of the nourseothricin acetyltrasnferase gene (*nat1*) as dominant marker for transformation of filamentous fungi. Fungal Genetics Newsletter 2006; 53: 9-11.

[33] Margolin B, Freitag M, Selker E. Improved plasmids for gene targeting at the his-3 locus of *Neurospora crassa* by electroporation. Fungal Genetics Newsletter 1997; 44: 34-6.

[34] Liu D, Coloe S, Baird R, Pederson J. Rapid mini-preparation of fungal DNA for PCR. J Clin Microbiol 2000; 38: 471.

[35] Sambrook J, Russell, D.W. Molecular Cloning: a Laboratory Manual. 2 ed. Painview, NY: Cold Spring Harbor Lab. Press 2001.

[36] Pfaffl MW, Horgan GW, Dempfle L. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res 2002; 30: e36.

[37] Druzhinina IS, Schmoll M, Seiboth B, Kubicek CP. Global carbon utilization profiles of wild-type, mutant, and transformant strains of *Hypocrea jecorina*. Appl Environ Microbiol 2006; 72: 2126-33.

[38] Sun M, Liu X. Carbon requirements of some nematophagous, entomopathogenic and mycoparasitic hyphomycetes as fungal biocontrol agents. Mycopathologia 2006; 161: 295-305.

[39] Vaaje-Kolstad G, Westereng B, Horn SJ*, et al.* An oxidative enzyme boosting the enzymatic conversion of recalcitrant polysaccharides. Science 2010; 330: 219-22.

[40] Beeson WT, Phillips CM, Cate JH, Marletta MA. Oxidative cleavage of cellulose by fungal copper-dependent polysaccharide monooxygenases. J Am Chem Soc 2012; 134: 890-2.

[41] Hemsworth GR, Henrissat B, Davies GJ, Walton PH. Discovery and characterization of a new family of lytic polysaccharide monooxygenases. Nature chemical biology 2014; 10: 122-6.

[42] White RJ. Control of amino sugar metabolism in *Escherichia coli* and isolation of mutants unable to degrade amino sugars. Biochem J 1968; 106: 847-58.

[43] Liu X, Guan D, ZLi Y, KD Y. Antibacterial action of chitosan and carboxymethylated chitosan. J Appl Polym Sci 2001; 79: 1324-35.

[44] Liu H, Du Y, Wang X, Sun L. Chitosan kills bacteria through cell membrane damage. Int J Food Microbiol 2004; 95: 147-55.

[45] Tikhonov VE, Stepnova EA, Babak VG*, et al.* Bactericidal and antifungal activities of a low molecular weight chitosan and its N-/2(3)-(dodec-2-enyl)succinoyl/-derivatives. Carbohydrate Polymers 2006; 64: 66-72.

[46] Bautista-Banos S, Hernandez-Lauzardo AN, Velazquez-del Valle MG*, et al.* Chitosan as a potential natural compound to control pre and postharvest diseases of horticultural commodities. Crop Prot 2006; 25: 108-18.

[47] Bell AA, Hubbard JC, Liu L, Davis RM, Subbarao KV. Effects of chitin and chitosan on the incidence and severity of *Fusarium* yellows of celery. Plant Dis 1998; 82: 322-8.

[48] Laflamme P, Benhamou N, Bussieres G, Dessureault M. Differential effect of chitosan on root rot fungal pathogens in forest nurseries. Can J Bot 1999; 77: 1460-8.

[49] Palma-Guerrero J, Jansson HB, Salinas J, Lopez-Llorca LV. Effect of chitosan on hyphal growth and spore germination of plant pathogenic and biocontrol fungi. J Appl Microbiol 2008; 104: 541-53.

[50] Plascencia-Jatomea M, Viniegra G, Olayo R, Castillo-Ortega MM, Shirai K. Effect of Chitosan and Temperature on Spore Germination of *Aspergillus niger*. Macromol Biosci 2003; 3: 582-6.

[51] Zakrzewska A, Boorsma A, Brul S, Hellingwerf KJ, Klis FM. Transcriptional response of *Saccharomyces cerevisiae* to the plasma membrane-perturbing compound chitosan. Eukaryot Cell 2005; 4: 703-15.

[52] Palma-Guerrero J, Huang IC, Jansson HB*, et al.* Chitosan permeabilizes the plasma membrane and kills cells of *Neurospora crassa* in an energy dependent manner. Fungal Genet Biol 2009; 46: 585-94.

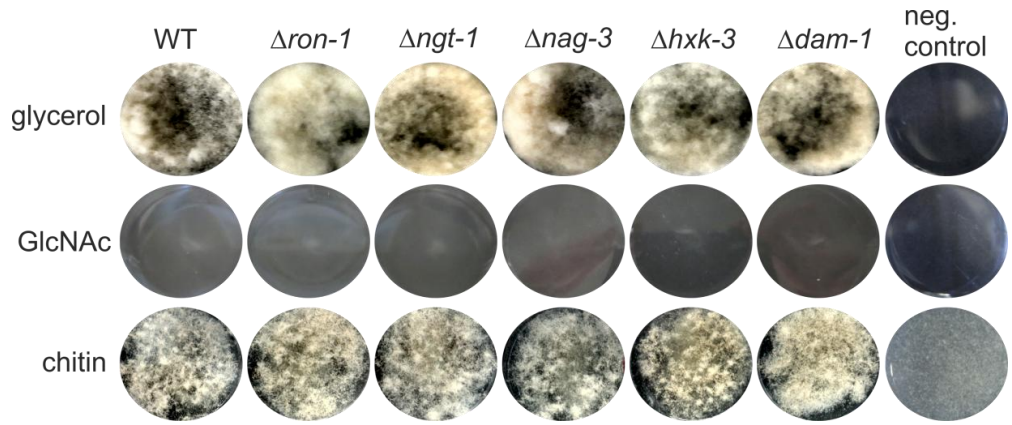
[53] Palma-Guerrero J, Lopez-Jimenez JA, Perez-Berna AJ*, et al.* Membrane fluidity determines sensitivity of filamentous fungi to chitosan. Mol Microbiol 2010; 75: 1021-32.

[54] Larkin MA, Blackshields G, Brown NP*, et al.* Clustal W and clustal X version 2.0. Bioinformatics 2007; 23: 2947-8.

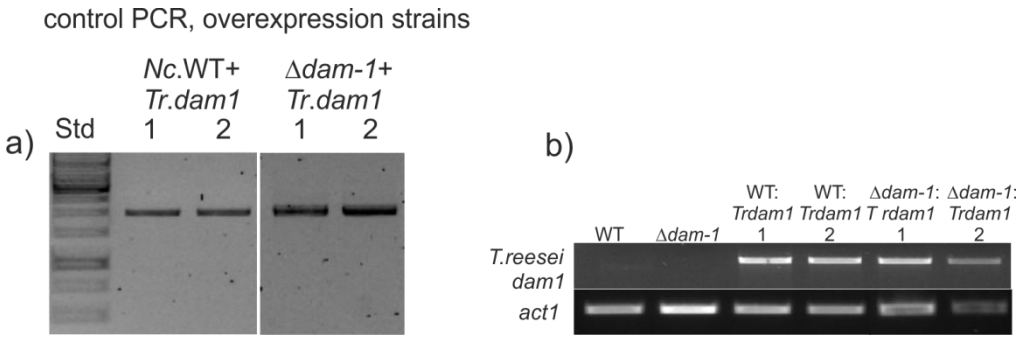
[55] Nicholas KB, Nicholas HBj, Deerfield DW. GeneDoc: Analysis and Visualization of Genetic Variation. EMBNEWNEWS 1997; 4.

Supplementary Table1. Primers and Protein IDs used in this study

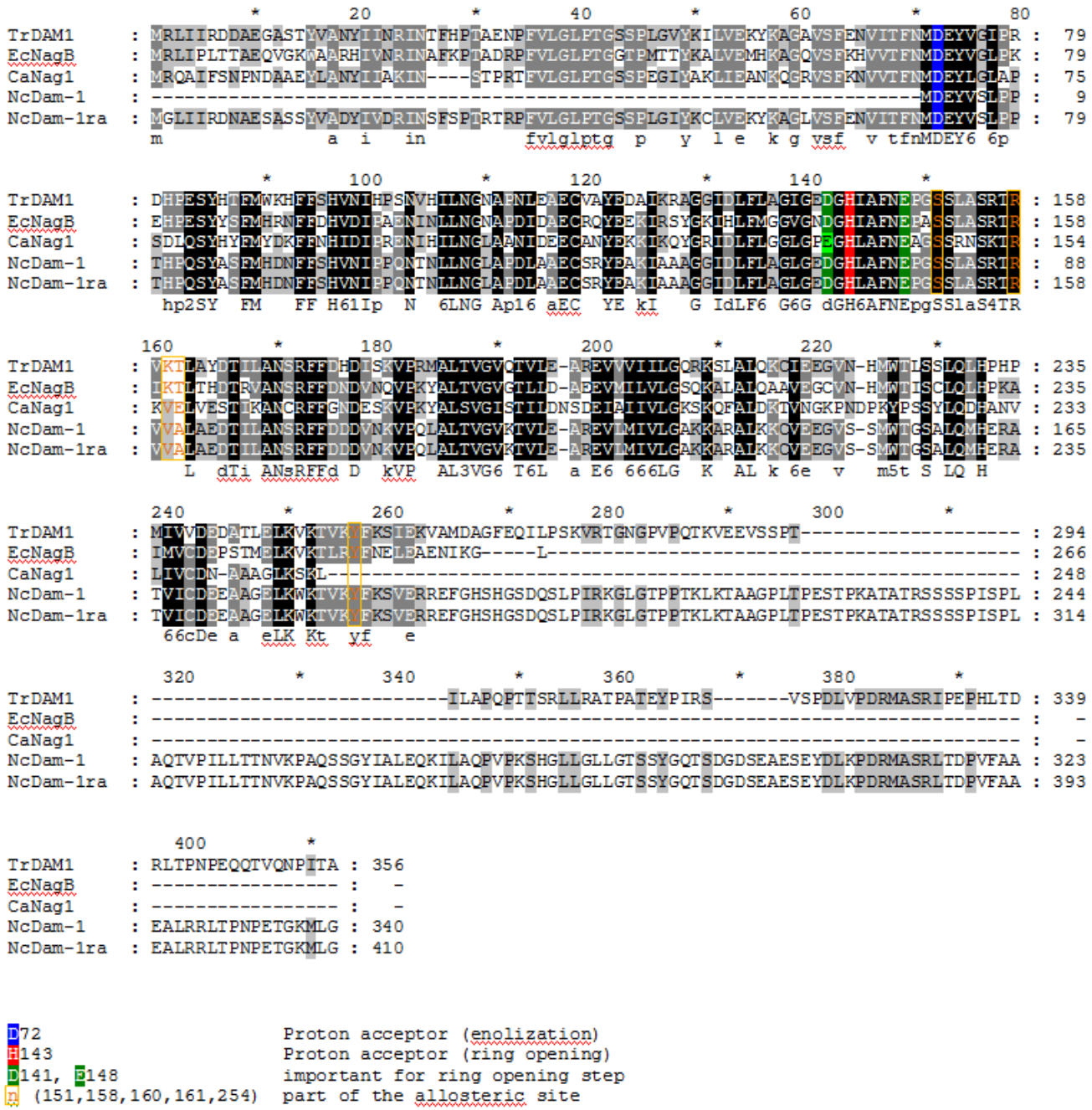
Primer for *T. reesei dam1* plasmid	
FW_INFU_DAM_T.r.	CACTTTCCTCGACGGATCCATGAGGTTGATC
RV_INFU_DAM_T.r.	GTAACGTTAAGTGGATCCAGGGTTCGGCTTTG
FW_pAL6_linear	GATCCACTTAACGTTACTG
RV_pAL6_linear	GGATCCGTCGAGGAAAG
FW_Trafo_Pccg1_DAM_nat	GGAGAAACAGGATGGG
RV_Trafo_Pccg1_DAM_nat	TCAGGGGCAGGGCATGC
Primer for Trafo check	
FW_Trafo_check_DAM	GACCGCATGGCCTCC
RV_Trafo_check_DAM	GGTTCCAGCCGGAGTAC
RT-Primer	
Tr_DAM_RTs	GCCTACGAGGACGCCATTAA
Tr_DAM_RTa	CCTTGGACGGCAGGATCTG
qPCR-Primer	
Nc_GH3_qPCRs	CAGCAACAGCAACAGCAACA
NC_GH3_qPCRa	TTCCACCACTTCCATTGCCA
Nc_DAC_qPCRs	TGAGGTTGAGGATCTGCTGG
Nc_DAC_qPCRa	TCACCAAAGCAGTCCCATCC
Nc_Dac2_qPCRs	TGAACTTTACCACTCCGCCC
Nc_Dac2_qPCRa	TTGGAGGGGAGAGAGGAAGG
Nc_TF_qPCRs	GAAGGAAACCCACGCCCTTA
Nc_TF_qPCRa	GGAGTAGCAGTCCTATTCATAGG
NC_DAM_qPCRs	CTAGCCCAACCCGTACCC
NC_DAM_qPCRa	ACGGGATCAGTTAGCCTGGA
Nc_HEX_qPCRs	CAGCCGCTTGAGCATCTG
Nc_HEX_qPCRa	CTTTCCTCCCAACAGACCCG
Nc_NGTL_qPCRs	GGATACCGTTGCCCTCGAAG
Nc_NGTL_qPCRa	GTCTCGCTACCACCACCATG
qPCR_Acts_Nc	GCGTGGTTACACCTTCTCCA
qPCR_Acta_Nc	CGGGAAGCTCGTAGGACTTC
Protein IDs	
Hxk3	NCU04728
Dac1	NCU04725
Dam1	NCU04727
Ron1	NCU04729
Nag3	NCU04726
Ngt1	NCU09771
Actin1	NCU04173



Suppl. Fig.1: Growth tests of *N. crassa* WT 74A and GlcNAc catabolic gene knockout strains (Δ*ngt-1,* Δ*nag-3*, Δ*ron-1*, Δ*hxk-3*, Δ*dam-1*). WT and knockout strains were grown for 7 days on VM containing 1% glycerol, 1% GlcNAc and 1% chitin as carbon source. Negative control was VM with the corresponding carbon source without spores.



Suppl. Fig.2: Verification of the constructed *N. crassa* WT 74A and Δ*dam-1 T. reesei dam1* overexpression strains. a) (Std) Gene Rule, 1 kb DNALadder (Fermentas). Genomic DNA was extracted from purified overexpression strains as described in the Material and Methods section and subjected to PCR with primers binding to a sequence in the selection marker cassette and a sequence in the *T.reesei dam1* gene (Suppl.Tab.1), generating an approximately 2 kb long PCR product in overexpression strains. **b)** Gene expression (RT-PCR) of *T.reesei dam1* from WT, Δ*dam-1* and overexpression strains (WT::*ccg1*:*Trdam1* and Δ*dam-1*::*ccg1*:*Trdam1*) are shown. *act1* was used as reference gene.



Suppl. Fig.3: Multiple sequence alignment of glucosamine-6-phosphate deaminase (DAM1) proteins. *T. reesei* DAM1 (TrDAM1), *E.coli* NagB (EcNagB), *C. albicans* Nag1(CaNag1), *N. crassa* Dam1 (NcDam-1) and *N. crassa* Dam1 with the correct annotation (NcDam1-ra). Alignments were created with the ClustalX program [54] and manually refined in GeneDoc [55].

Chapter 2: Functional and biochemical studies of members of the cerato-platanin protein family

Chapter 2.1

Cerato-platanins: a fungal protein family with intriguing properties and application potential. Gaderer, R., Bonazza, K., & Seidl-Seiboth, V. (2014). *Appl Microbiol Biotechnol, 98*(11), 4795-4803. doi: 10.1007/s00253-014-5690-y.

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MINI-REVIEW

Cerato-platanins: a fungal protein family with intriguing properties and application potential

Romana Gaderer · Klaus Bonazza · Verena Seidl-Seiboth

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Abstract Cerato-platanin proteins are small, secreted proteins with four conserved cysteines that are abundantly produced by filamentous fungi with all types of lifestyles. These proteins appear to be readily recognized by other organisms and are therefore important factors in interactions of fungi with other organisms, e.g. by stimulating the induction of defence responses in plants. However, it is not known yet whether the main function of cerato-platanin proteins is associated with these fungal interactions or rather a role in fungal growth and development. Cerato-platanin proteins seem to unify several biochemical properties that are not found in this combination in other proteins. On one hand, cerato-platanins are carbohydrate-binding proteins and are able to bind to chitin and *N*-acetylglucosamine oligosaccharides; on the other hand, they are able to self-assemble at hydrophobic/hydrophilic interfaces and form protein layers, e.g. on the surface of aqueous solutions, thereby altering the polarity of solutions and surfaces. The latter property is reminiscent of hydrophobins, which are also small, secreted fungal proteins, but interestingly, the surface-activity-altering properties of cerato-platanins are the opposite of what can be observed for hydrophobins. The so far known biochemical properties of cerato-platanin proteins are summarized in this review, and potential biotechnological applications as well as implications of these properties for the biological functions of cerato-platanin proteins are discussed.

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Introduction

Proteins belonging to the cerato-platanin protein (CPP) family are only found in filamentous fungi, i.e. fungi that produce hyphae as growth structures, or in fungi which have at least a pseudo-hyphal growth stage during their life cycle. The name-giving protein for this family was cerato-platanin (CP) from the plant pathogenic fungus *Ceratocystis platani*, which infects plane trees. CP was first described in 1999 (Pazzagli et al. 1999). Since then, CPPs have been reported from many different filamentous fungi, and it has been recognized that genes encoding CPPs can be found in the genomes of fungi with all kinds of lifestyles, including biotrophic and necrotrophic plant pathogens, human pathogens, mycoparasites, plant-beneficial fungi and saprotrophs (Frischmann et al. 2013; Chen et al. 2013). CPPs are small proteins (12 kDa) that are abundantly secreted into the culture filtrate, but remain also partially bound in the fungal cell wall (Seidl et al. 2006; Boddi et al. 2004; Gonzáléz-Fernandez et al. 2014).

Over the last decade, several studies revealed that CPPs are important players in interactions of fungi with other organisms. Many of the so far reported CPPs are from plant pathogenic fungi, and these proteins are able to act as virulence factors in fungal-plant interactions (Scala et al. 2004; Jeong et al. 2007; Frías et al. 2011). However, in fungi that positively interact with plants, e.g. *Trichoderma* spp., which are used as biocontrol fungi in agricultural applications, they act in a positive way as elicitors of plant defence responses (Djonovic et al. 2006). Further, a member of the CPP family from the human pathogen *Coccidioides immitis* has been described as an antigen (Pan and Cole 1995). Thus, CPPs

are readily perceived by other organisms and signal them the presence of a fungus.

With these insights in the effects of CPPs on fungal interactions, several questions came up that still need to be answered: Is the main function of CPPs related to the interaction of fungi with other organisms, or do they have a primary role in fungal growth? Or do they even have multiple roles? In order to answer these questions, a more detailed understanding of the biochemical properties of CPPs is necessary. The studies so far conducted on this topic revealed intriguing properties for this protein family ranging from carbohydrate-binding and carbohydrate-disrupting abilities to protein self-assembly and biofilm formation. Therefore, in this review, the so far known biochemical properties of CPPs, as well as similarities and differences to protein families with related properties, are discussed. Further, we examine what these properties can potentially tell us about the biological functions of CPPs and how these findings could be important for biotechnological applications.

Features of cerato-platanin proteins: carbohydrate binding and modification

CPPs are small proteins of ca. 12 kDa and contain a signal peptide that targets them to the secretory pathway. They seem to be predominantly secreted and can be readily found in the culture filtrates of fungi (Seidl et al. 2006; Pazzagli et al. 1999; González-Fernández et al. 2014; Frías et al. 2013b). In *C. platani* and *Botrytis cinerea*, CPPs were also already found in the fungal cell wall (Boddi et al. 2004; Frías et al. 2013b).

In the initial studies about CPPs, it was suspected that they are hydrophobin-like proteins (Pazzagli et al. 1999; Seidl et al. 2006), but structural analysis of CP from *C. platani* revealed that there are significant structural differences between CPPs and hydrophobins. CPPs are structurally rather related to expansins, which are proteins associated with carbohydrate-binding and loosening of the cellulose scaffolds in plant cell walls (de Oliveira et al. 2011). However, biochemical analysis of the properties of CPPs showed—somewhat surprisingly—that they have actually both carbohydrate-binding/carbohydrate-loosening properties, similar to expansins (Baccelli et al. 2014), but also the ability to self-assemble and change the polarity of surfaces and solutions, which are properties that are reminiscent of hydrophobins (Frischmann et al. 2013).

The first protein structure that was reported for this protein family was a NMR-derived solution structure for CP. It revealed a globular fold containing two alpha-helices and six beta-strands forming a six-stranded double ψβ-barrel (de Oliveira et al. 2011). The structural fold of CP turned out to be very stable and was preserved over a wide pH range (pH 3 to 9) and up to 76 °C. Further, an *N*-acetylglucosamine-binding pocket forming a shallow groove on one side of the barrel was detected. Another CPP for which structural data are available is SM1 from *Trichoderma virens*. The 3D structure of SM1 has been deposited in the Protein Data Bank (PDB) (accession number 3m3g), but no publication is associated with it yet. Recently, the structures of MpCP1, MpCP2, MpCP3 and MpCP5 from *Moniliophthora perniciosa* were determined (de O Barsottini et al. 2013). All of these CPPs comprise a single domain containing the double ψβ-barrel. This folding is remarkably similar to that found in plant and bacterial expansins, endoglucanases and the plant defence protein barwin (de O Barsottini et al. 2013). Expansins loosen the plant cell wall through a non-enzymatic mechanism (Sampedro and Cosgrove 2005), while endoglucanases catalyse the hydrolysis of cellulose. Barwin proteins belong to the plant pathogenesis-related protein-4 (PR4) family and are generally thought to be involved in plant defence responses (Bai et al. 2013).

For several CPPs (for *Trichoderma atroviride* EPL1, *C. platani* CP, *Ceratocystis populicola* Pop1 and *M. perniciosa* MpCP1-5), it was already shown that they bind to polymeric chitin—which consists of *N*-acetylglucosamine subunits and/or chitin oligomers (Frischmann et al. 2013; Baccelli et al. 2014; de O Barsottini et al. 2013). Interestingly, the chitin-binding site of MpCP5 is in a different region of the protein than in the other MpCPs, suggesting that there is a selective evolutionary pressure on MpCP5 to maintain its carbohydrate-binding properties (de O Barsottini et al. 2013). Chitin is an important structural component of the fungal cell wall, and the peptidoglycan of bacterial cell walls contains also *N*-acetylglucosamine sugars. However, so far, no binding to fungal or bacterial cell walls was detected (Frischmann et al. 2013). Further, none of the so far tested CPPs bind to cellulose, but nonetheless, for CP, expansin-like activity of cellulosic materials was reported (Baccelli et al. 2014). This included weakening of filter paper, fragmentation of crystalline cellulose and breakage of cotton fibres. These effects were also observed for Pop1, albeit weaker than for CP, and it will be interesting to test further CPPs in order to analyse their expansin-like activities in more detail. For MpCP2, it was reported that only an aggregated form, but not soluble protein, was able to fragment cellulose (de O Barsottini et al. 2013). Possibly, there is considerable differentiation among CPPs with respect to cellulose fragmentation. For EPL1, the filter paper assay setup used for CP and Pop1 (Baccelli et al. 2014) was unfortunately not feasible due to excessive foaming of the protein solution (R. Gaderer and V. Seidl-Seiboth, unpublished results).

The biological function of the chitin-binding properties is not clear yet. One possibility is that CPPs are fungal expansins, and their roles might therefore be related to expansin-like activities within the fungal cell wall. Another possibility is that their functions are related to the scavenging of chitin oligomers, e.g. during fungal-plant interactions,

which are released due to the action of plant chitinases and would otherwise induce plant defence responses in order to mask the presence of the fungus for the plant, similar to what has been reported for the LysM protein Ecp6 from the plant pathogen *Cladosporium fulvum* (de Jonge et al. 2010). However, since CPPs like EPL1 and its orthologues in several fungi are continuously secreted under many different growth conditions, we suggest that a more general role related to fungal growth, i.e. in the fungal cell wall, is more likely than a specialized function during fungal-plant interactions.

Features of cerato-platanin proteins: self-assembly and protein biofilm formation

Biochemical analysis of EPL1 from *T. atroviride* revealed that it readily forms protein biofilms at air/water interfaces (Frischmann et al. 2013). A first indication for the self-assembly properties of CPPs from *Trichoderma* were reports on the dimerization of these proteins (Seidl et al. 2006; Vargas et al. 2008). EPL1 is able to form relatively stable dimers, which were still observed after protein-concentration and protein-denaturing steps during 2D gel electrophoresis of a culture filtrate of *T. atroviride* (Seidl et al. 2006). Detailed mass spectrometric analysis of EPL1 showed that the dimer had a double-oxidized tryptophan residue. Subsequently, a comparison of the homologues SM1 from *T. virens* and EPL1 from *T. atroviride* revealed that, although these proteins are overall highly similar (83 % amino acid (aa) identities), *T. virens* has a glycosylation site which is not found in *T. atroviride* (Vargas et al. 2008). This glycan moiety also influences the dimerization tendencies of SM1, which is less prone to form dimers than the non-glycosylated EPL1. This is also of biological relevance because the monomeric form is more efficient in the induction of plant defence responses than the dimer.

An investigation of the self-assembly potential of EPL1 showed that protein layers can be readily observed under the microscope and are even macroscopically visible after a few minutes of incubation of a protein solution (Frischmann et al. 2013). Self-assembly of EPL1 is reversible, and these protein biofilms can be easily re-dissolved by pipetting or stirring of the solution. High-resolution imaging of EPL1 layers with atomic force microscopy (AFM) revealed that, in these protein layers, EPL1 assembles into rather irregular, meshwork-like structures (Frischmann et al. 2013). However, it should be noted that, by AFM imaging of these protein layers, rather large amounts of protein are deposited on the sample carrier. More recently, we applied in-solution AFM imaging to investigate the self-assembly of EPL1 in more detail, and we were able to show that, on solid/liquid interfaces, it indeed does form highly ordered protein monolayers (Bonazza et al. 2014). Figure 1 shows AFM images of an EPL1 protein layer that was imaged in situ during its formation in buffer. The riffled surface exhibits a periodicity of 6 nm which corresponds to the size of one monomer (Frischmann et al. 2013). In the height image (Fig. 1a), the measurement mode is strictly related to height proportions and reveals the actual 3D topography, but small height differences are difficult to discern in this imaging mode. In the amplitude error image (Fig. 1b), height proportions are more relative to each other, which limits some size measurement options, but circumvents the limitation of displaying just 1-Å-deep riffles beside a several-nanometer-high step in the substrate surface.

Beside the formation of protein biofilms, EPL1 protein solutions have also other surface-activity-altering properties (Frischmann et al. 2013). They exhibit strong foaming, and at higher concentrations, the protein solution shows the tendency to crawl up along needles and pipet tips. Further, it was shown that EPL1 solutions alter the contact angle of aqueous solutions, making them even more hydrophilic, and EPL1 protein layers that are deposited on surfaces can also enhance the polarity effects of surfaces.

For CP and MpCPs, the formation of amyloid-like aggregates was reported although these were observed after prolonged incubation times and harsher incubation conditions (Pazzagli et al. 2009; Sbrana et al. 2007; de O Barsottini et al. 2013). Rapid formation (less than 30 min) of Pop1 aggregates was observed upon contact with hydrophobic surfaces (Teflon beads), and it was shown that in vivo Pop1 is able to interact with the hydrophobic cuticle of leaves (Martellini et al. 2013). The authors therefore proposed that the interaction of CPPs with host plant cuticle waxes could induce their partial unfolding and thereby enhance the recognition of the proteins by the plant.

Differences and similarities between cerato-platanin proteins and hydrophobins

The ability to self-assemble at interfaces and to alter the polarity of solutions and surfaces is reminiscent of another protein family: hydrophobins. They are a family of small, secreted cysteine-rich proteins that, similar to CPPs, also solely occur in filamentous fungi (Linder et al. 2005; Wösten and Wessels 1997). These proteins self-assemble in the form of an insoluble amphipathic membrane at hydrophobic/hydrophilic interfaces, thereby forming protective surface coatings of fungal structures and aiding in their adherence to surfaces. Hydrophobins are found on the outer surfaces of cell walls of hyphae and conidia, where they mediate interactions between the fungus and the environment. Because the protein surface of hydrophobins contains large hydrophobic and hydrophilic patches, they are able to invert the polarity of surfaces. Biologically, this is for example relevant for fungal hyphae that emerge from an aqueous growth medium to form aerial hyphae and produce spores, which are then covered with a layer of hydrophobins that

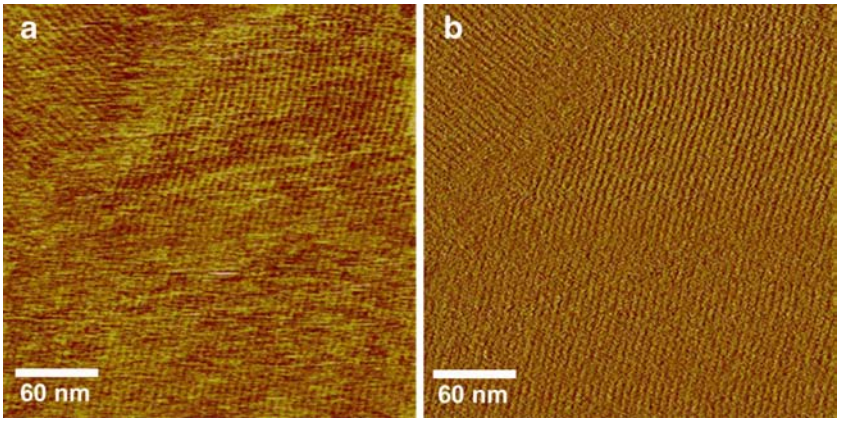

Fig. 1 AFM height (**a**) and amplitude error (**b**) images of a highly ordered EPL1 protein layer on a hydrophobic HOPG substrate. The average distance of protein rows is ca. 6 nm. Areas with homogenous protein orientation are in the micrometer range. The selected spot shows an orientation zone boundary and a terrace step of the substrate. Images were taken in tapping mode under PBS buffer, proteins self-assembled in situ. Data scale, 1 nm (**a**) or 10 mV (**b**) from dark to bright

render them hydrophobic. This facilitates the dispersal of fungal conidia (spores). Due to their unique properties, hydrophobins are also of interest for biotechnological applications, e.g. modification of surface properties and stabilization of foams and emulsions (Linder et al. 2005).

Biochemically, hydrophobins are characterized by the presence of eight positionally conserved cysteine amino acid residues (Linder et al. 2005). Hydrophobins are conventionally grouped into two classes (class I and II) according to their solubility in solvents, hydropathy profiles and spacing between the conserved cysteines. Overall, hydrophobins share only a few conserved residues besides the cysteine patterns. This also indicates that the cysteines are critical for structural reasons, while the other, variable residues give rise to protein variants with specific properties.

The cysteine spacing pattern of class I hydrophobins is $CX_{(6)}CCX_{(9–39)}CX_{(5–25)}CX_{(5)}CCX_{(8–17)}C$ and of class II hydrophobins, $CX_{(10)}CCX_{(11)}CX_{16}CX_{(8)}CCX_{(10)}C$ (Seidl-Seiboth et al. 2011; Wösten and Wessels 1997). The disulfide bridges connecting these cysteines span C1–C6, C2–C5, C3–C4, and C7–C8 (Linder et al. 2005). In contrast to that, analysis of the aa sequences of CPPs shows that, in this protein family, the general cysteine spacing pattern is $C_{(38)}CXXC_{(54)}C$. For most CPPs, the CXXC motif in this pattern can be confined to C–G–S/T–C. The disulfide bridges that connect the cysteines in CPPs span C1–C2 and C3–C4 (de Oliveira et al. 2011; de O Barsottini et al. 2013). Thus, there are no similarities in the cysteine spacing patterns between hydrophobins and CPPs and also no other sequence similarities. The high numbers of microbial genomes that have been sequenced in the past decade revealed that many fungi have small, secreted, cysteine-rich proteins (Templeton et al. 1994), of which hydrophobins and CPPs are two families that were now already characterized in more detail. However, there are certainly several more protein families among these small, secreted, cysteine-rich proteins that need to be described yet, and it will be interesting to compare the biochemical properties and functions among them.

The surface architecture and the exposure of polar and apolar patches of amino acids are also different between CPPs and hydrophobins. Modelling of EPL1 based on the structure of *T. virens* SM1 (PDB accession number 3m3g) shows that there are some hydrophobic residues on the surface (carbohydrate-binding pockets are also lined with aromatic residues), but there are no large hydrophobic patches on the surface (Fig. 2). On the contrary, the surface seems to be rather hydrophilic, which is in agreement with the good solubility of EPL1 in aqueous solutions.

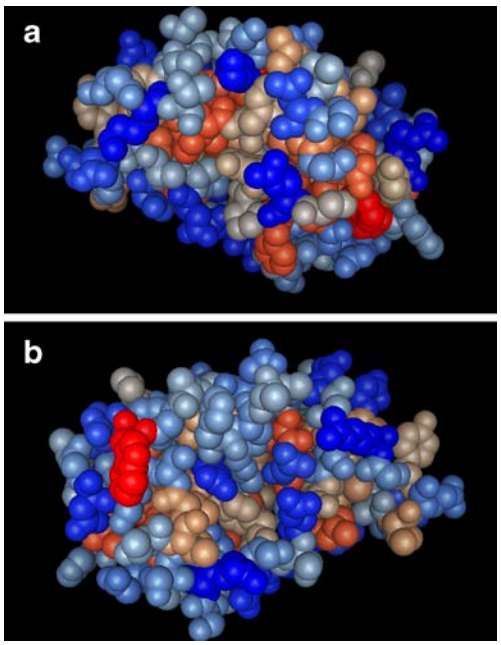

Fig. 2 **a**, **b** Modelled 3D structure of EPL1 using SM1 (PDB 3m3g) as template, generated with I-TASSER (Roy et al. 2010). C-score=1.86. Hydrophobic residues are shown in *shades of red*, hydrophilic residues in *shades of blue*

Another difference between hydrophobins and CPPs is that CPPs are better soluble than hydrophobins. While protein layers of the CPP EPL1 can be easily re-dissolved, the self-assembly of hydrophobin layers is mostly irreversible. Particularly, aggregates of class I hydrophobins are extremely stable and can only be dissolved in strong acids such as trifluoroacetic acid, while class II layers are soluble in aqueous dilutions of organic solvents (Linder et al. 2005).

Beside the differences between CPPs and hydrophobins with respect to their protein sequences and structure, it is important to note that also the surface-altering properties of these two protein families are complementary. EPL1 increases the polarity of surfaces and solutions, which is the opposite of what has been described for hydrophobins, and thus, CPPs could possibly rather be described as 'hydrophilins' in this respect. It will be interesting and necessary to determine these parameters for other proteins of the CPP family.

Gene numbers and gene expression of cerato-platanin proteins

Genome analysis showed that all filamentous fungi belonging to the phyla Ascomycota and Basidiomycota have CP-encoding genes, but their numbers vary strongly among different phyla (Chen et al. 2013). Basidiomycota have often many CP genes, e.g. the nectrotrophic plant pathogen *M. perniciosa*, the causal agent of witches' broom disease in cacao, has 12 CP genes (de O Barsottini et al. 2013). In contrast to that, fungi belonging to Ascomycota have only between one and three CP-encoding genes (Frischmann et al. 2013; Chen et al. 2013). Further, Ascomycota have usually one strongly conserved homologue of *T. atroviride* EPL1, which was already studied in more detail (see above). The protein sequences of EPL1-homologues are highly similar throughout the Ascomycota, and for several fungi, it was already shown that they are abundantly expressed during different types of growth conditions.

T. atroviride has three genes encoding CPPs: *epl1*, *epl2* and *epl3*. Transcriptional analysis of these genes showed that *epl1* is expressed during hyphal growth and mycelial development, while *epl2* expression occurs during sporulation and spore maturation, whereas almost no expression was detected for *epl3* (Frischmann et al. 2013). Similar findings were observed for the corresponding CP-encoding genes from *T. virens*: *sm1*, *sm2* and *sm3* (Gaderer 2013). Therefore, these genes are clearly not co-regulated, and these results indicate that the respective proteins are involved in different stages during fungal growth and development. In *C. platani*, it was reported that the expression levels of *cp* are associated with hyphal growth and the formation of chlamydospores, which are specialized, thick-walled, large spores (Baccelli et al. 2012). However, in *Trichoderma*, no connection between chlamydospore formation and *epl/sm*-gene expression was detected (Gaderer 2013; Frischmann et al. 2013). In other fungi, there is also evidence that CP genes, similar to *epl1*, are expressed during hyphal growth, e.g. in *B. cinerea*, *bcspl1* was found to be expressed under many different growth conditions, whereas no expression was found for *bcspl2*, a second CP gene (Frías et al. 2011). *MgSM1* from *Magnaporthe grisea* was also expressed during different fungal growth stages (Yang et al. 2009). In addition to these gene expression data, the protein EPL1 was found to be the predominant protein in the secretome of submerged *T. atroviride* cultivations with glucose as a carbon source (Seidl et al. 2006).

Despite these indications for a role of CPPs in fungal growth, so far, no specific biological function could be assigned to them. Gene knockout strains did not show any phenotypes related to hyphal growth and development (see below for details) (Frischmann et al. 2013; Djonovic et al. 2007; Frías et al. 2011; Jeong et al. 2007).

In the basidiomycete *M. perniciosa*, gene expression data of the 12 CP genes (*MpCP1-12*) showed complex transcriptional profiles throughout fungal development and specific stages of the pathogenic infestation of the plant, suggesting a specialization of the respective proteins in different biological processes. While the *MpCP1* gene was exclusively expressed during basidiocarp formation, *MpCP2* and *MpCP3* expression occurred in fast-growing mycelium and necrotic-infected seed and fruit, while *MpCP4*, *MpCP5*, *MpCP1l* and *MpCP12* were especially found to be expressed during the slow-growing biotrophic phase, when the fungus develops in the apoplast of the plant and the pathogen-host survival battle is established. For *MpCP 6*, *7*, *8*, *9*, and *10*, no gene expression was so far detected (de O Barsottini et al. 2013). With the large numbers of CPPs in some basidiomycetes, it will be interesting to test whether there are also homologues with similar functions or the specialization was rather separate in different fungi.

(Potential) biological roles of cerato-platanin proteins in fungal growth and dissemination

Despite several efforts to unravel the biological function of CPPs, their roles in fungal development are not clear yet. Their strong expression during hyphal growth and their chitin-binding properties together with their similarity to expansins suggest that they might be involved in fungal cell wall expansion and that this might actually be their primary function (see also the section "Features of cerato-platanin proteins: carbohydrate binding and modification"). However, unfortunately, there is so far still no direct evidence for that, and interestingly, the central question, whether the primary function of CPPs is related to fungal growth and development

or rather to the interaction of fungi with other organisms, still remains to be solved. Possibly, this is even different for individual CPPs, as gene expression data might indicate, but as long as we do not understand what—if anything—CPPs do in/on/at the fungal cell wall, we cannot definitely answer this question.

In *T. atroviride* and *T. virens*, *epl1/sm1* and *epl2/sm2* single knockout strains and even *epl1/epl2* double knockout strains were extensively tested for phenotypes related to fungal growth, including germination, hyphal elongation and branching, biomass formation and sporulation (Frischmann et al. 2013; Gaderer 2013; Djonovic et al. 2007). In none of these developmental stages were any differences between the wild-type and the knockout strains detected. Further, growth during different types of stress or the transition of hyphae, e.g. from liquid to solid media or surfaces, was also not different from that of the wild type. Based on the differential expression patterns of *epl1-3* and *sm1-3* in *T. atroviride* and *T. virens*, respectively (see above), it is not likely that the lack of phenotype is due to a compensation effect of other CP-encoding genes.

Also, in other fungi, e.g. *M. grisea*, *B. cinerea* and *Leptoshpaeria maculans*, so far no phenotypes of knockout strains related to fungal growth or sporulation were reported (Frías et al. 2011; Jeong et al. 2007; Wilson et al. 2002).

Another possibility for the functions of CPPs would be that they are connected to chitin utilization, but in *T. atroviride*, no direct inducibility of *epl1-3* genes by chitin was found, suggesting that the proteins EPL1-3 are not involved in aspects related to chitin degradation (Frischmann et al. 2013). Maybe the function of CPPs lies in between fungal growth and fungal interactions?

When *T. atroviride* hyphae grow out of a droplet of water/medium, they are covered with a film of water (Fig. 3). Although no changes in this water film were observed in knockout strains, it could be speculated that the secretion of CPPs influences the adherence of the hyphae to certain environments and with that the interaction of the fungus with its environment. By reducing the surface tension, substrates might become more easily accessible in aqueous environments for the fungus. However, they would not serve their purpose in fulfilling these roles if they would diffuse too far away from the fungus, and maybe due to their chitin-binding properties, they stay partially bound to the cell wall, serving as a reservoir to be partially released under the right growth conditions. It will be difficult to test some aspects of such a hypothesis, particularly since some of these effects might be more significant in the natural environment than under laboratory conditions, but in support of the general idea that CPPs influence the interaction of fungal hyphae with their environment, we recently obtained experimental data that showed that EPL1 is able to alter the surface properties of hydrophobin layers (Bonazza et al. 2014).

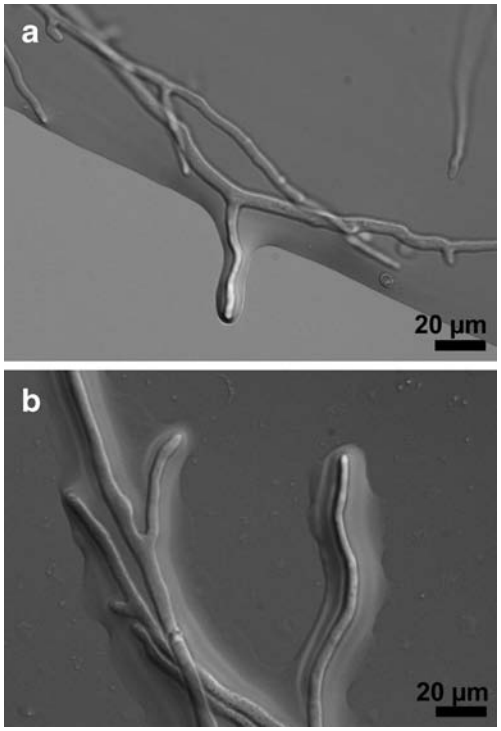

Fig. 3 **a**, **b** An aqueous film encloses hyphae of *Trichoderma reesei* that are growing out of a droplet of medium

(Potential) biological roles of cerato-platanin proteins in interactions between fungi and other organisms

In fungal-plant interactions, CPPs have been shown to act as virulence factors and elicitors of plant defence responses. For several plant pathogenic fungi, it was reported that CPPs are an important factor for virulence or induce necrosis in plant tissue (Frías et al. 2011, 2013a, b; Scala et al. 2004; Jeong et al. 2007). The eliciting activity of BcSpl1 from *B. cinerea* was mapped to a two-peptide motif on the protein surface (Frías et al. 2013b). A review discussing the plant-related aspects of CPPs in more detail has recently been published (Pazzagli et al. 2014).

Differential timing of defence-related responses induced by CP and Pop1 was found, which the authors suggested could be due to the structural differences between CP and Pop1, i.e. different hydrophobic index and different helix content (Lombardi et al. 2013). In addition to such biological differences between CPPs, which are undoubtedly present, overall, the question of the dose of applied, purified CPPs and/or expression levels in vivo should also be considered when different effects are observed for different CPPs. This might have a profound influence on the observed effects. For example, in *M. grisea*, knockout strains showed reduced virulence, while purified protein applied to wounded leaf tissue showed no phytotoxic effects (Jeong et al. 2007), and ectopic expression in *Arabidopsis thaliana* conferred broad-spectrum disease resistance (Yang et al. 2009).

The elicitation of defence responses by CPPs does not necessarily result in negative consequences such as necrosis, but can also strengthen the plants and confer resistance against microbial pathogens, as was shown for *Trichoderma* spp. *T. virens* SM1 and to a lesser extent *T. atroviride* EPL1 were shown to be an important inducer of plant defence responses (Djonovic et al. 2006; Vargas et al. 2008). Since *Trichoderma* spp. are plant-beneficial biocontrol fungi, the fungal-plant interaction as well as the effect of CPPs is in this case not associated with disease symptoms or necrosis in plants. The induction of defence responses by CPPs can also be conferred artificially by transgenic expression *in planta*. The CP MgSM1 from *M. grisea* was expressed in *A. thaliana*, and subsequently, the plants showed enhanced disease resistance and upregulation of defence-related genes (Yang et al. 2009).

The CPP AgCS (also called CS-Ag, *Coccidioides*-specific antigen) (Cole et al. 1989; Pan and Cole 1995) from the human pathogen *C. immitis*, which causes respiratory mycosis, has been reported to be an antigen. Antibodies of serum from patients with coccidioidomycosis reacted with AgCS that was isolated from the saprobic growth stage of *C. immitis*. In the initial study (Cole et al. 1989), AgCS, purified from culture filtrate, was reported to possess proteolytic activity. In the subsequent study (Pan and Cole 1995), AgCS was overexpressed in *Escherichia coli*, but protease activity of the purified protein was not reported in this follow-up paper. CPPs from other fungi were not tested for protease activity, and it might be something that should be kept in mind although the reported protease activity could have resulted from an impurity of the protein preparation.

In general, it can be concluded with respect to the roles of CPPs in the interactions between fungi and other organisms that CPPs are readily recognized, e.g. by plants or humans, probably due to their abundant secretion by fungi. We suggest that the presence of CPPs signals the other organism reliably the presence of a fungus, but probably dependent on other factors of this interaction, this can lead to different responses with positive or negative consequences for this interaction.

Possibilities for biotechnological applications of cerato-platanin proteins

Particularly, the surface-activity-altering effects of CPPs are potentially interesting for biotechnological applications, but we are not aware of any ongoing efforts in this respect yet. With native CPPs, stable coating of surfaces is not possible due to their good water solubility, but protein modification, coupling to or mixing with other proteins could enable this. We found, for example, that CPPs and hydrophobins form layers with mixed properties (Bonazza et al. 2014). Further, CPPs could be used to enhance the wettability properties of solutions, which can be of interest for applications where a uniform moistening of a moderately hydrophobic surface is of interest, e.g. spraying of plant protection products or in cleaning agents.

With an increasing understanding of the mode of action of CPPs, their possible use as additives for the induction of plant resistance and defence mechanisms in fertilizers would also be an interesting possibility.

The ability of CP and Pop1 to weaken cellulose—which still needs to be tested for other CPPs—should also be kept in mind for potential applications in cellulose degradation, e.g. bioethanol production from cellulosic plant waste material or paper processing (Himmel et al. 2007).

The strong foaming of the solution of CPP, as has been reported for Epl1 (Frischmann et al. 2013), could also be of interest for the stabilization of foams and emulsions. Further, foaming can be a problem during fermentation of filamentous fungi, which is carried out, for example, in large-scale enzyme production. Therefore, it could be tested whether knockout strains of CPP-encoding genes show favourable behaviour such as less foam formation during fungal fermentations.

Biochemical analysis of more CPPs will be necessary to elucidate the application potential of CPPs further and to reveal which properties are specific for individual CPPs and which properties can be generally found in all members of the CPP family.

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References

Baccelli I, Comparini C, Bettini PP, Martellini F, Ruocco M, Pazzagli L, Bernardi R, Scala A (2012) The expression of the cerato-platanin gene is related to hyphal growth and chlamydospores formation in *Ceratocystis platani*. FEMS Microbiol Lett 327(2):155–163. doi:10.1111/j.1574-6968.2011.02475.x

Baccelli I, Luti S, Bernardi R, Scala A, Pazzagli L (2014) Cerato-platanin shows expansin-like activity on cellulosic materials. Appl Microbiol Biotechnol 98(1):175–184. doi:10.1007/s00253-013-4822-0

Bai S, Dong C, Li B, Dai H (2013) A PR-4 gene identified from *Malus domestica* is involved in the defense responses against *Botryosphaeria dothidea*. Plant Physiol Biochem 62(0):23–32. doi:10.1016/j.plaphy.2012.10.016

Boddi S, Comparini C, Calamassi R, Pazzagli L, Cappugi G, Scala A (2004) Cerato-platanin protein is located in the cell walls of ascospores, conidia and hyphae of *Ceratocystis fimbriata f. sp. platani*. FEMS Microbiol Lett 233(2):341–346. doi:10.1016/j.femsle.2004.03.001

Bonazza K, Gaderer R, Przylucka A, Druzhinina IS, Friedbacher G, Seidl-Seiboth V (2014) Formation of hybrid protein layers of the cerato-platanin protein EPL1 and hydrophobins from the fungal genus *Trichoderma*. Manuscript submitted

Chen H, Kovalchuk A, Keriö S, Asiegbu FO (2013) Distribution and bioinformatic analysis of the cerato-platanin protein family in *Dikarya*. Mycologia 105(6):1479–1488. doi:10.3852/13-115

Cole GT, Zhu SW, Pan SC, Yuan L, Kruse D, Sun SH (1989) Isolation of antigens with proteolytic activity from *Coccidioides immitis*. Infect Immun 57(5):1524–1534

de Jonge R, van Esse HP, Kombrink A, Shinya T, Desaki Y, Bours R, van der Krol S, Shibuya N, Joosten MH, Thomma BP (2010) Conserved fungal LysM effector Ecp6 prevents chitin-triggered immunity in plants. Science 329(5994):953–955. doi:10.1126/science.1190859

de O Barsottini MR, de Oliveira JF, Adamoski D, Teixeira PJ, do Prado PF, Tiezzi HO, Sforca ML, Cassago A, Portugal RV, de Oliveira PS, de M Zeri AC, Dias SM, Pereira GA, Ambrosio AL (2013) Functional diversification of cerato-platanins in *Moniliophthora perniciosa* as seen by differential expression and protein function specialization. Mol Plant Microbe Interact 26(11):1281–1293. doi:10.1094/MPMI-05-13-0148-R

de Oliveira AL, Gallo M, Pazzagli L, Benedetti CE, Cappugi G, Scala A, Pantera B, Spisni A, Pertinhez TA, Cicero DO (2011) The structure of the elicitor cerato-platanin (CP), the first member of the CP fungal protein family, reveals a double ψβ-barrel fold and carbohydrate binding. J Biol Chem 286(20):17560–17568. doi:10.1074/jbc.M111.223644

Djonovic S, Pozo MJ, Dangott LJ, Howell CR, Kenerley CM (2006) Sm1, a proteinaceous elicitor secreted by the biocontrol fungus *Trichoderma virens* induces plant defense responses and systemic resistance. Mol Plant Microbe Interact 19(8):838–853. doi:10.1094/MPMI-19-0838

Djonovic S, Vargas W, Kolomiets M, Horndeski M, Wiest A, Kenerley C (2007) A proteinaceous elicitor Sm1 from the beneficial fungus *Trichoderma virens* is required for induced systemic resistance in maize. Plant Physiol 145(3):875–889. doi:10.1104/pp. 107.103689

Frías M, González C, Brito N (2011) BcSpl1, a cerato-platanin family protein, contributes to *Botrytis cinerea* virulence and elicits the hypersensitive response in the host. New Phytol 192(2):483–495. doi:10.1111/j.1469-8137.2011.03802.x

Frías M, Brito N, González C (2013a) The *Botrytis cinerea* cerato-platanin BcSpl1 is a potent inducer of systemic acquired resistance (SAR) in tobacco and generates a wave of salicylic acid expanding from the site of application. Mol Plant Pathol 14(2):191–196. doi:10.1111/j.1364-3703.2012.00842.x

Frías M, Brito N, González M, González C (2013b) The phytotoxic activity of the cerato-platanin BcSpl1 resides in a two-peptide motif in the protein surface. Mol Plant Pathol. doi:10.1111/mpp.12097

Frischmann A, Neudl S, Gaderer R, Bonazza K, Zach S, Gruber S, Spadiut O, Friedbacher G, Grothe H, Seidl-Seiboth V (2013) Self-assembly at air/water interfaces and carbohydrate-binding properties of the small secreted protein EPL1 from the fungus *Trichoderma atroviride*. J Biol Chem 288:4278–4287. doi:10.1074/jbc.M112.427633

Gaderer R (2013) Functional characterization of a fungal gene family encoding plant defense response-eliciting proteins in *Trichoderma* biocontrol species. Master Thesis. Vienna University of Technology, Austria

Gonzaléz-Fernandez R, Aloria K, Valero-Galván J, Redondo I, Arizmendi JM, Jorrín-Novo JN (2014) Proteomic analysis of mycelium and secretome of different *Botrytis cinerea* wild-type strains. Proteomics 97:195–221

González-Fernández R, Aloria K, Valero-Galván J, Redondo I, Arizmendi JM, Jorrín-Novo JV (2014) Proteomic analysis of mycelium and secretome of different *Botrytis cinerea* wild-type strains. J Proteomics 97(0):195–221. doi:10.1016/j.jprot.2013.06.022

Himmel ME, Ding SY, Johnson DK, Adney WS, Nimlos MR, Brady JW, Foust TD (2007) Biomass recalcitrance: engineering plants and enzymes for biofuels production. Science 315(5813):804–807. doi:10.1126/science.1137016

Jeong JS, Mitchell TK, Dean RA (2007) The *Magnaporthe grisea* snodprot1 homolog, MSP1, is required for virulence. FEMS Microbiol Lett 273(2):157–165. doi:10.1111/j.1574-6968.2007.00796.x

Linder MB, Szilvay GR, Nakari-Setälä T, Penttilä ME (2005) Hydrophobins: the protein-amphiphiles of filamentous fungi. FEMS Microbiol Rev 29(5):877–896. doi:10.1016/j.femsre.2005.01.004

Lombardi L, Faoro F, Luti S, Baccelli I, Martellini F, Bernardi R, Picciarelli P, Scala A, Pazzagli L (2013) Differential timing of defense-related responses induced by cerato-platanin and cerato-populin, two non-catalytic fungal elicitors. Physiol Plant 149(3):408–421. doi:10.1111/ppl.12041

Martellini F, Faoro F, Carresi L, Pantera B, Baccelli I, Maffi D, Tiribilli B, Sbrana F, Luti S, Comparini C, Bernardi R, Cappugi G, Scala A, Pazzagli L (2013) Cerato-populin and cerato-platanin, two non-catalytic proteins from phytopathogenic fungi, interact with hydrophobic inanimate surfaces and leaves. Mol Biotechnol 55(1):27–42. doi:10.1007/s12033-012-9618-4

Pan SC, Cole GT (1995) Molecular and biochemical characterization of a *Coccidioides* immits-specific antigen. Infect Immun 63(10):3994–4002

Pazzagli L, Cappugi G, Manao G, Camici G, Santini A, Scala A (1999) Purification, characterization, and amino acid sequence of cerato-platanin, a new phytotoxic protein from *Ceratocystis fimbriata f. sp. platani*. J Biol Chem 274(35):24959–24964. doi:10.1074/jbc.274.35.24959

Pazzagli L, Zoppi C, Carresi L, Tiribilli B, Sbrana F, Schiff S, Pertinhez TA, Scala A, Cappugi G (2009) Characterization of ordered aggregates of cerato-platanin and their involvement in fungus-host interactions. Biochim Biophys Acta 1790(10):1334–1344. doi:10.1016/j.bbagen.2009.07.014

Pazzagli L, Seidl-Seiboth V, Barsottini M, Vargas WA, Scala A, Mukherjee PK (2014) Cerato-platanins: elicitors and effectors. Plant Sci. doi:10.1016/j.plantsci.2014.02.009

Roy A, Kucukural A, Zhang Y (2010) I-TASSER: a unified platform for automated protein structure and function prediction. Nat Protoc 5:725–738. doi:10.1038/nprot.2010.5

Sampedro J, Cosgrove DJ (2005) The expansin superfamily. Genome Biol 6(12):242. doi:10.1186/gb-2005-6-12-242

Sbrana F, Bongini L, Cappugi G, Fanelli D, Guarino A, Pazzagli L, Scala A, Vassalli M, Zoppi C, Tiribilli B (2007) Atomic force microscopy images suggest aggregation mechanism in cerato-platanin. Eur Biophys J 36(7):727–732. doi:10.1007/s00249-007-0159-x

Scala A, Pazzagli L, Comparini C, Santini A, Tegli S, Cappugi G (2004) Cerato-platanin, an early-produced protein by *Ceratocystis fimbriata f. sp. platani*, elicits phytoalexin synthesis in host and non-host plants. J Plant Pathol 86:27–33. doi:10.4454/jpp.v86i1.934

Seidl V, Marchetti M, Schandl R, Allmaier G, Kubicek CP (2006) Epl1, the major secreted protein of *Hypocrea atroviridis* on glucose, is a member of a strongly conserved protein family comprising plant defense response elicitors. FEBS J 273(18):4346–4359. doi:10.1111/j.1742-4658.2006.05435.x

Seidl-Seiboth V, Gruber S, Sezerman U, Schwecke T, Albayrak A, Neuhof T, von Döhren H, Baker SE, Kubicek CP (2011) Novel hydrophobins from *Trichoderma* define a new hydrophobin subclass: protein properties, evolution, regulation and processing. J Mol Evol 72(4):339–351. doi:10.1007/s00239-011-9438-3

Templeton MD, Rikkerink Erik HA, Beever RE (1994) Small, cysteine-rich proteins and recognition in fungal-plant interactions. Mol Plant Microbe Interact 7:320–325. doi:10.1094/MPMI-7-0320

Vargas WA, Djonovic S, Sukno SA, Kenerley CM (2008) Dimerization controls the activity of fungal elicitors that trigger systemic resistance in plants. J Biol Chem 283(28):19804–19815. doi:10.1074/jbc.M802724200

Wilson LM, Idnurm A, Howlett BJ (2002) Characterization of a gene (sp1) encoding a secreted protein from *Leptosphaeria maculans*, the blackleg pathogen of *Brassica napus*. Mol Plant Pathol 3(6):487–493. doi:10.1046/j.1364-3703.2002.00144.x

Wösten HB, Wessels JH (1997) Hydrophobins, from molecular structure to multiple functions in fungal development. Mycoscience 38(3):363–374. doi:10.1007/bf02464099

Yang Y, Zhang H, Li G, Li W, Wang X, Song F (2009) Ectopic expression of MgSM1, a Cerato-platanin family protein from *Magnaporthe grisea*, confers broad-spectrum disease resistance in Arabidopsis. Plant Biotechnol J 7(8):763–777. doi:10.1111/j.1467-7652.2009.00442.x

Chapter 2: Functional and biochemical studies of members of the cerato-platanin protein family

Chapter 2.2

Sm2, a paralog of the *Trichoderma* cerato-platanin elicitor Sm1, is also highly important for plant protection conferred by the fungal-root interaction of *Trichoderma* with maize.

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Sm2, a paralog of the *Trichoderma* cerato-platanin elicitor Sm1, is also highly important for plant protection conferred by the fungal-root interaction of *Trichoderma* with maize

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Abstract

Background: The proteins Sm1 and Sm2 from the biocontrol fungus *Trichoderma virens* belong to the cerato-platanin protein family. Members of this family are small, secreted proteins that are abundantly produced by filamentous fungi with all types of life-styles. Some species of the fungal genus *Trichoderma* are considered as biocontrol fungi because they are mycoparasites and are also able to directly interact with plants, thereby stimulating plant defense responses. It was previously shown that the cerato-platanin protein Sm1 from *T. virens* - and to a lesser extent its homologue Epl1 from *Trichoderma atroviride* - induce plant defense responses. The plant protection potential of other members of the cerato-platanin protein family in *Trichoderma*, however, has not yet been investigated.

Results: In order to analyze the function of the cerato-platanin protein Sm2, *sm1* and *sm2* knockout strains were generated and characterized. The effect of the lack of Sm1 and Sm2 in *T. virens* on inducing systemic resistance in maize seedlings, challenged with the plant pathogen *Cochliobolus heterostrophus*, was tested. These plant experiments were also performed with *T. atroviride epl1* and *epl2* knockout strains. In our plant-pathogen system *T. virens* was a more effective plant protectant than *T. atroviride* and the results with both *Trichoderma* species showed concordantly that the level of plant protection was more strongly reduced in plants treated with the *sm2/epl2* knockout strains than with *sm1/epl1* knockout strains.

Conclusions: Although the cerato-platanin genes *sm1/epl1* are more abundantly expressed than *sm2/epl2* during fungal growth, Sm2/Epl2 are, interestingly, more important than Sm1/Epl1 for the promotion of plant protection conferred by *Trichoderma* in the maize-*C. heterostrophus* pathosystem.

Keywords: Cerato-platanin protein, *Trichoderma virens*, *Trichoderma atroviride*, Mycoparasitism, Biocontrol, Plant protection, Maize, *Cochliobolus heterostrophus*

Background

Fungi belonging to the ascomycete genus *Trichoderma* inhabit the soil and rhizosphere, where they interact with plant roots and with other fungi. Agricultural biocontrol applications take advantage of the well-known ability of *Trichoderma* spp. to attack and destroy fungal hosts, which is called mycoparasitism. The wide host range includes soil-borne plant pathogens such as *Rhizoctonia solani* or *Pythium ultimum*, which make *Trichoderma* spp. biological plant protectants. In addition, the interaction of *Trichoderma* with roots primes the plant's immune system for better resistance against pathogens [1-4]. Due to this induced systemic resistance, *Trichoderma* spp. are able to protect plants against some foliar pathogens, in addition to soil-borne pathogens. Plants recognize proteins secreted by the fungus, and such microbe associated (in this case fungal-

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associated) molecular patterns activate systemic resistance. The first such secreted protein to be studied in detail in *Trichoderma*-plant interactions was a small secreted cysteine-rich protein belonging to the cerato-platanin protein (CPP) family, named Sm1/Epl1 in *Trichoderma virens* and *Trichoderma atroviride*, respectively [5,6]. The *Trichoderma* genomes analyzed so far contain three genes encoding CPPs [7]. Gene expression was analyzed in *T. atroviride* and revealed that *epl1* is expressed during hyphal growth, *epl2* expression was only detected during spore maturation, and hardly any expression was found for *epl3* [8]. Single and double knockout strains of *epl1* and *epl2* did not reveal any phenotype related to hyphal growth or development.

T. atroviride and *T. virens* belong to distant clades within the genus *Trichoderma*, for which so far already more than 200 species have been described [9]. Analysis of the genomes of *T. virens* and *T. atroviride* revealed numerous differences in the genome inventory of these two species, which each have more than 2500 genes that do not occur in the other species [7]. Further, even strongly conserved genes, e.g. chitinases, have already been shown to be differentially expressed in *T. atroviride* and *T. virens* [10]. However, the biological consequences of these findings on the lifestyle of *T. atroviride* and *T. virens* have, as yet, only been partially understood. It is therefore important to note that it is not always valid to draw direct conclusions from the results in one species to the other. Rather, one can study protein families in both of them in order to better elucidate the similarities and differences between *T. atroviride* and T. *virens*.

One example of the differences between these two species are the CPP orthologues Sm1 and Epl1. *T. virens* Sm1 was shown to induce plant defense responses, but this ability is far weaker for its homologue *T. atroviride* Epl1 [11]. This was explained by different tendencies of these proteins to dimerize. Only monomers of Sm1 and Epl1 were shown to efficiently induce plant defense responses. While Epl1 is readily able to form dimers, Sm1 has a single glycosylation site that is not present in Epl1 and is predominantly found in its monomeric form, which is more effective in the induction of plant defense responses [11].

The ability of CPP family members of *Trichoderma* from other phylogenetic branches to promote systemic resistance in plants has not been studied yet. In this study, we generated knockout strains of *T. virens sm1* and *sm2* and analyzed them for developmental phenotypes and for their ability to induce resistance of maize to the Southern corn leaf blight pathogen *Cochliobolus heterostrophus*. Plant experiments were also carried out with *T. atroviride epl1* and *epl2* knockout strains [8].

Results

Gene expression of CPP-encoding genes in *T. virens*

The genome of *T. virens* Gv29-8 contains three genes, *sm1*, *sm2* and *sm3*, encoding CPPs (http://genome.jgi-psf.org/TriviGv29_8_2/TriviGv29_8_2.home.html and [7]). This is in analogy to the genomes of *T. atroviride* and *T. reesei*, which also contain three CPP genes [6,8]. The genes *sm2* and *sm3* and their respective proteins have not been studied yet. In order to assess the transcriptional profiles of all three genes encoding CPPs in *T. virens*, their expression was first analyzed with RT-PCR. While the *sm1* gene was found to be expressed during hyphal growth, no expression was observed for *sm2* and *sm3* at these time points (Figure 1a). In biomass harvested from sporulating cultures grown on potato dextrose agar (PDA) plates during different stages of spore maturation, ranging from mycelium covered with white conidia to first light green and then dark green conidia, *sm2* was found to be strongly upregulated, but *sm1* was also found to be expressed (Figure 1b). For *sm3* no expression was detected under the tested growth conditions.

Generation and characterization of *sm1* and *sm2* knockout strains

Based on these gene expression results, gene knockout strains of *sm1* and *sm2* were generated in *T. virens* I10 Δ*tku70* [12] as described in the methods section (Additional file 1: Figure S1). Since Sm1 was previously shown to be far more potent in inducing plant defense responses than Epl1 [11] and the lack of *sm1* strongly reduces the ability of *T. virens* to induce plant defense responses [13], we were interested to test whether Sm2 has a similar function in *T. virens*. Phylogenetically Sm2 belongs to a different branch of CPPs [6] and none of these proteins have been studied so far. Since *sm1* is expressed throughout different growth stages and a possible function of CPPs in fungal growth has been discussed in the literature [14], the generated knockout strains were tested for phenotypic alterations with respect to the following properties related to fungal growth: growth on agar plates (Figure 2), formation of

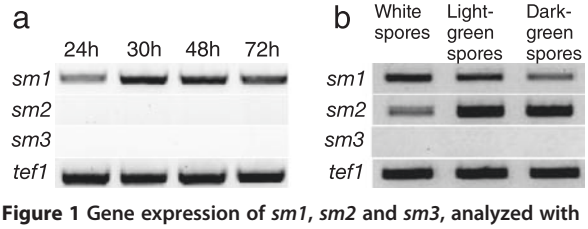

Figure 1 Gene expression of *sm1*, *sm2* and *sm3*, analyzed with RT-PCR. (a) Biomass harvested from shake flask cultivations with glucose as carbon source **(b)** Biomass harvested from sporulating fungal cultures grown on agar plates (PDA). *Tef1* was used as reference gene.

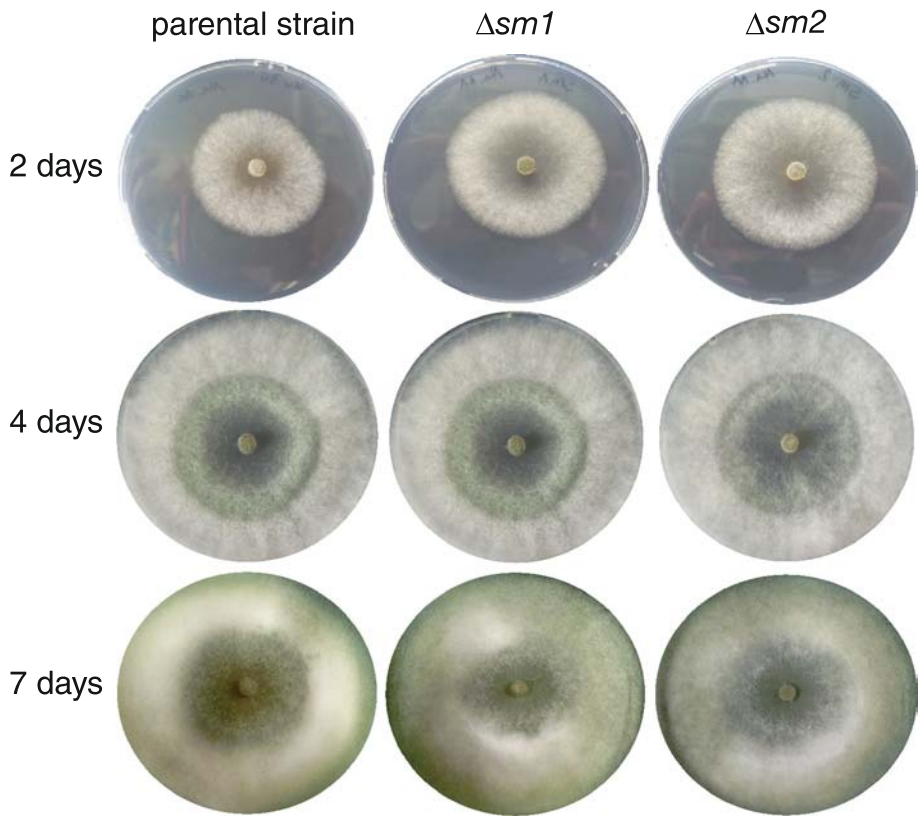

Figure 2 Colony growth of *T. virens* parental strain Δ*tku70, sm1* and *sm2* knockout strains on agar plates (PDA).

aerial hyphae, growth along (moist) surfaces, bridging of gaps between two agar blocks, and transition of hyphae between solid/liquid interfaces. Different types of desiccation stress, e.g. drying of water droplets and drying of thin agar plates, were also examined. Furthermore, we analyzed conidiation, biomass formation in shake flask cultivations, germination efficiency, hydrophobicity of the mycelium, chlamydospore formation, osmotic stress, and cell wall stress (i.e. addition of Calcofluor white and Congo Red, both of which interfere with the construction and stress response of the cell wall [15]). No morphological differences between the parental strain and the knockout strains were found in *T. virens,* which is similar to *T. atroviride,* where no phenotype had been detected in *epl1* and *epl2* single and double knockout strains [8]. At advanced time points (48 h and 72 h) of shake flask cultivations of *T. virens* abundant formation of chlamydospores was detected (Additional file 1: Figure S2). A correlation between chlamydospore formation and *cp* (*cerato-platanin*) gene expression had been reported for *Ceratocystis platani* [16], but no differences were detected between the parental strain and the knockout strains in *T. virens*.

The mycoparasitic potential of the knockout strains was also not altered based on confrontation assays of *T. virens* against *R. solani* (Additional file 1: Figure S3) and *Botrytis cinerea* (data not shown).

We had found in a previous study that the expression of *T. atroviride epl1,* the homologue of *T. virens sm1*, is not constant during hyphal growth but its expression level is strongly dependent on parameters that influence the growth rate (e.g. medium composition and growth temperature) [8]. We therefore paid particular attention to this aspect in the knockout strains and assessed the gene expression of *sm1* quantitatively with qPCR in the *sm2* knockout strain and the parental strain Δ*tku70* (control strain). The results (Figure 3) showed that *sm1* has a slightly different expression profile in the *sm2* knockout strain compared to the parental strain, but we were not able to elucidate this further due to the lack of any detectable morphological changes in the knockout strains.

T. virens strain I10 is a Q-strain

T. virens strains can be grouped into P- and Q-strains, based on their antibiotic profiles. Strains of the Q-group produce the antibiotic gliotoxin and are generally considered to be more effective biocontrol agents [17]. The spectrum of secondary metabolites that is produced has a profound effect on the plant protection potential and

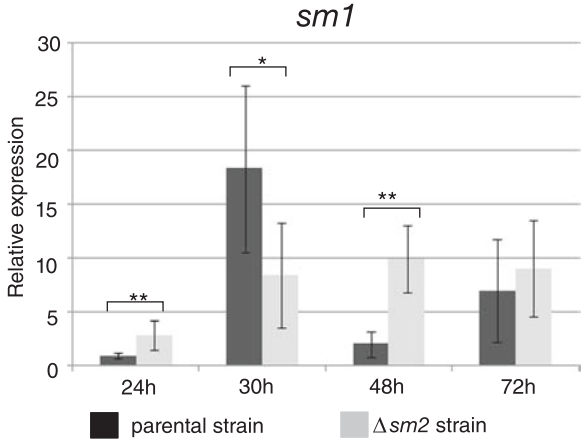

Figure 3 Gene expression (qPCR) of *sm1* in the parental strain and *sm2* knockout strain. Samples were taken at the indicated time points from shake flask cultivations with glucose as carbon source. All samples were normalized to the 24 h sample of the parental strain. *Tef1* was used as reference gene. Bars indicate the SEM (*, ** and *** indicate significance at $P < 0.05$, 0.01 and 0.001).

mycoparasitic activity of *T. virens* [18,19]. Previous studies on Sm1 were carried out in strain Gv29-8, which is a Q-strain [19]. In order to relate our experiments on the role of Sm2 (and Sm1) in the interaction of *T. virens* with plants (see below) more directly to previous studies [5,13], we were interested whether strain I10, which we used for our studies [12], is a P or a Q-strain. *T. virens sm1* – but not *sm2* – knockout strains have been previously studied in strain Gv29-8. Both strains, I10 and Gv29-8, were grown for 36 h in shake flask cultivations in liquid potato dextrose broth medium and gliotoxin production was measured from filtered culture supernatants. The results (Additional file 1: Figure S4) showed that I10 produced 18.5 mg/l gliotoxin, an amount similar to strain Gv29-8 (17.5 mg/l), and strain I10 can therefore also be attributed to the group of Q-strains of *T. virens,* which is of particular relevance for the discussion of our plant experiments (see below).

Sm1 and *sm2* knockout strains show reduced levels of plant protection

In order to test whether Sm2 is involved in the interaction of *Trichoderma* with plants we analyzed whether the lack of *sm2* leads to an altered potential to protect plants against fungal pathogens. For this, the interaction of *C. heterostrophus* with its host, maize, was used as a model pathosystem. We have recently standardized this system (N.L.L. and B.A.H., unpublished results) and it was used previously with *Trichoderma asperellum* ([20]). The *T. virens* parental and *sm1* knockout strains were also included, and in addition experiments were performed with *T. atroviride* wild-type, *epl1* and *epl2* knockout strains generated in a previous study [8]. Lesion sizes on maize plants, whose roots were co-cultured with the *T. virens* parental strain Δ*tku70* or *sm1* and *sm2* knockout strains, were measured (Figure 4a, b). Colonization of maize roots by the *T. virens* parental strain significantly decreased symptoms by more than 40%. Plants colonized by Δ*sm1* showed impaired resistance in comparison to plants treated with the *T. virens* parental strain, but were still significantly different from control plants, showing about 30% decrease in lesion size. Knock-out of *sm2*, however, led to a much more dramatic decrease in the ability of the fungus to induce resistance in maize, resulting in large lesions which were similar to the lesions of the control plants (no *Trichoderma*). In order to assess the gene expression of *sm*-genes in *T. virens* during plant interaction, root biomass was harvested from plant experiments four days post inoculation with *T. virens* conidia and analyzed by qPCR. Loss of one *sm*-gene might affect the expression of the other through feedback in the signaling network. The results (Figure 4c) showed, overall, no strong changes in gene expression of the other *sm*-gene. The expression of *sm1* was not altered in the Δ*sm2* strain. *sm2* expression tended to be higher in Δ*sm1* than in the parental strain, but this was not statistically significant.

Plant experiments were also carried out with *T. atroviride epl1* and *epl2* single and double knockout strains that were generated in a previous study [8]. While plants treated with the *T. virens* parental strain showed more than 40% reduction in lesion size, the lesion size in plants treated with the *T. atroviride* IMI206040 wild-type strain was only 13% smaller than in the control plants (Figure 4d). Despite the relatively poor plant protection potential of *T. atroviride*, we observed exactly the same trend regarding ability of Δ*epl1* and Δ*epl2* strains to protect plants as for the corresponding mutants in *T. virens.* In addition, lesion sizes on plants colonized by the double knockout strain Δ*epl1*Δ*epl2* were not significantly different from Δ*epl2* treated plants and control plants (Figure 4d).

Discussion

In this study the gene expression of the three genes encoding CPPs in *T. virens* was analyzed. In analogy to previous results from *T. atroviride*, *sm1* was found to be expressed during hyphal growth, i.e. under growth conditions when sufficient nutrients are available and fast biomass formation occurs. For *sm2* gene expression was detected in mycelium that was harvested from sporulating cultures. Since the formation of conidia is associated with differentiation of the hyphae to form conidiophores and phialides, *sm2* gene expression could also be associated with these structures, but it is unfortunately not possible to separate them efficiently. It should be noted that in shake flask cultivations, where, according to our microscopic observations, large amounts of chlamydospores - which directly split off from hyphae - but no

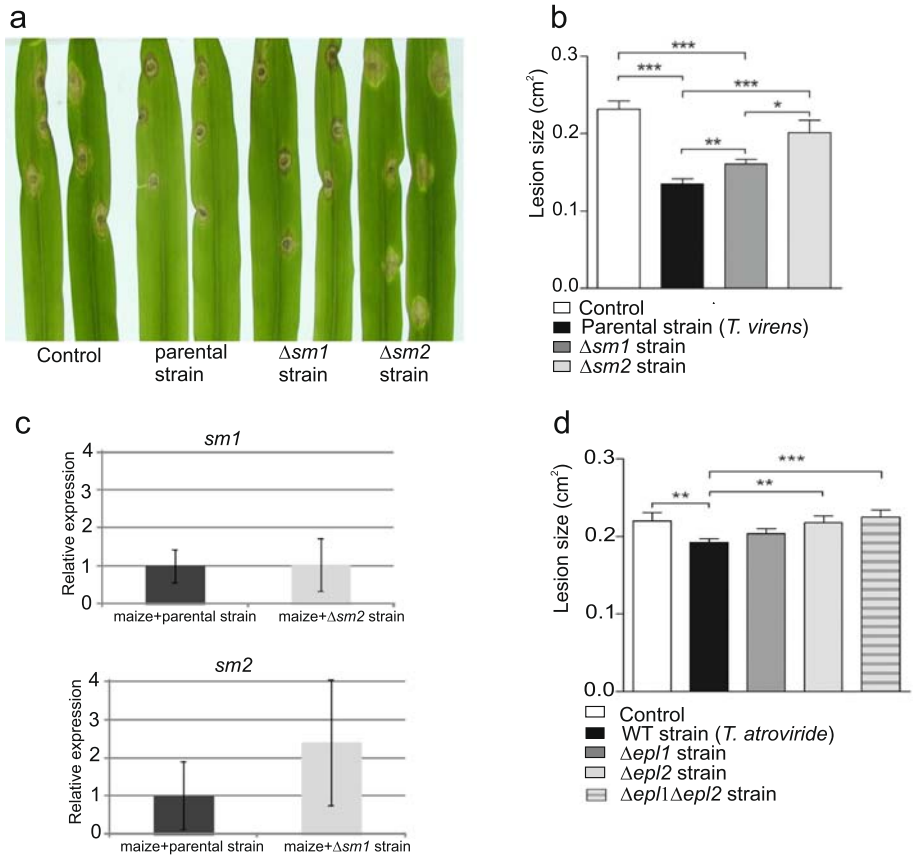

Figure 4 Effect of *T. virens* (parental strain, Δ*sm1* and Δ*sm2*) on plant protection in maize seedlings challenged with the maize pathogen *C. heterostrophus*. (a) Lesion development in leaves of *T. virens*-induced maize, two days after pathogen challenge. **(b)** Lesion size measured from photographed leaves. Number of lesions measured in control plant, 33; *T. virens* parental strain, 23; Δ*sm1*, 24; Δ*sm2*, 23. **(c)** Gene expression (qPCR) of *sm1* and *sm2* in maize seedlings infected with the pathogen *C. heterostrophus* and treated with the *T. virens* parental strain, *sm1* and *sm2* knockout strain. Number of lesions measured in control plant, 24; *T. atroviride* wild-type, 27; Δ*epl1*, 23; Δ*epl2*, 23; Δ*epl1*Δ*epl2*, 24. Data from (b) and (d) represent the combined data from plants taken from two beakers. The experiment was carried out independently twice, with two biological repeats, each one containing 12 plants, with the same results within the variability. Bars indicate the SEM (standard error of the mean). *, ** and *** indicate significance at $P < 0.05$, 0.01 and 0.001. **(d)** Effect of *T. atroviride* (wild-type, Δ*epl1*, Δ*epl2*, and Δ*epl1*Δ*epl2*) on plant protection in maize seedlings challenged with the maize pathogen *C. heterostrophus*. Lesion size measured from photographed leaves.

conidia, were formed, no expression of *sm2* was found (Figure 1 and Figure S2 in supplemental material). This indicates that *sm2* gene expression is connected to the formation and maturation of conidia but not to other types of spores in *T. virens*. It should be noted that, due to morphological differences of the mycelium on agar plates between *T. atroviride* and *T. virens*, in *T. atroviride* the harvested biomass from sporulating cultures consists mainly of spores, whereas in *T. virens* I10 the mycelium is fluffier and the harvested biomass is therefore a mixture of spores and hyphae. This probably explains why in *T. virens* expression of *sm1* and *sm2* was detected in these samples, whereas in *T. atroviride* strong *epl2* expression but only weak *epl1* expression was found. *Sm2* is also expressed in co-culture with maize (Figure 4).

Our transcriptional data for *sm1* are in agreement with findings by Djonovic et al. [5], who reported expression under all tested conditions, including sporulating and non-sporulating mycelia. In other fungi there is also evidence that homologues of *sm1* are expressed during hyphal growth, e.g. in *B. cinerea*, *bcspl1* was found to be expressed under many different growth conditions, whereas no expression was found for *bcspl2*, a second CP gene [21]. *MgSM1* from *Magnaporthe grisea* was also expressed during different fungal growth stages [22]. In addition to these gene expression data, the protein Epl1 was found to be the predominant protein in the secretome of submerged *T. atroviride* cultivations with glucose as a carbon source [6]. In the plant pathogenic basidiomycete *Moniliophthora perniciosa* gene expression data of the 12 CP genes (*MpCP1-12*) showed complex transcriptional profiles throughout fungal development and pathogenic infestation of the plant, suggesting a specialization of the respective proteins in different biological processes

[23]. In ascomycetes, gene expression data are so far limited to homologues of *sm1* except for *epl2* and *epl3* from *T. atroviride* and *bcspl2* from *B. cinerea* (see above). It will be of interest for future studies to obtain more expression data for these genes, in particular considering the strong effect of the lack of *sm2* on the ability of *Trichoderma* to protect maize from *C. heterostrophus* in our plant experiments (Figure 4).

The mycoparasitic potential of *sm*-knockout strains against *R. solani* and *B. cinerea* was not altered and it can be anticipated that this would also be the case for other host fungi. Nonetheless *sm*-knockout strains have a strong effect on the biocontrol properties of *T. virens* via direct effects on the *Trichoderma*-plant interaction. In the *T. virens* Gv29.8-maize - *Colletotrichum graminicola* interaction, loss of Sm1 resulted in complete loss of the capacity to reduce lesion size on the leaves [13]. A single protein might seem unlikely to be responsible, alone, for the induction of systemic resistance. However, it seems that in the maize *C. graminicola* interaction Sm1 is indeed the dominant player. In the maize - *Cochliobolus* assay used in this study, on the other hand, knock-out of *sm1* reduced the plant protection potential of *T. virens*, but in this pathosystem the lack of Sm2 had an even greater effect and lesion size of maize leaves was statistically not different from the control (no *Trichoderma*). Although the colonization efficiency of maize roots by *T. virens* was not directly measured, it should be noted that upon harvesting of plant roots for biomass extraction for qPCR experiments, no obvious phenotypes in fungal growth and the appearance of the colonized roots were observed. In plant experiments with *T. atroviride* plant protection levels were overall lower than with *T. virens*, but the same trend was observed for *epl1* and *epl2* knockout strains as for *sm1* and *sm2* knockout strains, confirming that Sm2/Epl2 are, in the *C. heterostrophus*-maize pathosystem, more important for plant protection than Sm1/Epl1. This is also relevant because it underlines that the observed effect is not due to any unwanted, genetic side-effects of the knockout strains or due a particular feature of the *T. virens* strain used. The *T. atroviride* double knockout strain Δ*epl1*Δ*epl2* appeared slightly (albeit not significantly) less effective than the Δ*epl2* strain (Figure 4b, d). These data are compatible with an additive contribution of Sm1 and Sm2, and the contribution of these two paralogs to the induction of resistance is similar for *T. virens* and *T. atroviride*. The data in Figure 4 provide genetic evidence that for maximal induction of resistance, both paralogs need to be present. In contrast to maize- *C. graminicola*, where Sm1 dominates, Sm2 appears to be the dominant one in this particular assay. Since *sm1* expression was not found to be altered in the *sm2* deletion strain in plant experiments it can be concluded that reduction of the capacity to protect the plant was directly due to the absence of Sm2.

When comparing the *C. graminicola* to the *C. heterostrophus* pathosystem assays, it is important to note that the assay here was done using hydroponic cultures rather than soil-grown plants, which could affect the relative extent of colonization, intensity of ISR and contribution of specific secreted proteins. To maximize the potential of *Trichoderma* spp. to protect plants, expression of different combinations of CPP family members, at different levels, will need to be tested, in different pathosystems.

Conclusions

CPPs are potent inducers of plant defense responses in plant pathogenic fungi as well as plant-beneficial fungi such as *Trichoderma* species. In this study we showed that *T. virens sm2* knockout strains were more impaired in the protection of maize seedlings against the pathogen *C. heterostrophus* than *sm1* knockout strains. *T. atroviride* was overall less effective in plant protection than *T. virens*, but the same trend was observed for the respective *epl2* and *epl1* knockout strains. These findings advance our understanding of the diversified functions of CPPs in fungi and of the pool of molecules that are involved in the beneficial interaction of *Trichoderma* with plants. Our results show that the paradigm of Sm1 as the main or exclusive inducer of plant systemic resistance triggered by *Trichoderma*-root interactions needs to be generalized. As we have shown that in the particular interaction studied here Sm2 is even more important than Sm1, it seems likely that even more elicitors remain to be discovered in *Trichoderma*.

Methods

Generation of knockout strains and phenotype analysis

Knockout strains were generated using *T. virens* I10 Δ*tku70* [12] as a parental strain. A schematic representation of the *sm1* and *sm2* knockout loci is shown in Additional file 1: Figure S1. All primers used for generation and verification of knockout strains are listed (see Additional file 1: Table S1). For construction of the *sm1* deletion vector 900 bp of the 5'- and 860 bp of the 3'-flanking regions of *sm1* were amplified from genomic DNA of *T. virens* I10 with the primers sm1-5'-fw/sm1-5'-rv and sm1-3'-fw/sm1-3'-rv, respectively. The PCR products were cloned into a pBS (Bluescript SK+) vector containing a selection hph-cassette (*hph* gene, conferring resistance to hygromycin, under control of the *T. reesei pki* promoter and *cbh2* terminator) [24]. The obtained plasmid was first linearized with XhoI and the *sm1*-5'flanking region was inserted with the In-Fusion cloning kit (Clontech, Mountain View, CA, USA). Then the resulting plasmid was again linearized with EcoRV and the *sm1*-3' flanking region was inserted. For identification

of knockout strains primers sm1-promupstream-fw and sm1-hph-cass-rv were used, yielding a 2 kb PCR product for positive knockout strains. The purification of the knockout strains and thus absence of the *sm1* gene was verified with primers sm1-promupstream-fw and sm1-term-rv, yielding a PCR product with the size of 2 kb for the *sm1*-wild-type.

For generation of the *sm2* deletion vector the *Aspergillus oryzae ptrA* gene was used as a selection marker, conferring resistance against pyrithiamine [25]. The resistance marker cassette, containing the native promoter and terminator of the *ptr* gene, was amplified from a plasmid (kindly obtained from B. Seiboth) and inserted into a pBS (Bluescript SK+) vector that was previously linearized with XhoI and HindIII via In-Fusion cloning (Clontech). The 5'-flanking region of *sm2* was amplified with the primers sm2-5'-fw/sm1-5'-rv and the 3'-flanking region was amplified with the primers sm2-3'-fw/sm2-3'-rv. The flanking regions were inserted into plasmid pBS-ptr that was linearized with XhoI for the s*m2*-5' region and HindIII for the *sm2*-3' region via In-Fusion cloning (Clontech). Knockout strains were identified by PCR with the primers sm2-promupstream-fw and sm2-ptr-cass-rv, yielding a 2 kb band for the *sm2* knockout locus. After single spore isolations, the absence of the *sm2* gene was verified with the primers sm2-promupstream-fw and sm2-term-rv, yielding a 2.2 kb band for the *sm2* wild-type locus.

Fungal transformation, carried out with the PCR-amplified transformation cassettes, protoplast generation, preparation of selection media and purification of fungal transformants were performed as described in [12]. For selection on pyrithiamine (1 μg/ml) *T. virens* transformants were grown on ISM medium; 0.68 g/L KH_2PO_4, 0.87 g/L K_2HPO_4, 1.7 g/L $(NH_4)_2SO_4$, 0.2 g/L $CaCl_2$, 0.2 g/L KCl, 0.2 g/L $MgSO_4.7H_2O$, 5 mg/L $FeSO_4.7H_2O$, 2 mg/L $ZnSO_4.7H_2O$, 2 mg/L $MnSO_4.7H_2O$ [6] in order to facilitate the differentiation between transformants and background growth.

Mycoparasitism assays were performed on potato dextrose agar (PDA) plates. *T. virens* and a host fungus (*R. solani* or *B. cinerea*) were placed on opposite sides of the agar plate and incubated at 28°C with a 12 h/12 h light/dark cycle. Images of the confrontation assays were taken every 24 h to record the antagonism and overgrowth of the host fungi by *T. virens*.

Phenotype analysis of the knockout strains was carried out as described for *T. atroviride* in [8]. All experiments were carried out with at least two independent biological replicates.

Fungal cultivations and gene expression analysis

Shake flask cultivations were carried out with ISM medium [6] containing 1% glucose and 0.05% peptone. Media were inoculated with 1×10^6 conidia/ml and cultivated at 25°C and 200 rpm. Mycelia were harvested at the time points indicated in the results section and frozen in liquid nitrogen. For gene expression analysis from conidia at different maturation stages (based on the appearance of the mycelium covered with conidia, ranging from white to first light green and then dark green conidia), sporulated mycelia were scraped from PDA plates with a spatula and frozen in liquid nitrogen. For RNA isolation the samples were ground to a fine powder under liquid nitrogen and total RNA was isolated using the guanidinium thiocyanate method [26]. Isolated RNAs were treated with DNAse I (Fermentas, St Leon-Rot, Germany), and cDNAs were generated with the Revert Aid H-minus cDNA synthesis kit (Fermentas). RT–PCR (25 cycles) was performed using the gene-specific primers listed (see Additional file 1: Table S1). Accession numbers of the *sm*-genes in the JGI *T. virens* genome database; http://genome.jgi-psf.org/TriviGv29_8_2/TriviGv29_8_2.home.html) are: *sm1* 110852, *sm2* 111830 and *sm3* 32154. The corresponding accession numbers in the NCBI database are EHK25601 for Sm1, EHK20677 for Sm2, and EHK25819 for Sm3. The *tef1* gene (translation elongation factor 1 alpha, protein ID 83874 in the JGI database and in the NCBI database EHK22702) was used as reference gene.

qPCR reactions were performed in an Eppendorf Realplex thermal cycler. The reaction mix contained 12.5 μl SYBR green Supermix (Bio-Rad Laboratories, Hercules, CA, USA), 8.5 μl pure water, 6.25 μM forward and 6.25 μM reverse primer, and 2 μl 1:50 diluted template cDNA (5 μg of RNA/reaction were reverse-transcribed using the Revert Aid H-minus cDNA synthesis kit (Fermentas)). Reactions were performed in triplicates. Primer efficiency was calculated using a dilution series from 1:5 to 1:5000 with the PCR baseline-subtracted mode. The amplification protocol consisted of an initial denaturation step for 3 min at 95°C followed by 40 cycles of denaturation (95°C for 15 s), annealing, and elongation (60°C for 15 s). Oligonucleotides are listed in Additional file 1: Table S1. The *tef1* gene was used as a reference. Expression data were evaluated using REST software [27]. Cultivations for gene expression analysis were carried out with at least two independent biological replicates.

Gliotoxin measurements

LC-MS/MS screening of fungal metabolites was performed with a QTrap 5500 LC-MS/MS System (Applied Biosystems, Foster City, CA) equipped with a TurboIonSpray electrospray ionization (ESI) source and a 1290 Series HPLC System (Agilent, Waldbronn, Germany). Chromatographic separation was performed at 25°C on a Gemini® C_{18}-column, 150 × 4.6 mm i.d., 5 μm particle size, equipped with a C_{18} 4 × 3 mm i.d. security guard cartridge (all from Phenomenex, Torrance, CA, US). The chromatographic

method, as well as chromatographic and mass spectrometric parameters for 186 of the investigated analytes, is as described by Vishwanath et al. [28]. In the meantime, the method has been further expanded to cover 320 metabolites.

ESI-MS/MS was performed in the time-scheduled multiple reaction monitoring (MRM) mode both in positive and negative polarities in two separate chromatographic runs per sample by scanning two fragmentation reactions per analyte. The MRM detection window of each analyte was set to its expected retention time ± 27 seconds and ± 48 seconds in the positive and the negative mode, respectively. The target cycle time was 1 second. Confirmation of positive analyte identification was obtained by the acquisition of two MRMs per analyte (with the exception of moniliformin and 3-nitropropionic acid, that exhibit only one fragment ion), which yielded 4.0 identification points according to commission decision 2002/657/EC. In addition, the LC retention time and the intensity ratio of the two MRM transition agreed with the related values of an authentic standard within 0.1 min and 30% rel., respectively.

For further confirmation of the identity of gliotoxin, Enhanced Product Ion scans were performed using the third quadrupole as linear ion trap, applying a collision energy of 35 V, a collision energy spread of 15 V and a dynamic fill time of the trap. Spectra were obtained by averaging 20 scans of a scan speed of 1000 amu/sec and a scan range of 50–820 amu.

Plant assays for induced systemic resistance

We used a hydroponic system [5,29] to evaluate the resistance response of maize seedlings stimulated by *T. virens* or *T. atroviride*. 600 ml glass beakers were filled with 200 ml plant nutrient solution (half-strength Murashige and Skoog basal medium, 2.5 mM MES buffer pH = 5.7). A perforated stand for supporting the seeds was made from a 200 μl tip holder. Maize seeds (Royalty, local hybrid, purchased from Ben Shachar, Tel Aviv) were surface sterilized by dipping them in 10% H_2O_2 for three hours, followed by three washes with sterile water. Treated seeds were dried on sterile Whatman #1 paper, placed in sterile Petri dishes containing half-strength Murashige and Skoog agar and incubated in the dark for three days at 30°C to allow germination. 12 germinated seeds with similar-sized roots and shoots were placed on the stands in each aseptic beaker. The plants were maintained in a controlled environment at 23°C and a 16 h photoperiod with moderate shaking on an orbital shaker (100 rpm). After four days of growth in the beakers, plants were inoculated with *Trichoderma* spore suspension to a final concentration of 5×10^3 spores/ml. Roots and fungus were allowed to interact for four more days before pathogen challenge. For pathogen challenge, plants - with their roots - were taken out of the beakers and the second leaf of each plant was attached to a tray from the edges of the leaf. The roots of each set of plants according to treatment were wrapped separately in wet paper towels.

The maize pathogen *C. heterostrophus* (strain C4) was grown for seven days on complete xylose medium [30] in the same controlled environment as the plants. The second leaf was inoculated with 7 μl droplets of 0.02% Tween 20 in double distilled water containing 1000 spores. Trays were closed in clear plastic bags to keep the plants moist and kept in the controlled environment. Pictures of the challenged leaves were taken after 48 h and lesions were measured using ImageJ software (http://imagej.nih.gov/ij/). For each treatment the data represent at least eight leaves, each with three lesions from two biological repeats.

For analysis of gene expression, roots with adhering *Trichoderma* were harvested from hydroponic cultures grown in parallel to those used for the ISR assays. The roots were washed gently with culture medium, frozen and ground to a fine powder in liquid nitrogen. RNA was extracted using Tri Reagent (MBC Molecular Research Center, Cincinnati, OH, USA) following the manufacturer's protocol, and cDNA was synthesized as described above.

Supporting data

The data sets supporting the results of this article are included within the article and its additional files.

Additional file

Additional file 1: Figure S1. Generation of *sm1* and *sm2* knockout strains. **Figure S2.** Chlamydospore formation in *T. virens* shake flask cultivations. **Figure S3.** Mycoparasitism confrontation assays of *T. virens* against *R. solani*. **Figure S4.** Gliotoxin measurements of *T. virens* strains I10 and Gv29-8. **Table S1.** Primers used in this study.

Abbreviations

CPP: Cerato platanin protein; CP: Cerato platanin; PDA: Potato dextrose agar.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

RG carried out the gene expression analysis, generation and morphological characterization of knockout strains, NLL performed the plant experiments, AF contributed to the generation of knockout strains, MS and RK carried out the gliotoxin measurements, BAH participated in the design and writing of the manuscript, VSS designed the study, coordinated the experiments and writing of the manuscript. All authors read and approved the final manuscript.

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References

1. Druzhinina IS, Seidl-Seiboth V, Herrera-Estrella A, Horwitz BA, Kenerley CM, Monte E, et al. *Trichoderma*: the genomics of opportunistic success. Nat Rev Microbiol. 2011;9:749–59.
2. Harman GE, Howell CR, Viterbo A, Chet I, Lorito M. *Trichoderma* species - opportunistic, avirulent plant symbionts. Nat Rev Microbiol. 2004;2:43–56.
3. Lorito M, Woo SL, Harman GE, Monte E. Translational research on *Trichoderma*: from 'omics to the field. Annu Rev Phytopathol. 2010;48:395–417.
4. Mukherjee PK, Horwitz BA, Herrera-Estrella A, Schmoll M, Kenerley CM. *Trichoderma* research in the genome era. Annu Rev Phytopathol. 2013;51:105–29.
5. Djonovic S, Pozo MJ, Dangott LJ, Howell CR, Kenerley CM. Sm1, a proteinaceous elicitor secreted by the biocontrol fungus *Trichoderma virens* induces plant defense responses and systemic resistance. Mol Plant Microbe Interact. 2006;19:838–53.
6. Seidl V, Marchetti M, Schandl R, Allmaier G, Kubicek CP. Epl1, the major secreted protein of *Hypocrea atroviridis* on glucose, is a member of a strongly conserved protein family comprising plant defense response elicitors. FEBS J. 2006;273:4346–59.
7. Kubicek CP, Herrera-Estrella A, Seidl-Seiboth V, Martinez DA, Druzhinina IS, Thon M, et al. Comparative genome sequence analysis underscores mycoparasitism as the ancestral life style of *Trichoderma*. Genome Biol. 2011;12:R40.
8. Frischmann A, Neudl S, Gaderer R, Bonazza K, Zach S, Gruber S, et al. Self-assembly at air/water interfaces and carbohydrate binding properties of the small secreted protein EPL1 from the fungus *Trichoderma atroviride*. J Biol Chem. 2013;288:4278–87.
9. Atanasova L, Druzhinina IS, Jaklitsch WM. Two hundred Trichoderma species recognized based on molecular phylogeny. In: Mukherjee PK, Sigh US, Horwitz BA, Schmoll M, Mukherjee M, editors. *Trichoderma*: Biology and Applications. Wallingford, UK: CABI; 2013. p. 10–42.
10. Gruber S, Kubicek CP, Seidl-Seiboth V. Differential regulation of orthologous chitinase genes in mycoparasitic *Trichoderma* species. Appl Environ Microbiol. 2011;77:7217–26.
11. Vargas WA, Djonovic S, Sukno SA, Kenerley CM. Dimerization controls the activity of fungal elicitors that trigger systemic resistance in plants. J Biol Chem. 2008;283:19804–15.
12. Catalano V, Vergara M, Hauzenberger JR, Seiboth B, Sarrocco S, Vannacci G, et al. Use of a non-homologous end-joining-deficient strain (delta-*ku70*) of the biocontrol fungus *Trichoderma virens* to investigate the function of the laccase gene *lcc1* in sclerotia degradation. Curr Genet. 2011;57:13–23.
13. Djonovic S, Vargas WA, Kolomiets MV, Horndeski M, Wiest A, Kenerley CM. A proteinaceous elicitor Sm1 from the beneficial fungus *Trichoderma virens* is required for induced systemic resistance in maize. Plant Physiol. 2007;145:875–89.
14. Gaderer R, Bonazza K, Seidl-Seiboth V. Cerato-platanins: a fungal protein family with intriguing properties and application potential. Appl Microbiol Biotechnol. 2014;98:4795–803.
15. Ram AF, Klis FM. Identification of fungal cell wall mutants using susceptibility assays based on Calcofluor white and Congo red. Nat Protoc. 2006;1:2253–6.
16. Baccelli I, Comparini C, Bettini PP, Martellini F, Ruocco M, Pazzagli L, et al. The expression of the cerato-platanin gene is related to hyphal growth and chlamydospores formation in *Ceratocystis platani*. FEMS Microbiol Lett. 2012;327:155–63.
17. Howell CR. Selective isolation from soil and separation in vitro of P and Q strains of *Trichoderma virens* with differential media. Mycologia. 1999;91:930–4.
18. Mukherjee PK, Horwitz BA, Kenerley CM. Secondary metabolism in *Trichoderma*–a genomic perspective. Microbiology. 2012;158:35–45.
19. Vargas WA, Mukherjee PK, Laughlin D, Wiest A, Moran-Diez ME, Kenerley CM. Role of gliotoxin in the symbiotic and pathogenic interactions of *Trichoderma virens*. Microbiology. 2014;160:2319–30.
20. Enhancement of plant disease resistance by the biocontrol agent *Trichoderma* [http://www.weizmann.ac.il/Biology/open_day_2006/book/Abstracts/Ilan_Chet.pdf]
21. Frías M, González C, Brito N. BcSpl1, a cerato-platanin family protein, contributes to *Botrytis cinerea* virulence and elicits the hypersensitive response in the host. New Phytol. 2011;192:483–95.
22. Yang Y, Zhang H, Li G, Li W, Wang X, Song F. Ectopic expression of MgSM1, a Cerato-platanin family protein from *Magnaporthe grisea*, confers broad-spectrum disease resistance in *Arabidopsis*. Plant Biotechnol J. 2009;7:763–77.
23. de O Barsottini MR, de Oliveira JF, Adamoski D, Teixeira PJ, do Prado PF, Tiezzi HO, et al. Functional diversification of cerato-platanins in *Moniliophthora perniciosa* as seen by differential expression and protein function specialization. Mol Plant Microbe Interact. 2013;26:1281–93.
24. Mach RL, Schindler M, Kubicek CP. Transformation of *Trichoderma reesei* based on hygromycin B resistance using homologous expression signals. Curr Genet. 1994;25:567–750.
25. Kubodera T, Yamashita N, Nishimura A. Transformation of *Aspergillus* sp. and *Trichoderma reesei* using the pyrithiamine resistance gene (*ptrA*) of *Aspergillus oryzae*. Biosci Biotechnol Biochem. 2002;66:404–6.
26. Sambrook J, Russell DW. Molecular Cloning: a Laboratory Manual. 2nd ed. Painview, NY: Cold Spring Harbor Lab. Press; 2001.
27. Pfaffl MW, Horgan GW, Dempfle L. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res. 2002;30:e36.
28. Vishwanath V, Sulyok M, Labuda R, Bicker W, Krska R. Simultaneous determination of 186 fungal and bacterial metabolites in indoor matrices by liquid chromatography/tandem mass spectrometry. Anal Bioanal Chem. 2009;395:1355–72.
29. Yedidia II, Benhamou N, Chet II. Induction of defense responses in cucumber plants (*Cucumis sativus* L.) by the biocontrol agent *Trichoderma harzianum*. Appl Environ Microbiol. 1999;65:1061–70.
30. Turgeon BG, Condon B, Liu J, Zhang N. Protoplast transformation of filamentous fungi. In: Sharon A, editor. Methods Mol Biol. New York: Humana/Springer; 2010. p. 3–19 [Walker J (Series Editor): Springer Protocols: Methods in Molecular Biology].

Chapter 2: Functional and biochemical studies of members of the cerato-platanin protein family

Chapter 2.3

The fungal cerato-platanin protein EPL1 forms highly ordered layers at hydrophobic/hydrophilic interfaces.

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The fungal cerato-platanin protein EPL1 forms highly ordered layers at hydrophobic/hydrophilic interfaces

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Cerato-platanin proteins (CPPs) and hydrophobins are two classes of small, secreted proteins that are exclusively found in fungi. CPPs are known as chitin-binding proteins, and were recently also shown to form protein layers at air/water interfaces, but the features of these layers were not investigated on the molecular level yet. In this study, by means of atomic force microscopy (AFM), EPL1, a member of the CPP family was shown to form highly ordered monolayers at a hydrophobic surface/liquid-interface. Furthermore, two new hydrophobins were analysed, and the influence of EPL1 on hydrophobin layers was studied *in situ*. Hydrophobins are amphiphilic proteins that are able to self-assemble at hydrophobic/hydrophilic interfaces, thereby inverting the polarity of the surface. This renders fungal growth structures such as spores water repellent. The combination of AFM data and wettability experiments led to the conclusion that in presence of both, hydrophobins and EPL1, a previously unknown hybrid layer is formed. This mixed protein layer is on one hand not inverting but enhancing the hydrophobicity of HOPG (highly oriented pyrolytic graphite), typical for EPL1, and on the other hand, it is stable and water insoluble, which is reminiscent of hydrophobin layers.

Introduction

Self-assembly of molecules into ordered structures is one of the most remarkable preconditions for life. One of the most prominent three-dimensional examples for oriented molecular assemblies are lipid monolayers in cell walls. Scientists of multiple disciplines have investigated self-assembly phenomena and even the fundamental studies of aliphatic self-assembled monolayers (SAM), such as octadecylsilane (ODS) or octadecyltrichlorosilane (OTS),[1–3] which are meanwhile the most understood systems, can be seen in this general context. While SAMs are often covalently bound to the surface, as shown by reflection infrared spectroscopy,[3,4] Langmuir Blodget (LB) films, which grow at air–liquid interfaces prior to be deposited onto a substrate, usually lack anchor groups. However, it has been shown by atomic force microscopy (AFM) that also ODS layers first assemble in liquid and then attach to the surface as preformed flakes.[4]

Another type of amphiphilic surface active molecules that has already been studied in considerable detail is the hydrophobin protein family. Hydrophobins are small secreted fungal proteins, which were also found to self-assemble at hydrophobic/hydrophilic interfaces.[5,6] Due to hydrophobic and hydrophilic amino-acid patches, creating an amphiphilic protein surface, they are able to invert the polarity of surfaces on which they self-assemble very effectively. Therefore, they are handled as candidates for large scale applications, spanning from non-wetting coatings to biocompatible surfactants. Biologically their surface activity-altering properties are for example relevant for fungal hyphae that emerge from an aqueous growth environment to form aerial hyphae and produce spores, which are then covered with a non-wettable layer of hydrophobins. This facilitates the dispersal of fungal conidia (spores). The common feature of all these layers is that they consist of amphiphilic molecules which are uniformly oriented at hydrophilic/hydrophobic interfaces; hence their growth is self-terminating, generating monolayers.

Here, a member of the cerato-platanin protein family (CPP), namely EPL1 from the fungus *Trichoderma atroviride*, was studied in view of its ability to form ordered self-assembled layers. CPPs are not related to hydrophobins with respect to their protein sequences or structure, but we recently found that

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EPL1 is also able to form protein biofilms at air/water interfaces.[7] However, EPL1 rather enhances the polarity effects of surfaces and solutions instead of inverting them, which is the opposite of what has been described for hydrophobins.

Similar to hydrophobins, CPPs can only be found in filamentous fungi (moulds), *i.e.* fungi that produce hyphae as growth structures, or in fungi which have at least a pseudohyphal growth stage.[8] They are small, secreted proteins that are released into the culture filtrate, but they have also been found within the cell wall of fungal hyphae and spores.[9–11] CPPs are important factors in fungal–plant interactions. In plant pathogenic fungi they have been reported to act as phytotoxins and were also shown to induce plant defence responses in plant-beneficial fungi of the genus *Trichoderma*.[12–15] However, the presence and abundant expression of CPPs in fungi with all types of life-styles suggests that the main biological functions are not solely related to fungal–plant interactions but to other, more general aspects of fungal growth.[7,16,17]

Structural analyses revealed that CPPs have a protein fold that is similar to expansins.[18] Expansins are non-enzymatic proteins that aid in plant cell wall extension and plant growth by loosening up the cellulose scaffold of the cell wall.[19]

In analogy to that, CPPs can bind carbohydrates but are also not enzymatically active. They were found to have an *N*-acetylglucosamine binding pocket. Binding to chitin, which is a biopolymer consisting of *N*-acetylglucosamine subunits, was already shown for some CPPs including EPL1.[7,20,21] Since chitin is a structural component of the fungal cell wall, it was suggested that CPPs might exhibit similar functions in the fungal cell wall as expansins do in plants.[7,20]

In addition to these carbohydrate-binding properties, we were recently able to show that EPL1 readily self-assembles at air/water interfaces and is able to form protein layers on the surface of aqueous liquids.[7]

In this study we investigated protein layers of EPL1 and two new hydrophobins HFB9a and HFB9b with tapping mode atomic force microscopy (TM-AFM). The results from this study significantly increase our understanding of EPL1 protein layers and reveal that upon self-assembly they indeed form a regularly patterned protein biofilm surface. Furthermore, since both, CPPs and hydrophobins, can be found on/in fungal cell walls, we tested whether the formation and patterning of protein layers of hydrophobins might be influenced or perturbed by EPL1 and *vice versa*.

Results

Features of EPL1 protein layers on HOPG

In a previous study we showed that EPL1 forms irregular, meshwork-like or large granular structures upon drying of a droplet of protein solution (0.06 μg mL^{-1}) on a mica surface.[7] Following up on these results we investigated the topography of EPL1 protein layers on HOPG (highly orientated pyrolytic graphite) by preparation and imaging in air as well as *in situ* preparation an imaging directly in liquid (PBS buffer). It turned out that such irregular, meshwork-like protein patches can also be observed on HOPG when imaging samples prepared by drying a droplet in air (Fig. 1a).

In order to circumvent limitations in imaging quality on such uneven surfaces (see also Fig. 1b), the following experiment was performed: HOPG was first imaged with AFM under PBS buffer, then a small amount of concentrated EPL1 solution (120 nmol mL^{-1}) was added *in situ* to achieve a final concentration of approximately 30 nmol mL^{-1} in the AFM liquid cell. As shown in Fig. 2, the protein immediately formed highly ordered, thin layers (most probably monolayers as indicated by

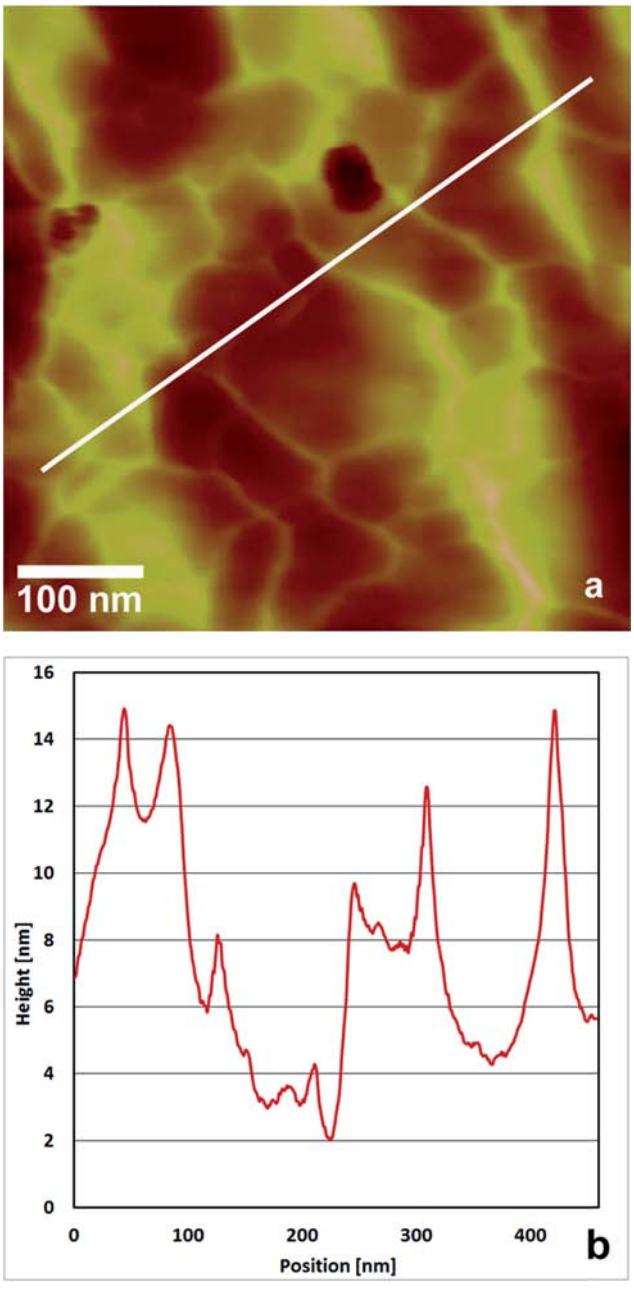

Fig. 1 (a) TM-AFM image of the dried liquid–air interface, taken after total dehydration of an EPL1 droplet on HOPG and (b) cross sectional profile along the line shown in (a). An irregular meshwork with a strongly corrugated surface can be seen. Image taken in air, height scale: 20 nm from dark to bright.

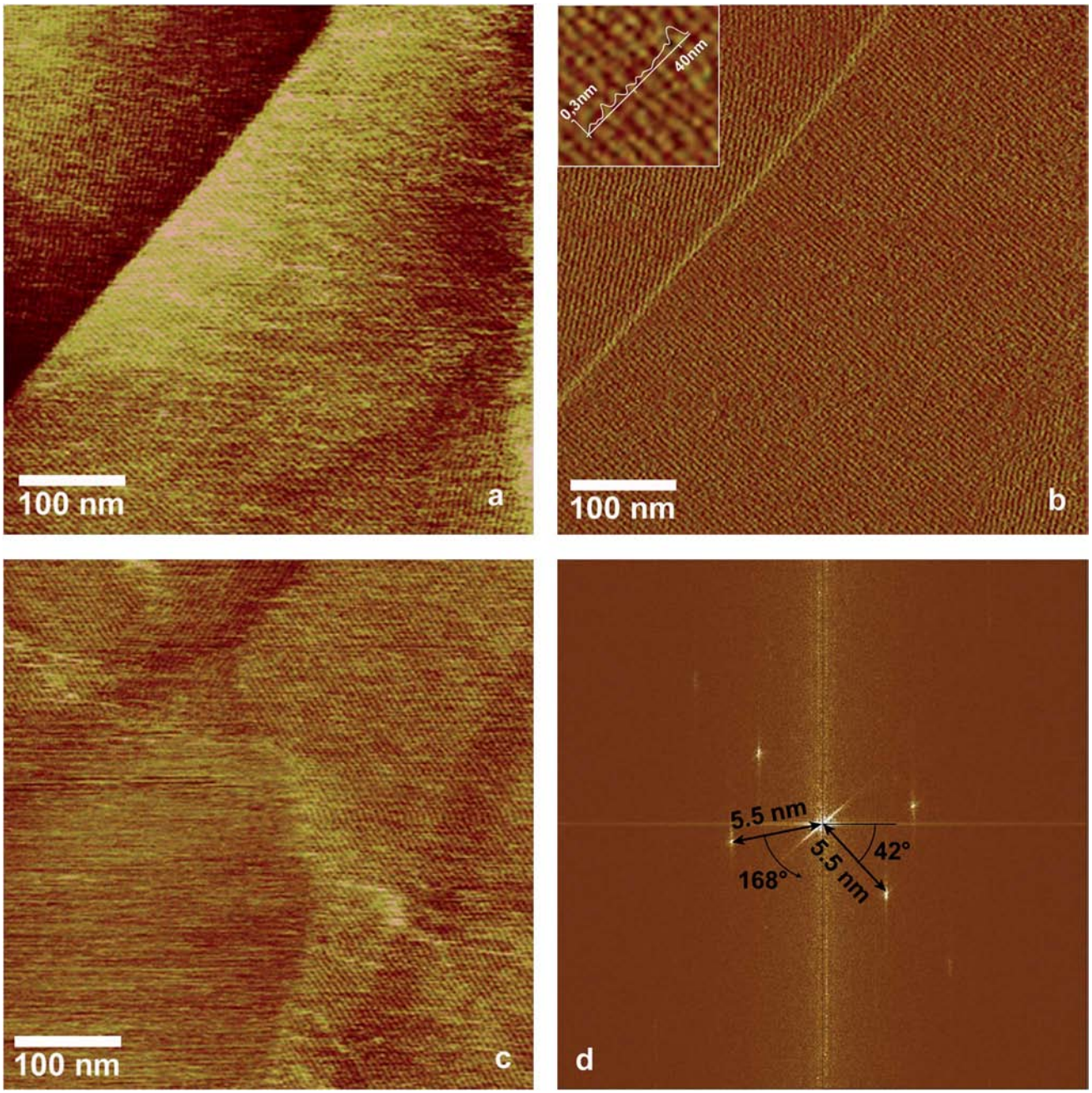

Fig. 2 TM-AFM images of EPL1 on HOPG. (a) Topography image and (b) amplitude error image of the same area. The layers have been prepared *in situ* in the AFM liquid cell and imaging was performed under PBS buffer. The height profile in the inset (zoom) is derived from the topographical image. (c) Image across a margin of an ordered domain, suggesting that a monolayer is observed. (d) The periodicity of 5.5 nm and two main orientation angles can be extracted from the Fourier transformation of (a). Data scale: (a) 1 nm (topography) and (b and c) 10 mV (amplitude error) from dark to bright.

the image of an edge of such an ordered EPL1 domain in Fig. 2c) on the hydrophobic HOPG surface. Fig. 2a shows a topographical image and Fig. 2b an amplitude error image of the same area. It can clearly be seen that the recognisability of the ordered domains is strongly increased in the amplitude error image. This is particularly valid when *e.g.* steps on the substrate surface are encountered in an image. As shown in the height profile (inset in Fig. 2b) the grooves between protein rows have a depth of only 0.2–0.3 nm.

For that reason only amplitude error images are shown in the following figures. In contrast to the experimental approach shown in Fig. 1, where during drying of the droplet rather large amounts of protein are deposited on the surface, the images shown in Fig. 2 were recorded directly in liquid and here solely a thin layer of EPL1 is deposited on an atomically flat surface. This facilitates high-resolution imaging of the formed protein layer. The Fourier transformation in Fig. 2d shows a periodicity of ~5.5 nm and the height profile in Fig. 2b displays a groove

depth of 0.2–0.3 nm. Previous works reported that hydrophobin layers adhere much stronger to HOPG surfaces than to mica,[22] indicating that the hydrophobic interactions are stronger than their hydrophilic counterparts. This is in accordance with our experiments insofar as no formation of EPL1 protein layers could be observed on mica under liquid. *Ex situ* AFM measurements performed on EPL1 layers on hydrophilic mica and on a hydrophobic gold (111) surface in air yielded no ordered structures.

Influence of EPL1 on hydrophobin protein layers

Some members of the classes I and II of the hydrophobin family have been shown to form ordered monolayers,[5,6,22,23] but most hydrophobins that were identified in fungal genomes in the last years have not been characterized by high resolution imaging methods. In this study, two hydrophobins, HFB9a and HFB9b from *Trichoderma virens*, belonging to a phylogenetically new section of class I hydrophobins in ascomycete fungi,[24] were analysed in this respect. Self-assembled layers of these two hydrophobins were produced using the droplet method, which is explained in the experimental section.

AFM images showed that both hydrophobins are able to form highly ordered lattices on hydrophobic surfaces (Fig. 3a and b). The surface pattern exhibits a riffled morphology, very similar to the EPL1 layers described above, with a separation of 5.3 nm and 5.5 nm (extracted from the Fourier transformed images) between the protein rows, which reflects the similar molecular mass of the mature proteins (EPL1 11 kDa, HFB9a 13 kDa, HFB9b 11 kDa). Since both, EPL1 and HFBs, are present in/on fungal cell walls, we were interested whether the formation and patterning of protein layers of hydrophobins might be influenced or perturbed by EPL1. In order to elucidate this hypothesis, the effect of EPL1 on HFB9a and HFB9b was observed *in situ*. First, the effect of EPL1 on pre-formed hydrophobin layers was tested. As a representative example of several independent replications of these experiments, Fig. 3c and d show the samples already shown in Fig. 3a and b, respectively, after addition of EPL1 solution to the AFM's liquid cell. Significant structural changes could not be observed even after one hour of scanning. Thus, it can be concluded that EPL1 neither destroys ordered HFB layers nor sticks onto their surface to grow layers on top of them. Evidently, already existing HFB layers are stable under this treatment. Next, the effect of EPL1 on the growth of HFB9a/b layers was studied. The respective hydrophobins were mixed with EPL1 in a molar ratio of 1 : 1 and the droplet method (see Experimental section) was applied. As shown in Fig. 4, periodic structures were formed, which, at least by AFM, cannot be distinguished from those of either pure HFB9a/b or EPL1 alone. Image analysis by Fourier transformation (analogous to Fig. 2d) revealed a periodicity of 6.2 nm and 5.3 nm and the height profiles showed groove depths <1 nm.

Wettability experiments. Despite their topographical similarity to pure hydrophobins, layers composed of HFB9a/EPL1 or HFB9b/EPL1 mixtures, exhibited unprecedented macroscopic properties: on one hand they were stable upon washing of the surface with ultrapure water. This was also found to be the case for HFB9a/b layers, whereas EPL1 layers are readily re-solubilized (this study and[7]). On the other hand the mixed layers were interestingly not inverting the hydrophobicity of a HOPG surface anymore (Fig. 5), which is reminiscent of EPL1 layers. Fig. 5 shows different shapes of water drops deposited on pure HOPG and HOPG substrates covered either with pure HFB9a/b or with mixed HFB9a/EPL1 and HFB9b/EPL1 layers. It can be seen that pure HFB9a/b significantly increased the wettability of HOPG, while in case of layers mixed with EPL1 the substrate remains hydrophobic. These observations were further verified by contact angle measurements using 2 μL droplets of ultrapure water. In agreement with the macroscopic observations, the results (Table 1) showed that hydrophobin layers on HOPG strongly reduced contact angles, whereas hybrid protein layers were more water repellent and yielded larger contact angles with similar values as HOPG without protein. These results indicate that a new type of hybrid layer was formed. Due to the solubility of EPL1 in water, measurements with EPL1 layers could not be performed. Preliminary experiments with MALDI-TOF-MS (matrix assisted laser desorption ionization – time of flight – mass spectrometry in the positive linear ion mode) confirmed the presence of two different proteins (hydrophobin and EPL1) on the surface but were not conclusive in terms of a further characterization of the layers (data not shown).

Discussion

In this study, we analysed the formation of EPL1 protein layers *in situ* during their formation in buffer, *i.e.* at solid/liquid interfaces. Up to now it was only known that EPL1 self-assembles at air/water interfaces[7] but highly ordered structures on the molecular level were not observed. We were able to show that upon self-assembly of EPL1 indeed highly ordered layers, most likely monolayers (see Fig. 2c) are formed. In the light of the structural analysis of CPPs,[18,21] which did not reveal any significant similarities to the amphiphilic surface architecture of hydrophobins,[18] it is a curiosity that EPL1 nevertheless shows surface active properties. The coarse meshwork-like structure of the EPL1 protein layer observed in Fig. 1 might result from drying of the EPL1 protein droplet and could be caused by a rather stiff protein film that is already formed on the surface of the droplet (prior to adsorption on the substrate) which is subsequently deformed by the shrinkage of the droplet during drying and adsorption. The stiffness of this surface film is indicative for a stable protein assembly at the air/water interface.

In this study also two new hydrophobins were analysed, and the influence of EPL1 on hydrophobin layers was observed. Both, EPL1 and HFBs used in this study, were derived from the fungal genus *Trichoderma*. Hydrophobins are conventionally grouped into two classes (class I and II) according to their solubility in solvents, hydropathy profiles, and spacing between their conserved cysteines. In general, protein aggregates of class I hydrophobins are more stable and can only be dissolved in strong acids such as TFA, while class II aggregates can be dissolved using aqueous dilutions of organic solvents.[25] Previously, in ascomycete fungi only class II hydrophobins were detected, but the wealth of fungal genomes that became available in the

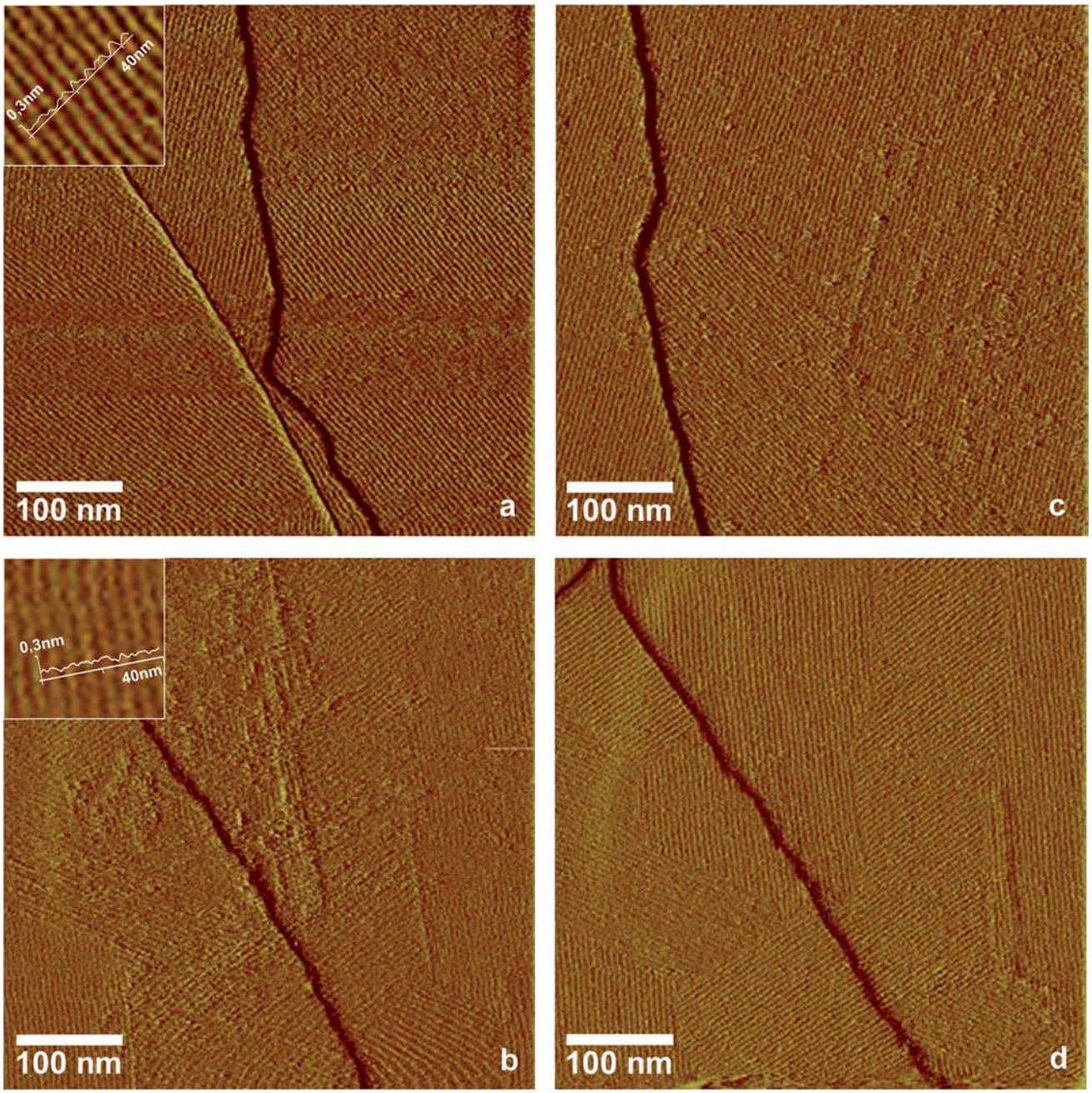

Fig. 3 AFM amplitude error images of hydrophobin layers produced by the "drop method" imaged under PBS buffer. (a) HFB9a and (b) HFB9b. Height profiles in the insets (zooms) are derived from the corresponding topographical images. (c) HFB9a protein layer of (a) after *in situ* addition of EPL1 and (d) HFB9b protein layer of (b) after *in situ* addition of EPL1. Morphological changes upon addition of EPL1 cannot be observed. The ~5 nm high steps running across the images are terraces of the underlying, cleaved HOPG substrate. Data scale: 10 mV from dark to bright.

past few years led to the discovery of large numbers of new hydrophobins. This included the description of a novel sub-set of class I hydrophobins in ascomycetes, such as *Trichoderma*[24] and *Aspergillus* species.[26] These hydrophobins show also the typical eight cysteines which are characteristic for hydrophobin sequences. Further, structural predictions revealed the typical amphipathic surface that is responsible for the biochemical properties of hydrophobins.[24] We found that, in analogy to what has been shown for other hydrophobins before, also HFB9a and HFB9b are able to form highly ordered (mono-)layers. These layers dramatically increase the wettability of HOPG surfaces, as was shown by the strong alteration of droplet shapes (Fig. 5). This can be explained much more straightforward than in the case of EPL1, because these proteins indeed have hydrophilic and hydrophobic surface patches and therefore behaved like typical hydrophobins.

With respect to EPL1 layers, several aspects of our results indicate that the formed protein layers have a monomolecular thickness. At the margins of the highly ordered EPL1 layers (see Fig. 2c) higher step heights which would be indicative for

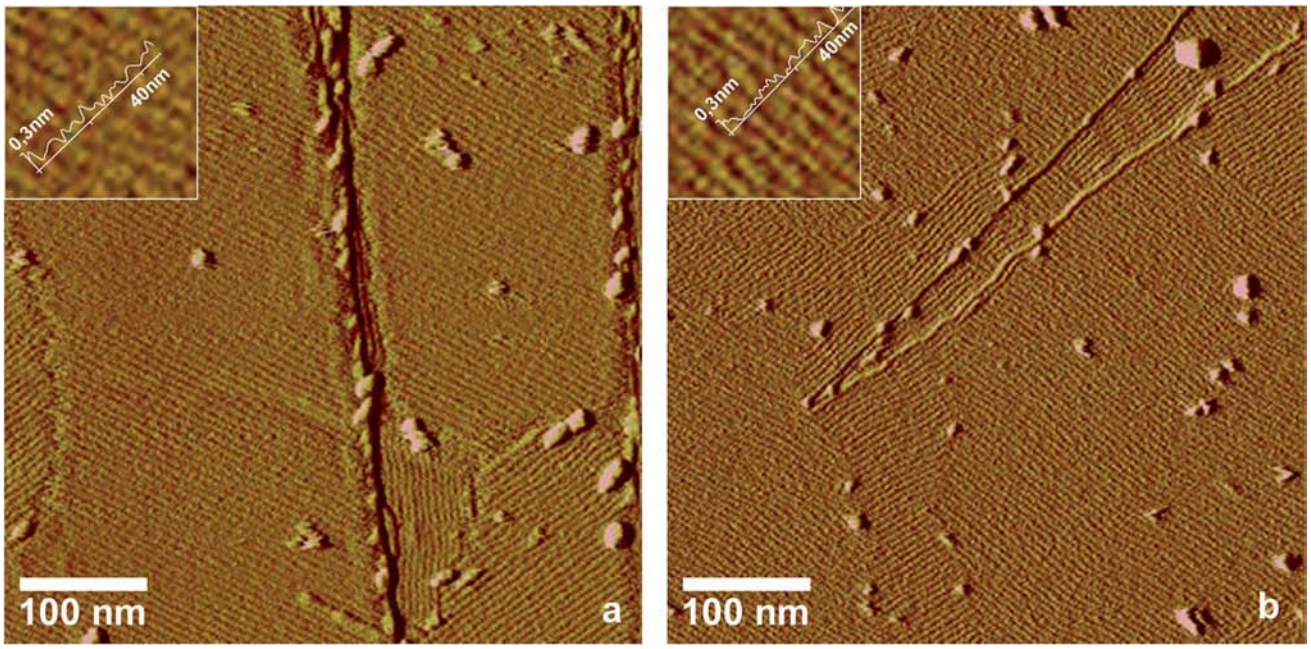

Fig. 4 AFM amplitude error images of hybrid layers of (a) EPL1/HFB9a and (b) EPL1/HFB9b. Ordered layers which cannot be distinguished from pure EPL1 or HFB9 layers are observed. Height profiles in the insets (zooms) are derived from the corresponding topographical images. Data scale: 10 mV from dark to bright.

thicker layers could not be observed. Moreover, the flat films with an exceptional long distance order (up to micrometers) suggest a self-terminating growth, most likely leading to monolayers. Furthermore, in case of the amphiphilic hydrophobins the monolayer nature has already been shown.[22] Nevertheless, for EPL1 layers a less probable alternative assembly (*e.g.* double layers) cannot be fully excluded.

The addition of an EPL1 solution had no effect on preformed hydrophobin layers, whereas when HFB9a/9b and EPL1 were present in the adsorption solution simultaneously, the resulting layers showed interesting, mixed properties. We suggest that an alternating pattern of single proteins, subunits or protein rows is more likely than a sandwich stack of hydrophobins and EPL1, because sandwiches would probably also be formed if EPL1 was added to an already formed hydrophobin layer, which was not the case.

The distances of periodical rows of HFB9a- and HFB9b-surfaces is 5.3 nm and 5.5 nm, and therefore slightly smaller than those of previously published HFB I and II surfaces, which had lattice parameters ranging from 5.9 nm to 6.1 nm.[22,23] EPL1 structures revealed a very similar periodicity of 5.5 nm. The HFB9a/EPL1 hybrid layers had a slightly increased distance between protein rows (6.2 nm) in contrast to the HFB9b/EPL1 hybrids (5.3 nm). However the differences are not significant enough to allow any interpretations on the organization of these hybrid layers. This is also the case for height differences, as profiles show similar sub-nanometer grooves for both, pure-protein and hybrid-layers. The organisation and distribution of the two protein species in those hybrid layers will be an important and interesting aspect for further investigations. The fact that the wettability of hybrid layers was the opposite of what was observed for hydrophobin layers suggests the orientation of hydrophilic/hydrophobic patches of the proteins was at least slightly altered. Protein–protein interactions between EPL1 and the hydrophobins are the likely driving force for that, but detailed investigations on the single-molecule level will probably be necessary to elucidate that further.

The observation of hybrid layers has interesting implications for potential biotechnological applications as well as for the biological roles of EPL1 and CPPs in general. Hybrid layers, which are not as water-soluble as pure EPL1 layers, could be used to enhance the wettability-properties of surfaces in applications where a uniform moistening of a moderately hydrophobic surface is of interest, *e.g.* in cleaning agents or in spraying applications of plant protection products.

Concerning the biological functions of CPPs it has previously been suggested that they might play a role in fungal growth and development due to their chitin-binding properties and abundant expression during many different growth conditions.[7,16,21] Interestingly the *epl1* gene in *T. atroviride*, as well as its orthologues in other fungi, are abundantly expressed during hyphal growth whereas expression of hydrophobins is usually rather related to sporulation, but this has not been tested for the specific case of *hfb9a* and *hfb9b* yet. However, since EPL1 is in our experience relatively stable, also in fungal cultivations, and in addition other CPPs such as *epl2* are also expressed during sporulation,[7] it is presumable that CPPs and hydrophobins also interact *in vivo*. In this context it is interesting that EPL1 does not affect existing hydrophobin layers, but modifies surface properties in a more subtle way. The biological consequence of mixed hydrophobin/CPP layers would be that fungal growth structures could, under certain conditions, be covered with a protein biofilm which does not invert the polarity of the surface.

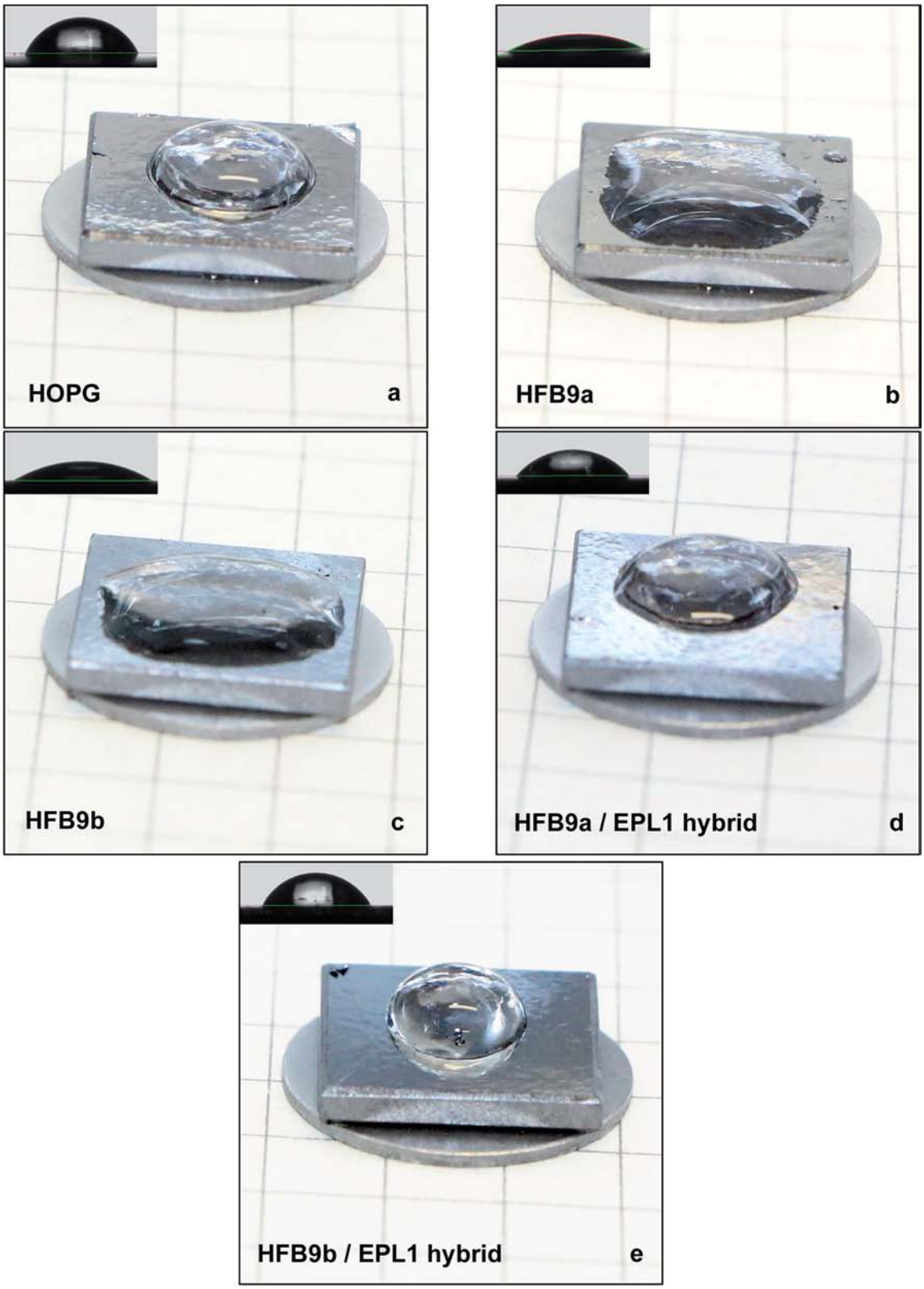

Fig. 5 Photographs of 50 μL and 2 μL water drops deposited on (a) pure HOPG, (b) a HFB9a layer, (c) a HFB9b layer, (d) a HFB9a/EPL1 hybrid layer, and (e) a HFB9b/EPL1 hybrid layer. The hydrophobins clearly invert the hydrophobicity of HOPG, rendering the surface more hydrophilic, while in case of the hybrid layers the surface remains hydrophobic. The insets show 2 μL droplets on the respective surfaces. The images were taken during contact angle measurements with the camera integrated in the contact angle device.

Table 1 Contact angles of 2 μL water droplets on HOPG coated with hydrophobin or hybrid protein layers

Type of layer	Contact angle [°]
HOPG surface (without protein)	59 ± 10
HFB9a	22 ± 9
HFB9b	27 ± 5
Hybrid layer EPL1/HFB9a	53 ± 8
Hybrid layer EPL1/HFB9b	61 ± 7

For polar or hydrophilic molecules, such as those found in the fungal cell wall (carbohydrates and proteins), this would mean an increase of the wettability and hydrophilicity. Most fungi prefer moist growth conditions and therefore an enhancement of the wettability of fungal hyphae might be advantageous for the fungus. Such properties could also aid in the adherence of hyphae to certain surfaces and thus in a better adaptation of the fungus to a growth environment that has zones with varying water content, as might be found in natural environments such as soil.

With respect to the potential roles of CPPs and/or mixed hydrophobin/CPP layers in fungal growth it has to be mentioned that, *e.g.* for *epl1* gene knockout strains so far no growth defects or phenotypes related to the formation of hyphae or spores were detected, also not during stress growth conditions such as osmotic stress.[7] This shows that *epl1* is a non-essential gene despite the abundant production of EPL1 during fungal growth. In contrast to that, *epl1* knockout strains, as well as knockout strains of its orthologue in *T. virens*, *sm1*, show a reduced induction of plant defense responses.[27,28] Whether this is solely due to the fact that CPPs such as EPL1 or SM1 effectively signal the presence of a fungus for plants or whether CPPs are directly involved in the physical interaction of fungal hyphae with plant roots remains to be investigated. While *Trichoderma* species are not pathogenic for plants but rather plant-beneficial fungi, in plant pathogens it was already reported that knockout strains of CP-genes showed reduced virulence and necrosis of plant tissue.[15,17,29] The attachment and interaction of fungal hyphae with plants, *e.g.* during the infection process of plant leaves or roots by plant pathogenic fungi could be influenced by either mixed layers of hydrophobins and CPPs, as shown in this paper, which would have a direct effect on the attachment-abilities of hyphae, or possibly also by the interaction of CPPs with plant surface proteins, which could alter the surface properties of the plant leave, thereby aiding in the pathogenic attack of the fungus. The findings of this study provide a possible starting point towards understanding the mechanistic effects of CPPs in fungal–plant interactions in more detail.

Experimental

Production and purification of EPL1

The protein EPL1 was purified from culture supernatants of *Trichoderma atroviride* P1 as described in ref. 9. Briefly, culture supernatants were concentrated *via* ultrafiltration using a membrane with 10 kDa cut-off and subsequently purified *via* cation-exchange chromatography. Purification steps were checked with SDS-PAGE.[30] The protein was stored at 4 °C in sodium acetate buffer, pH 4.5. This pH is close to that of *T. atroviride* cultivations from which EPL1 is purified and the protein was found to be very stable under these conditions.

Overexpression and purification of hydrophobins

The hydrophobins that were used in this study are *Trichoderma virens* HFB9a and HFB9b. The respective NCBI/EMBL/DDBJ accession number, derived from the genome sequencing project of *T. virens* Gv29-8 v2.0 (http://genome.jgi-psf.org/TriviGv29_8_2/TriviGv29_8_2.home.html) are EHK16816 for HFB9a and EHK25899 for HFB9b. In order to amplify cDNA fragments of *T. virens hfb9a* and *hfb9b* the primers hfb9a-fw (CAACAAGGGCAAAGGTGGCAA), hfb9a-rv (TCGTAGATGTTGATGGTGATGGG) and hfb9b-fw (CAACAACAACTGGCAGAGCAAC) and hfb9b-rv (GTAAACGACCTTGGACTGTCCG) were used. The hydrophobin genes were fused between an N-terminal pelB leader directing the proteins to the bacterial periplasm and a C-terminal 6xHis-Tag for rapid purification by affinity chromatography. Overexpression of hydrophobins was carried out in *Escherichia coli* BL21 DE3 strain (GE Healthcare, Amersham, England). Bacterial strains were cultivated in LB broth containing 40 μg mL^{-1} kanamycin at 37 °C and 170 RPM, expression was induced by the addition of isothiopropyl-β-D-galactoside at a final concentration of 0.05 mM. The culture was incubated for 5 hours and the cells were harvested by centrifugation (5000*g*, 4 °C, 10 min). Cell pellets were resuspended in 5 mL buffer and purified using the HisTALON™ Gravity Column Purification Kit (Takara Bio Company, Mountain View, CA, USA). Purification was carried out according to the manufacturer's instructions. Sonification was used to lyse the cells (10 cycles; 30 s pulse with 1 min on ice between pulses) and the supernatant separated by centrifugation at 5000*g* for 30 min at 4 °C. The pellet was washed twice with the equilibration buffer containing 2 M urea. After the final centrifugation (5000*g*, 10 min at 4 °C) inclusion bodies were solubilised in the equilibration buffer containing 8 M urea and refolded while bound to the column. The proteins were loaded onto 2 mL of Co^{2+}-charged affinity resin (TALON® Metal Affinity Resin, Takara Bio Company, Mountain View, CA, USA) and subsequently washed with 10 column volumes of equilibration buffer containing 8 M urea. An on-column refolding was performed using a step-wise gradient from 8 M to 0 M urea using a refolding buffer that contained 1 mM reduced glutathione and 0.1 mM oxidized glutathione. For each step 2 column volumes were used until the buffer was free of urea. The beads were then washed with 10 column volumes of equilibration buffer containing 20 mM imidazole to remove histidine-rich impurities. The elution was performed using 300 mM imidazole elution buffer. PD-10 desalting columns (GE Healthcare, Amersham, England) were used to exchange the buffer to 100 mM K_2HPO_4/KH_2PO_4 buffer at pH 7. Protein concentration was determined using the Bio-Rad Protein Assay (Biorad, Hercules, CA, USA) and bovine serum albumin as standard. The purified proteins were analysed with SDS-PAGE and staining was performed with Coomassie Brilliant Blue R-250.[30]

AFM sample preparation and imaging

Samples were prepared by 3 different methods: (i) completely drying a drop of protein solution on the HOPG substrate, (ii) droplet method: ordered hydrophobin layers were obtained by the following procedure: a 50 μL drop of 1 nmol mL^{-1} solution was first pipetted onto a freshly cleaved HOPG surface. After 2 h of incubation in humid environment the hydrophobin layer, which had self-assembled on the drop surface, was transferred and thereby inversed, by gently touching the drop with another freshly cleaved HOPG substrate. Before imaging, the samples were extensively washed with buffer. (iii) *In situ* preparation: ordered layers of EPL1 were generated *in situ* adding a small amount of concentrated EPL1 solution (120 nmol mL^{-1}) to the liquid cell to achieve a final concentration of approximately 30 nmol mL^{-1}. For this purpose scanning was interrupted for approximately 30 seconds at a tip-sample separation of 300 nm.

AFM images where recorded in tapping mode with a NanoScope V (Bruker, Santa Barbara, CA, USA) either in air or under PBS (phosphate buffered saline) buffer. In both cases the same type of cantilevers was used in order to increase comparability: etched single crystal silicon probes (NCH from Nanoworld, Neuchatel, Switzerland) with a spring constant of 42 N m^{-1}. A free oscillating amplitude of approximately 50–100 mV and a drive frequency of either ~180 kHz (liquid) or 298 kHz (air) were chosen. Images were taken with set-points corresponding to a damping of approximately 90% of the free amplitude. The pixel size of 500 nm × 500 nm images was set to 512 × 512. In order to enhance the contrast and to increase the visibility of the ordering in the domains, instead of topography images amplitude error images have been chosen for presentation in this paper. The scanner was frequently calibrated and confirmed to have a tolerance of less than 5%. When periodical surface structures were imaged, artefacts due to oscillation were excluded by controlling the correct size scaling and by changing the scanning angle. AFM experiments described in this study were repeated at least two times on independent samples and representative images are shown.

Surface contact angle measurements

Contact angle measurements were performed on a contact angle device DSA 100 (KRÜSS, Hamburg, Germany) using the sessile drop method. For the analysis of the surface properties of protein layers, 2 μL drops of ultrapure water were set down on a HOPG substrate which was either freshly cleaved or coated with hydrophobins or hybrid layers. Drop shapes were modelled with the software program DSA1 (KRÜSS) using a polynomial function. From each surface at least 2 independent samples were prepared, the formation of layers was checked by AFM and 7–15 drops were analysed.

Conclusions

In this study we showed with AFM that the CPP EPL1 readily forms highly ordered (mono)layers at liquid/solid interfaces which is remarkable, considering that this molecule has no evidently amphiphilic structural features. This is a clear indication for fast and efficient self-assembly of EPL1. Furthermore, from a phenomenological point of view it was observed that hybrid layers with mixed properties are formed upon simultaneous presence of both, hydrophobins and EPL1, in solution, whereas pre-formed hydrophobin layers are not perturbed by subsequent addition of EPL1. This unprecedented mixture of properties provides a promising starting point for future investigations of their detailed structure and organisation as well as for potential biotechnological applications of CPPs. Furthermore, CPPs might act as antagonists for hydrophobins by subtly modifying their surface activity-altering properties.

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Notes and references

1 R. Resch, M. Grasserbauer, G. Friedbacher, T. Vallant, H. Brunner, U. Mayer and H. Hoffmann, *Appl. Surf. Sci.*, 1999, **140**, 168–175.
2 T. Leitner, G. Friedbacher, T. Vallant, H. Brunner, U. Mayer and H. Hoffmann, *Microchim. Acta*, 2000, **133**, 331–336.
3 J. Foisner, A. Glaser, T. Leitner, H. Hoffmann and G. Friedbacher, *Langmuir*, 2004, **20**, 2701–2706.
4 T. Vallant, H. Brunner, U. Mayer, H. Hoffmann, T. Leitner, R. Resch and G. Friedbacher, *J. Phys. Chem. B*, 1998, **102**, 7190–7197.
5 M. B. Linder, G. R. Szilvay, T. Nakari-Setälä and M. E. Penttilä, *FEMS Microbiol. Rev.*, 2005, **29**, 877–896.
6 H. A. B. Wösten, *Annu. Rev. Microbiol.*, 2001, **55**, 625–646.
7 A. Frischmann, S. Neudl, R. Gaderer, K. Bonazza, S. Zach, S. Gruber, O. Spadiut, G. Friedbacher, H. Grothe and V. Seidl-Seiboth, *J. Biol. Chem.*, 2013, **288**, 4278–4287.
8 H. Chen, A. Kovalchuk, S. Keriö and F. O. Asiegbu, *Mycologia*, 2013, **105**, 1479–1488.
9 V. Seidl, M. Marchetti, R. Schandl, G. Allmaier and C. P. Kubicek, *FEBS J.*, 2006, **273**, 4346–4359.
10 S. Boddi, C. Comparini, R. Calamassi, L. Pazzagli, G. Cappugi and A. Scala, *FEMS Microbiol. Lett.*, 2004, **233**, 341–346.
11 R. González-Fernández, K. Aloria, J. Valero-Galván, I. Redondo, J. M. Arizmendi and J. V. Jorrín-Novo, *J. Proteomics*, 2014, **97**, 195–221.
12 S. Djonovic, M. J. Pozo, L. J. Dangott, C. R. Howell and C. M. Kenerley, *Mol. Plant-Microbe Interact.*, 2006, **19**, 838–853.

13 W. A. Vargas, S. Djonovic, S. A. Sukno and C. M. Kenerley, *J. Biol. Chem.*, 2008, **283**, 19804–19815.
14 M. Frías, N. Brito and C. González, *Mol. Plant Pathol.*, 2013, **14**, 191–196.
15 A. Scala, L. Pazzagli, C. Comparini, A. Santini, S. Tegli and G. Cappugi, *J. Plant Pathol.*, 2004, **86**, 27–33.
16 I. Baccelli, C. Comparini, P. P. Bettini, F. Martellini, M. Ruocco, L. Pazzagli, R. Bernardi and A. Scala, *FEMS Microbiol. Lett.*, 2012, **327**, 155–163.
17 M. Frías, C. González and N. Brito, *New Phytol.*, 2011, **192**, 483–495.
18 A. L. de Oliveira, M. Gallo, L. Pazzagli, C. E. Benedetti, G. Cappugi, A. Scala, B. Pantera, A. Spisni, T. A. Pertinhez and D. O. Cicero, *J. Biol. Chem.*, 2011, **286**, 17560–17568.
19 J. Sampedro and D. J. Cosgrove, *Genome Biol.*, 2005, **6**, 242.
20 I. Baccelli, S. Luti, R. Bernardi, A. Scala and L. Pazzagli, *Appl. Microbiol. Biotechnol.*, 2014, **98**, 175–184.
21 M. R. d. O. d. Barsottini, J. F. de Oliveira, D. Adamoski, P. J. Teixeira, P. F. do Prado, H. O. Tiezzi, M. L. Sforca, A. Cassago, R. V. Portugal, P. S. de Oliveira, M. Z. A. C. de, S. M. Dias, G. A. Pereira and A. L. Ambrosio, *Mol. Plant-Microbe Interact.*, 2013, **26**, 1281–1293.
22 G. R. Szilvay, A. Paananen, K. Laurikainen, E. Vuorimaa, H. Lemmetyinen, J. Peltonen and M. B. Linder, *Biochemistry*, 2007, **46**, 2345–2354.
23 A. Paananen, E. Vuorimaa, M. Torkkeli, M. Penttilä, M. Kauranen, O. Ikkala, H. Lemmetyinen, R. Serimaa and M. B. Linder, *Biochemistry*, 2003, **42**, 5253–5258.
24 V. Seidl-Seiboth, S. Gruber, U. Sezerman, T. Schwecke, A. Albayrak, T. Neuhof, H. von Döhren, S. E. Baker and C. P. Kubicek, *J. Mol. Evol.*, 2011, **72**, 339–351.
25 M. B. Linder, G. R. Szilvay, T. Nakari-Setälä and M. E. Penttilä, *FEMS Microbiol. Rev.*, 2005, **29**, 877–896.
26 B. G. Jensen, M. R. Andersen, M. H. Pedersen, J. C. Frisvad and I. Søndergaard, *BMC Res. Notes*, 2010, **3**, 344.
27 S. Djonovic, W. Vargas, M. Kolomiets, M. Horndeski, A. Wiest and C. Kenerley, *Plant Physiol.*, 2007, **145**, 875–889.
28 R. Gaderer, N. L. Lamdan, A. Frischmann, M. Sulyok, R. Krska, B. A. Horwitz and V. Seidl-Seiboth, *BMC Microbiology*, 2015, DOI: 10.1186/s12866-014-0333-0.
29 J. S. Jeong, T. K. Mitchell and R. A. Dean, *FEMS Microbiol. Lett.*, 2007, **273**, 157–165.
30 J. Sambrook and D. W. Russell, *Molecular Cloning: a Laboratory Manual*, Cold Spring Harbor Laboratory Press, Plainview, NY, 2nd edn, 2001, pp. 7.1–7.74 and A.78.40–A.78.51.

Concluding remarks

This doctoral thesis focused on the GlcNAc-catabolism in ascomycete filamentous fungi, especially *Trichoderma* spp. and *Neurospora crassa*. We found out that the respective genes, encoding the enzymes essential for GlcNAc-catabolism, are clustered in these fungi, as already described for *Candida albicans* (Konopka, 2012). Furthermore filamentous fungi possess an Ndt80-like transcription factor, designated RON1, which is responsible for regulation of this cluster. Additional a gene encoding a GH family 3 protein, which we have annotated as *nag3*, can often be found in the GlcNAc gene cluster. NAG3 exhibits similarities to bacterial GH3 β-*N*-acetylhexosaminidases ((Cheng and Li, 2000; Li et al., 2002; Litzinger et al., 2010; Mayer et al., 2006; Tsujibo et al., 1994) and http://www.cazy.org/GH3.html)) (chapter 1.1 and 1.2).

Another insight into the GlcNAc-catabolism is that the filamentous fungus *N. crassa* in contrast to *Trichoderma* spp. is not able to grow on medium containing GlcNAc, although *N. crassa* possesses all genes of the GlcNAc-catabolism cluster. One hypothesis for this phenomenon is that there is a defect in the GlcNAc-catabolism itself. This is corroborated by findings in *C. albicans* where deletion strains of *dac1* and *nag1* (*N. crassa dam-1*) were not able to grow on medium containing GlcNAc, even in the presence of an additional carbon source (Naseem et al., 2011), which is a hint that GlcNAc-6-phosphate deacetylase and GlcN-6-phosphate deaminase are bottlenecks in this pathway. Interestingly, *N. crassa* was readily able to utilize the polymer chitin. An explanation could be that instead of chitinases, non-hydrolytic enzymes, e.g. lytic polysaccharide monooxygenases are mainly responsible for chitin degradation in *N. crassa*, leading to chitin-degradation products other than GlcNAc, which would not be catabolised via the GlcNAc pathway and therefore not lead to accumulation of the growth-inhibitory intermediates. This is supported by our findings that, although, *N. crassa* is able to utilize chitin as carbon source, expression of the GlcNAc-cluster is absent (chapter 1.3).

In contrast, transcription of the GlcNAc gene cluster was highly inducible by GlcNAc, similar to *T. reesei*, which very efficiently uses GlcNAc as carbon source (Kappel et al., 2015).

The results from these studies contribute to new important insights in GlcNAc-catabolism. However, further investigations will be necessary to get a better understanding of the function and action of chitin metabolizing enzymes which will also help to increase their productivity and specificity via directed genetic engineering. This in turn furthers the production of chitinolytic enzymes and hence reduces their costs, which can help to develop a cheaper alternative to chemical processing of chitin for industrial applications.

Current applications of chitin comprise wound dressings, separation membranes, antibacterial coatings for stents and tissue engineering scaffolds (Bhattarai et al., 2010; Crompton et al., 2007; Noel et al., 2010). Due to the high antibacterial properties of chitin and chitooligomers, they can assist regeneration of human tissue after injury. GlcNAc itself was discovered to be a valuable pharmacological agent in the treatment of a wide variety of maladies in the past decade such as treatment in joint damage including arthritic diseases, cartilage- or joint injury and degenerative joint diseases (Burtan, 1998; Burton, 1990; Burton and Freeman, 1993; Karzel and Domenjoz, 1971; Mönnikes et al., 2006). Another field of application is cosmetics for example hyaluronic acid is widely used to treat skin and mucosal damages despite inefficient absorption (Chen et al., 2008; Sayo et al., 2004).

The second focus of this doctoral study was laid on the chitin-binding protein family denominated cerato-platanins. In addition to their established characteristics we have discovered new, intriguing properties of the CPPs from *T. atroviride* and *T. virens*, EPLs and SMs respectively. CPPs are associated with the induction of defense responses in plants, especially CPPs from the genus *Trichoderma* are key factors in the fungus-plant interaction. In the maize – *Cochliobolus heterostrophus* assay used in this study knock-outs of *sm1* reduced the plant protection potential of *T. virens*, but in this pathosystem the lack of *sm2* had an even greater effect and lesion size of maize leaves was statistically not different from the control (without addition of *Trichoderma*). The results showed that although the CP gene *sm1* is more abundantly expressed than *sm2* during fungal growth, Sm2 is, interestingly, more important than Sm1 for the promotion of plant protection. Additional analyses showed that *T. virens* was in general a more effective plant protectant compared to *T. atroviride* (chapter 2.2). These findings advance our understanding of the diversified functions of CPPs in fungi and of the pool of molecules that are involved in the beneficial interaction of *Trichoderma* with plants.

Further it is known that CPPs, in contrast to hydrophobins, enhance the polarity of solutions and surfaces and self-assemble at air/water interfaces. We detected that EPL1 forms highly ordered monolayers at surface/liquid-interface and furthermore a combination of EPL1 and hydrophobins resulted in a new unknown layer which comprise features of both, EPL1 and hydrophobins (chapter 2.1 and 2.3).

One possible biotechnological application for CPPs could be to enhance the wettability properties of solutions, which can be of interest for applications where a uniform moistening of a moderately hydrophobic surface is of interest, e.g. spraying of plant protection products or in cleaning agents.

With an increasing understanding of the mode of action of CPPs, their possible use as additives for the induction of plant resistance and defence mechanisms in fertilizers would also be an interesting possibility.

Biochemical analysis of more CPPs will be necessary to elucidate the application potential of CPPs further and to reveal which properties are specific for individual CPPs and which properties can be generally found in all members of the CPP family.

References

Bhattarai, N., Gunn, J. and Zhang, M., 2010. Chitosan-based hydrogels for controlled, localized drug delivery. Adv Drug Deliv Rev, 62(1): 83-99.

Burtan, A.F., 1998. N-Acetyl Glucosamine as a Cytoprotective Agent.

Burton, A.F., 1990. *N*-acetyl glucosamine as a cytoprotective agent. EP 0372730 A2. Google Patents.

Burton, A.F. and Freeman, H.J., 1993. *N*-acetyl glucosamine as gastroprotective agent. WO 1993023055 A1. Google Patents.

Chen, R.H., Hsu, C.N., Chung, M.Y., Tsai, W.L. and Liu, C.H., 2008. Effect of Different Concentrations of Collagen, Ceramides, *N*-Acetyl Glucosamine, or their Mixture on Enhancing the Proliferation of Keratinocytes, Fibroblasts and the Secretion of Collagen and/or the Expression of mRNA of Type I Collagen. J. Food Drug Anal. , 16: 66-74.

Cheng, C.Y. and Li, Y.K., 2000. An *Aspergillus* chitosanase with potential for large-scale preparation of chitosan oligosaccharides. Biotechnol Appl Biochem, 32 (Pt 3): 197-203.

Crompton, K.E., Goud, J.D., Bellamkonda, R.V., Gengenbach, T.R., Finkelstein, D.I., Horne, M.K. et al., 2007. Polylysine-functionalised thermoresponsive chitosan hydrogel for neural tissue engineering. Biomaterials, 28(3): 441-9.

Kappel, L., Gaderer, R., Flipphi, M. and Seidl-Seiboth, V., 2015. The *N*-acetylglucosamine catabolic gene cluster in *Trichoderma reesei* is controlled by the Ndt80-like transcription factor RON1. Mol Microbiol.

Karzel, K. and Domenjoz, R., 1971. Effects of hexosamine derivatives and uronic acid derivatives on glycosaminoglycane metabolism of fibroblast cultures. Pharmacology, 5(6): 337-45.

Konopka, J.B., 2012. *N*-acetylglucosamine (GlcNAc) functions in cell signaling. Scientifica (Cairo).

Li, H., Morimoto, K., Katagiri, N., Kimura, T., Sakka, K., Lun, S. et al., 2002. A novel beta-*N*-acetylglucosaminidase of *Clostridium paraputrificum* M-21 with high activity on chitobiose. Appl Microbiol Biotechnol, 60(4): 420-7.

Litzinger, S., Fischer, S., Polzer, P., Diederichs, K., Welte, W. and Mayer, C., 2010. Structural and kinetic analysis of *Bacillus subtilis N*-acetylglucosaminidase reveals a unique Asp-His dyad mechanism. J Biol Chem, 285(46): 35675-35684.

Mayer, C., Vocadlo, D.J., Mah, M., Rupitz, K., Stoll, D., Warren, R.A. et al., 2006. Characterization of a beta-*N*-acetylhexosaminidase and a beta-*N*-acetylglucosaminidase/beta-glucosidase from *Cellulomonas fimi*. FEBS J, 273(13): 2929-41.

Mönnikes, H., Schmidtmann, M. and van der Voort, I.R., 2006. [Drug therapy for irritable bowel syndrome. What works, what doesn't work and for whom? Internist (Berl), 47(10): 1073-6, 1078-83.

Naseem, S., Gunasekera, A., Araya, E. and Konopka, J.B., 2011. *N*-acetylglucosamine (GlcNAc) induction of hyphal morphogenesis and transcriptional responses in *Candida albicans* are not dependent on its metabolism. J Biol Chem, 286(33): 28671-28680.

Noel, S.P., Courtney, H.S., Bumgardner, J.D. and Haggard, W.O., 2010. Chitosan sponges to locally deliver amikacin and vancomycin: a pilot *in vitro* evaluation. Clin Orthop Relat Res, 468(8): 2074-2080.

Sayo, T., Sakai, S. and Inoue, S., 2004. Synergistic effect of *N*-acetylglucosamine and retinoids on hyaluronan production in human keratinocytes. Skin Pharmacol Physiol, 17(2): 77-83.

Tsujibo, H., Fujimoto, K., Tanno, H., Miyamoto, K., Imada, C., Okami, Y. et al., 1994. Gene sequence, purification and characterization of *N*-acetyl-beta-glucosaminidase from a marine bacterium, *Alteromonas* sp. strain O-7. Gene, 146(1): 111-5.

Curriculum vitae

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Publications

Frischmann A, Neudl S, Gaderer R, Bonazza K, Zach S, Gruber S, Spadiut O, Friedbacher G, Grothe H, Seidl-Seiboth V. Self-assembly at air/water interfaces and carbohydrate binding properties of the small secreted protein EPL1 from the fungus *Trichoderma atroviride*. J Biol Chem 2013; 288: 4278-87.

Gaderer R, Bonazza K, Seidl-Seiboth V. Cerato-platanins: a fungal protein family with intriguing properties and application potential. Appl Microbiol Biotechnol 2014; 98: 4795-803.

Bonazza K, Gaderer R, Neudl S, Przylucka A, Allmaier G, Druzhinina IS, Grothe H, Friedbacher G, Seidl-Seiboth V. The fungal cerato-platanin protein EPL1 forms highly ordered layers at hydrophobic/hydrophilic interfaces. Soft Matter 2015; 11: 1723-32.

Gaderer R, Lamdan NL, Frischmann A, Sulyok M, Krska R, Horwitz B.A, Seidl-Seiboth V. Sm2, a paralog of the *Trichoderma* cerato-platanin elicitor Sm1, is also highly important for plant protection conferred by the fungal-root interaction of *Trichoderma* with maize. BMC Microbiol 2015, 15.

Kappel L, Gaderer R, Flipphi M, Seidl-Seiboth V. The *N*-acetylglucosamine catabolic gene cluster in *Trichoderma reesei* is controlled by the Ndt80-like transcription factor RON1. Mol Microbiol 2015.

Romana Gaderer, Verena Seidl-Seiboth and Lisa Kappel. Chitin and *N*-acetylglucosamine metabolism in fungi - a complex machinery harnessed for the design of chitin-based high value products. Curr Biotech. 2016. Under review.

Romana Gaderer, Verena Seidl-Seiboth and Lisa Kappel. *N*-acetylglucosamine, the monomeric building block of chitin, inhibits growth of *Neurospora crassa*. 2016. Manuscript in preparation for submission.

Talk

Romana Gaderer, Susanna Zach, Alexa Frischmann, Verena Seidl-Seiboth (2012). Functional analysis of cerato- platanin encoding genes in the fungus *Trichoderma atroviride*. Talk at the 4th ÖGMBT Annual Meeting, September 17th-19th, Graz, Austria.

Posters

Romana Gaderer, Verena Seidl-Seiboth (2013). Biochemical characterization of fungal chitinases. Poster at the 5thÖGMBT Annual Meeting, September 25th-27th, Innsbruck, Austria.

Romana Gaderer, Klaus Bonazza, Netta L. Lamdan, Benjamin A. Horwitz, Verena Seidl-Seiboth (2014). CPPs in *Trichoderma*: Properties of protein layers and analysis of cerato-platanin gene knockout strains. Poster at the 6thÖGMBT Annual Meeting, September 15th-18th September, Vienna, Austria.

Lisa Kappel, Romana Gaderer, Verena Seidl-Seiboth. Characterization of the *N*-acetylglucosamine catabolism gene cluster in filamentous fungi. Poster at Austrian Association of Molecular Life Sciences and Biotechnology- Annual Meeting. September 15-18, 2014Vienna, Austria.

Romana Gaderer, Klaus Bonazza, Netta L. Lamdan, Benjamin A. Horwitz, Verena Seidl-Seiboth (2014). Properties of protein layers of the cerato-platanin protein EPL1 and analysis of cerato-platanin gene knockout strains in *Trichoderma* spp. Poster at the 12th European Conference on Fungal Genetics, March 23th-27th, Seville, Spain.

Lisa Kappel, Romana Gaderer, Verena Seidl-Seiboth. Characterization of the *N*-acetylglucosamine catabolism gene cluster in filamentous fungi. Poster at 12th European Conference on Fungal Genetics, March 23-27, Sevilla, Spain.

Romana Gaderer, Lisa Kappel and Verena Seidl-Seiboth (2015). Metabolism of the chitin monomer *N*-acetylglucosamine in *Neurospora crassa.* Poster at the 28thFungal Genetics Conference, March 17th-22th 2015, Pacific Grove, CA.

Lisa Kappel, Romana Gaderer and Verena Seidl-Seiboth (2015). Regulation of the *N*-acetylglucosamine catabolism gene cluster by an NDT80-like transcription factor in filamentous fungi. Poster at the 28thFungal Genetics Conference, March 17th-22th 2015, Pacific Grove, CA.

Lisa Kappel, Romana Gaderer Verena Seidl-Seiboth (2015). *N*-acetylglucosamine catabolism in fungi. Poster at 12th International Conference of the European Chitin Society and 13th International Conference on Chitin and Chitosan. August 30th- September2nd Münster, Germany.

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