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DISSERTATION

Molecular and physiological investigations of biocontrol by the genus *Hypocrea/Trichoderma*

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Contents

Kurzfassung der Dissertation

Spezies des filamentösen Pilzes *Hypocrea/Trichoderma* werden in der Landwirtschaft als biologische Pflanzenschutzmittel verwendet. Der Einsatz von *Hypocrea/Trichoderma* beruht auf der mykoparasitischen Wirkung gegen pflanzenpathogene Pilze und positiven Effekten von *Hypocrea/Trichoderma* auf das Pflanzenwachstum und die Wurzelbildung. Um den Einsatz von *Hypocrea/Trichoderma* als Pflanzenschutzmittel im Feld und während der Lagerung von Feldfrüchten zu optimieren, ist ein detailliertes Verständnis der Wirkungsweise und Limitierungen der Anwendung von *Hypocrea/Trichoderma* notwendig.

Der mykoparsitische Angriff beinhaltet mehrere Schritte wie zum Beispiel das Umwickeln der Wirtshyphen, die Bildung von Appressorien und den enzymatischen Angriff der Wirtszellwand. Die Appressorienbildung geht mit lokalen Änderungen der intrazellulären Polyol-Konzentrationen einher, welche bisher in *Hypocrea/Trichoderma* noch nicht studiert wurden. Daher wurden die Polyol-Konzentrationen von *H. atroviridis* während normalem Hyphenwachstum und verschiedenen Arten von osmotischem Stress untersucht. Unter osmotischem Stress erhöhte *H. atroviridis* die intrazelluläre Glyzerin-Konzentration, und die Biosynthese von Glyzerin erfolgte mittels der Glyzerindehydrogenase (Gld1). Der STRE-(Stress-Element) bindende Transkriptionsfaktor Seb1 war im Gegensatz zu den Hefe-Orthologen Msn2/4 zwar in die osmotische Stressreaktion involviert, jedoch nicht essentiell für diese.

H. jecorina wird industriell für die Produktion von Enzymen für den Abbau von Zellulose und Hemizellulosen und für die Herstellung von rekombinanten Proteinen verwendet, wurde jedoch bisher noch nicht auf seine Eigenschaften als biologisches Pflanzenschutzmittel untersucht. *H. jecorina* QM9414 zeigte gegen *Pythium ultimum* einen starken Antagonismus in Plattenkonfrontationstests und auch statistisch signifikante, obwohl eher schwache, Biokontrollwirkung in Pflanzenversuchen, aber nicht gegen *Rhizoctonia solani*. Eine Zellulase-negative *H. jecorina* Mutante zeigte ähnlichen Antagonismus auf Agarplatten und ähnliche Pflanzenschutzwirkung und eine Kohlenstoff-dereprimierte *H. jecorina* Mutante antagonisierte *P. ultimum* auf Agarplatten besser und erhöhte auch die Überlebensrate von mit *P. ultimum* beimpften Zucchinipflanzen im Vergleich zu *H. jecorina* QM9414. Die Tatsache dass eine große Anzahl von *H. jecorina* Gen-knock-out Mutanten verfügbar sind und die eher schwache Biokontrollwirkung von *H. jecorina* machen diese

Spezies zu einem interessanten Modell, um positive und negative Einflüsse von diversen Genen auf die Biokontrollwirkung von *H. jecorina* zu studieren.

Chitin ist der Hauptbestandteil der Zellwand von pflanzenpathogenen Pilzen wie z.B. *R. solani*, *Botrytis cinerea* und *Sclerotinia sclerotiorum*. Die *H. jecorina* Genomdatenbank wurde benutzt, um einen umfangreichen Einblick in das chitinolytische Potential von *Hypocrea/Trichoderma* zu bekommen. Das *H. jecorina* Genom enthält 18 Gene die für Chitinasen kodieren, welche alle zur Glykosid-Hydrolase Familie 18 gehören. Elf dieser Gene kodieren für bisher nicht beschriebene Chitinasen. Fünf neue Chitinase-Gene wurden aus *H. atroviridis* kloniert. Die Transkription von *chi18-10* (das zu Gruppe C, einer neuen phylogenetischen Untergruppe von Chitinasen mit Ähnlichkeit zu *Kluyveromyces lactis* Killertoxinen gehört) und *chi18-13* (das zur neuen Untergruppe B gehört) war spezifisch bei Wachstum auf *R. solani* Zellwänden und bei Plattenkonfrontationen mit *R. solani* erhöht.

Um zu studieren welche Arten von Kohlenstoffquellen, die auch in Pilzzellwänden enthalten sind, die Expression von β-*N*-Acetylglucosaminidasen und Chitinasen hervorrufen und die Bildung von chitinolytischen Enzymen in *Hypocrea/Trichoderma* induzieren, wurden β-*N*-Acetylglucosaminidase-Aktivitäten auf 95 verschiedenen Kohlenstoffquellen analysiert. Die Ergebnisse zeigten, dass die Expression von β-*N*-Acetylglucosaminidasen, eine Mischung der beiden Enzyme Nag1 und Nag2, nicht auf Mykoparasitismus beschränkt ist, denn β-*N*-Acetylglucosaminidase-Aktivitäten konnten bei Wachstum auf den meisten Kohlenstoffquellen nachgewiesen werden, und es konnte gezeigt werden, dass die Aktivität von der Wachstumsrate abhängt. Außerdem waren die β-*N*-Acetylglucosaminidase-Aktivitäten auf Kohlenstoffquellen mit strukturellen Ähnlichkeiten erhöht, besonders auf α-Glukanen (z.B. Glykogen, Dextrin und Maltotriose) und auf Oligosacchariden die Galaktose enthielten. Die Reduktion der β-*N*-Acetylglucosaminidase-Aktivitäten in einer *H. atroviridis* Δ*nag1* Mutante war stark Kohlenstoffquellen- und wachstumsabhängig, was darauf hindeutet, dass diese Gene verschiedene physiologische Aufgaben besitzen. Auf Kohlenstoffquellen die erhöhte β-*N*-Acetylglucosaminidase-Aktivitäten zeigten, war auch die Transkiption von *nag1* und *nag2* erhöht.

Die Ergebnisse dieser Doktorarbeit zeigten neue Richtungen für zukünftige Forschungsprojekte mit *Hypocrea/Trichoderma* auf, warfen neue Fragen zu den untersuchten Themengebieten auf und verdeutlichten das bisher nur teilweise genutzte Potential für die industrielle Nutzung der vielfältigen Enzyme von *Hypocrea/Trichoderma* und die landwirtschaftliche Anwendung von *Hypocrea/Trichoderma.*

Summary

Species of the filamentous fungus *Hypocrea/Trichoderma* are used in agriculture as biocontrol agents due to their mycoparasitic action against plant pathogenic fungi and their beneficial effects on plant growth and root development. To achieve an optimal application of *Hypocrea/Trichoderma* for the control of plant diseases during cultivation and storage, a detailed understanding of the biocontrol agents' modes of action and their limitations is essential.

The mycoparasitic attack involves steps such as coiling around the host, formation of appressoria and lysis of the host cell wall. The stage of appressoria formation is accompanied by localized changes in polyol pools, which were not investigated in *Hypocrea/Trichoderma* yet. The polyol pools of *H. atroviridis* during normal growth and different kinds of osmotic stress were analysed. When *H. atroviridis* was subjected to osmotic stress it responded by raising its intracellular glycerol level and glycerol was synthesized via the glycerol dehydrogenase (Gld1). The STRE (stress element)-binding transcription factor Seb1 was only involved in, but not essential for osmotic stress response, which is in contrast to the yeast orthologues Msn2/4 of Seb1.

H. jecorina is widely used for the production of cellulolytic and hemicellulolytic enzymes and recombinant proteins, but was not tested for its biocontrol potential yet. *H. jecorina* displayed against *Pythium ultimum* good antagonism in plate confrontation assays and also statistically significant, albeit rather weak, biocontrol action in greenhouse experiments, but not against *Rhizoctonia solani*. A cellulase negative *H. jecorina* mutant showed a similar range of antagonism in plates and plant protection and a carbon catabolite derepressed *H. jecorina* mutant antagonized *P. ultimum* on plates more actively and also increased the survival rates of *P. ultimum*-inoculated zucchini plants. The fact that a number of *H. jecorina* gene knock-out mutants are available and the rather weak biocontrol properties of *H. jecorina* make it an interesting target to study positive and negative influences of selected genes on the biocontrol potential of *H. jecorina*.

Chitin is a major component of the cell wall of plant pathogenic fungi like e.g. *R solani*, *Botrytis cinerea* and *Sclerotinia sclerotiorum*. The *H. jecorina* genome database was used to obtain a comprehensive insight into the chitinolytic potential of *Hypocrea/Trichoderma*. The *H. jecorina* genome contains 18 ORFs encoding putative

chitinases, all of them belonging to glycoside hydrolase family 18. Eleven of them encode yet undescribed chitinases. Five novel chitinase genes were subsequently cloned from *H. atroviridis*. Transcription of *chi18-10* (belonging to group C, a novel phylogenetic subgroup of chitinases with similarity to *Kluyveromyces lactis* killer toxins) and *chi18-13* (belonging to a novel clade in group B) was triggered upon growth on *R. solani* cell walls, and during plate confrontation tests with *R. solani*.

To study which types of carbon sources, derived from fungal cell walls, can possibly trigger β-*N*-acetylglucosaminidase and chitinase expression and act as inducers for the formation of chitinolytic enzymes in *Hypocrea/Trichoderma*, β-*N*-acetylglucosaminidase activities were analysed on a set of 95 different carbon sources. The results showed that the expression of β-*N*-acetylglucosaminidases, which is a mixture of the activity of the two enzymes Nag1 and Nag2, was not restricted to mycoparasitism-related growth conditions but could be found on most carbon sources and was shown to be dependent on the growth-rate. Additionally, β-*N*-acetylglucosaminidase activities were enhanced on carbon sources sharing certain structural properties, especially on α-glucans (e.g. glycogen, dextrin and maltotriose) and oligosaccharides containing galactose. Reduction of β-*N*-acetylglucosaminidase levels in a *H. atroviridis* Δ*nag1* mutant was strongly carbon source and growth phase dependent, indicating distinct physiological roles of those genes. Transcript abundance of *nag1* and *nag2* was increased on carbon sources with elevated β-*N*-acetylglucosaminidase activities.

The results from this work pointed out directions for future *Hypocrea/Trichoderma* research projects, raised new questions about the investigated topics and alluded to the yet only partially used potential for the industrial utilization of the large range of versatile *Hypocrea/Trichoderma* enzymes and agricultural applications of *Hypocrea/Trichoderma*.

Introduction

The genus *Hypocrea/Trichoderma*

The anamorphic fungal genus *Trichoderma* (*Hypocreales*, *Ascomycota*) is cosmopolitan in soils and on decaying wood and other forms of plant organic matter (Samuels 1996; Klein and Eveleigh 1998). *Trichoderma* species are among the most widely distributed and common fungi in nature and exist in climates ranging from the tundra to the tropics. This may be attributable to their diverse metabolic capability and aggressively competitive nature (Samuels 1996; Klein and Eveleigh 1998).

Rapid growth rates in culture and the production of numerous spores (conidia) that are mostly varying shades of green characterize fungi in this genus. A growing number of teleomorphs in *Hypocrea* have been linked to commonly occurring *Trichoderma* anamorphs, but most strains of *Trichoderma* are classified as imperfect fungi because they have not been associated with a sexual state (Gams and Bissett 1998). The taxonomy of *Hypocrea/Trichoderma* is rather difficult and complex due to the plasticity of characters if classical approaches, based on morphological criteria, are applied. The use of molecular phylogenetic markers has refined *Hypocrea/Trichoderma* taxonomy significantly and phylogenetic analysis of the large number of *Hypocrea/Trichoderma* spp. is still a field of active ongoing research (Druzhinina and Kubicek 2005).

Hypocrea/Trichoderma spp. have a number of remarkable mechanisms for survival and proliferation including physical attack of other fungi and degradation and utilization of complex carbohydrates. For the most part they are beneficial to man's economic interests and are used for commercial applications. *Hypocrea jecorina* (= *Trichoderma reesei*) is an economically important producer of cellulases and hemicellulases and is used for heterologous protein expression (Kubicek and Penttilä 1998). *H. lixii* (= *T. harzianum*), *H. atroviridis* (= *T. atroviride*) and *T. asperellum* are applied as biocontrol agents against plant pathogenic fungi (Hjeljord and Tronsmo 1998) for a wide variety of crops and climates. However, there are also negative effects of *Trichderma*: due to their high cellulolytic potential they degrade cotton fabrics, strains of *T. aggressivum* are pathogenic on commercial mushrooms like *Agaricus* and *Pleurotus* (Seaby 1998), and more recently *T. longibrachiatum* was reported to be an opportunistic pathogen of immunocompromised mammals including humans (Kredics et al. 2003).

Biocontrol

The reason for plant disease epidemics is that modern agriculture is an ecologically unbalanced system, which is based on growing one or a few crop cultivars on large areas. Prevention of such epidemics has so far been mainly achieved through use of chemical fungicides, but farmers are increasingly often confronted with pathogens resistant to available chemical plant protectants. Additionally, consumers are becoming gradually more concerned about chemical pollution of the environment and pesticide residues in food and there is an increasing demand for products coming from sustainable agriculture and eco-farming. Thus both, customers and industry, are highly interested in finding alternative methods of disease control.

Replacement or reduction of chemical applications has been achieved through use of biologically based pesticides, a concept included in the broad definition of biocontrol proposed by Cook and Baker (1983): *"Biological control is the reduction of the amount of inoculum or disease-producing activity of a pathogen accomplished by or through one or more organisms other than man."* This broad definition includes use of less virulent variants of the pathogen, more resistant cultivars of the host and microbial antagonists "*that interfere with the survival or disease producing activities of the pathogen*".

The advantages of biological pest management are the safety of handling, the self perpetuation and therefore a less frequent need of application and a high degree of host specificity. On the other hand biological control agents are, like any other organism, affected by abiotic and biotic factors such as weather, disease pressure and competition from the indigenous microflora. Chemical pesticides are less prone to such influences and thus the inconsistent performance of biocontrol agents is a major disadvantage of biological pest management.

In greenhouse systems environmental conditions such as temperature and relative humidity are tightly controlled. The high economic value of greenhouse crops can absorb higher inputs to control disease. Because of the reduced area and high density of planting, less inoculum is needed than in treating a field. Additionally, the continuous harvesting of many crops, which puts the workers at greater risk of fungicide exposure, makes the application of many commercial fungicides infeasible. Therefore, biological control of plant diseases in greenhouses is a unique niche and attractive alternative to chemical pesticides. Biological

control of greenhouse insects is already the predominant method in e.g. the United Kingdom, but the application of biological fungicides is still a developing market (Paulitz and Belanger 2001).

A large area of interest in biocontrol is the reduction of plant diseases caused by soil-borne and foliar plant pathogenic fungi. Roughly 70% of all the major crop diseases are caused by fungi, or the fungus-like Oomycota (Deacon 1997). Notorious examples are species belonging to the genera *Rhizoctonia*, *Botrytis*, *Phytophthora*, *Pythium*, *Sclerotinia* and *Fusarium*. Most of the formulations of commercially available biocontrol products against plant pathogenic fungi contain the bacteria *Pseudomonas* and *Bacillus* or fungi belonging to the genus *Hypocrea/Trichoderma* (Paulitz and Belanger 2001).

Biocontrol by *Hypocrea/Trichoderma*

The potential of *Hypocrea/Trichoderma* species as biocontrol agents of plant diseases was first recognized by Weindling in the early 1930s (Weindling 1934) who described the mycoparasitic action of *Hypocrea/Trichoderma* on *Rhizoctonia* and *Sclerotinia* and its beneficial effects for plant disease control. This has stimulated research on this topic and also the commercial use of several *Hypocrea/Trichoderma* species for the protection and growth enhancement of a number of crops. Commercially available formulations are e.g. RootShield™ , BioTrek 22G™ , T-22G™ , T-22HB™ (Bio-Works, USA), Supresivit™ (Borregaard BioPlant, Denmark), Binab™ (Bio-Innovation, Sweden), Trichopel™ , Trichojet™ , Trichodowels™, Trichoseal™ (Agimm, New Zealand), Trieco™ (Ecosense Labs, India), Gliomix™ (Verdera Oy, Finland), Trichodex™ (Makhteshim, Israel) SoilGuard™ (Thermo Trilogy, USA) or Promot™ (J.H. Biotech, USA). However, not all of these products are registered as biocontrol agents, but are marketed as plant growth promoters, plant strengtheners, or soil conditioners. These designations have enabled the products to get to the marketplace with less stringent toxicology or efficacy testing than would be required for plant protectants (Paulitz and Belanger 2001).

One of the most interesting aspects of the research field of biological control is the study of the mechanisms employed by biocontrol agents to accomplish disease control. Past research indicates that the mechanisms are many and varied, even within the genus *Hypocrea/Trichoderma*. To achieve an optimal application of *Hypocrea/Trichoderma* for the control of plant diseases during cultivation and storage, a detailed understanding of the biocontrol agents' modes of action and their limitations is essential.

Biocontrol mechanisms of *Hypocrea/Trichoderma*

Biocontrol by *Hypocrea/Trichoderma* results from different mechanisms acting synergistically to achieve disease control. Those involve the competition for nutrients and living space with plant pathogenic organisms, the direct attack and destruction of the pathogens (antagonism, mycoparasitism) and promotion of plant beneficial processes such as enhancement of plant growth and induction of systemic and localized resistance.

Competition for nutrients and living space: *Hypocrea/Trichoderma* spp. have a rapid growth rate, persistent conidia and a broad spectrum of substrate utilization which makes them very efficient in the competition for nutrients and living space (Hjeljord and Tronsmo 1998). Furthermore *Hypocrea/Trichoderma* spp. are able to produce and/or resist metabolites that either impede spore germination (fungistasis), kill the cells (antibiosis) or modify the rhizosphere, e.g. by acidifying the soil, so that pathogens cannot grow (Benitez et al. 2004). Starvation is the most common cause of death for microorganisms and competition has turned out to be especially important for the biocontrol of phytopathogens such as *B. cinerea*, the main pathogenic agent during pre- and post-harvest in many countries, which is particularly sensitive to the lack of nutrients (Benitez et al. 2004).

Attack and decomposition of the pathogens: The direct interaction between *Hypocrea/Trichoderma* spp. and the pathogen is called mycoparasitism. The events leading to mycoparasitism are complex and different between various species, but the mycoparasitic attack generally follows the same scheme: *Hypocrea/Trichoderma* strains detect other fungi and grow straightly towards them; remote sensing is at least partially responsible for the sequential expression of hydrolytic, cell wall-degrading enzymes (Cortes et al. 1998; Zeilinger et al. 1999; Kullnig et al. 2000). Once the fungi come into contact, *Hypocrea/Trichoderma* spp. attach to the host, coil around the host hyphae and form appressoria on the host surface (Inbar and Chet 1992; Rocha-Ramirez et al. 2002). Then the *Hypocrea/Trichoderma* spp. produce a range of fungitoxic, hydrolytic enzymes such as chitinases, glucanases and proteases and other toxic compounds and/or peptaibol antibiotics (Schirmbock et al. 1994; Lorito et al. 1996a; Szekeres et al. 2005). Penetration of the host cell wall is achieved by a synergistic action of the hydrolytic enzymes and antibiotics (Schirmbock et al. 1994; Lorito et al. 1996a; Kubicek et al. 2001; Szekeres et al. 2005). The invasive process is locally restricted to the sites of the appressoria, where holes are produced in the host cell wall, and direct entry of *Hypocrea/Trichoderma* hyphae into the lumen of the target fungus occurs (Inbar and Chet 1992).

Plant beneficial processes: *Hypocrea/Trichoderma* spp. can also exert positive effects on plants, which cause an increase in plant growth and root development (biofertilization) and stimulate plant-defense mechanisms (Harman et al. 2004). Some *Hypocrea/Trichoderma* strains (e.g. of the species *H. atroviridis* and *H. lixii*) were shown to establish robust and long-lasting colonizations of root surfaces and to penetrate into the epidermis. They are opportunistic, avirulent plant symbionts and produce a variety of compounds that induce plant defense mechanisms. Interestingly, *Hypocrea/Trichoderma* spp. are even able to induce systemic resistance, which is characterized by the occurrence of disease control in the plant at a site distant from the location of *Hypocrea/Trichoderma*. They stimulate the production of low-molecular weight compounds that have antimicrobial activity like e.g. phytoalexins which are normally produced by plants in response to an attack by pathogens. Additionally, proteome analysis of *H. lixii* identified homologues of the avirulence genes Avr4 and Avr9 from *Cladosporium fulvum*. The protein products of avirulence genes have been identified in a variety of fungal and bacterial plant pathogens. They usually function as race- or pathovar specific elicitors that are capable of inducing hypersensitive responses and other defense-related reactions in plant cultivars that contain the corresponding resistance gene (Harman et al. 2004).

The (hydro) lytic enzyme system of *Hypocrea/Trichoderma*

Hypocrea/Trichoderma spp. produce a wide range of enzymes for degradation of homo- and heterpolysaccharides, which are designative for their broad spectrum of substrate utilization and their ubiquitous occurrence in nature. Furthermore they possess a wide spectrum of proteases which help them in the defense of their habitats and the competition for nutrients with other microorganisms. Sequencing of the *H. jecorina* genome allowed a more detailed and extensive analysis of the genes coding for those enzymes and their regulation and revealed and even larger number of hydrolytic enzymes, such as e.g. 12 genes encoding cellulases, than previously suspected. With the currently ongoing genome sequencing of the mycoparasitic species *H. atroviridis* the study of lytic enzymes, but also other gene products like Avrs, in biocontrol will be more complete and greatly facilitated. However, even without a sequenced genome a wide variety of hydrolytic enzymes were already cloned from various mycoparasitic *Hypocrea/Trichoderma* spp., their enzymes characterized and their regulation studied.

Chitinases

Chitin, the (1-4)-β-linked homopolymer of *N*-acetyl-D-glucosamine, is one of the most abundant polymers in the biosphere, and chitinolytic enzymes are found among all kingdoms, e.g., protista, bacteria, fungi, plants, invertebrates and vertebrates, including humans (Cabib 1987; Gooday 1990; Sahai and Manocha 1993). Enzymatic degradation of chitin is generally involved in many biological processes, such as autolysis (Vessey and Pegg 1972), morphogenesis and nutrition (Griffin 1994) and plays in addition to mycoparasitism also a role in relationships between fungi and other organisms such as plant-fungus and insect-fungus interactions (St. Leger et al. 1987; Mauch et al. 1988).

Chitinolytic enzymes can be divided into *exo*- and *endo*-acting enzymes based on their reaction end products and catalytic mechanism.

β-*N*-acetylglucosaminidases (NAGases, EC 3.2.1.52) catalyze the hydrolysis of terminal non reducing *N*-acetyl-D-glucosamine (GlcNAc) residues. The tolerance of NAGases for the aglycon moiety is generally quite high which enables the detection of NAGases in enzyme assays with chromogenic substrates (Horsch et al. 1997). NAGases have also already been shown to catalyze transglycosilation reactions and are used in polymer chemistry to synthesize regio- and stereo-selective polymers (Kobayashi et al. 1997).

Additionally to the *exo*-acting NAGases, *endo*-β-*N*-acetylglucosaminidases (EC 3.2.1.96) exist, which catalyze the hydrolysis of the *N*, *N*'-diacetylchitobiosyl unit in high-mannose glycopeptides and glycoproteins containing the [Man$(GlcNAc)_2$]Asn-structure, with one GlcNAc residue remaining attached to the protein and the rest of the oligosaccharide being released intact (Horsch et al. 1997).

Chitinases (EC 3.2.1.14) catalyze random hydrolysis of *N*-acetyl-β-glucosaminide 1,4-β-linkages in chitin and chito-oligomers according to an endo-mechanism with $(GlcNAc)_2$ and some $(GlcNAc)_3$ as the only end products. By using those definitions it is important that a NAGase, that effects a processive degradation of chito-oligomers (i.e. $(GlcNAc)_{n\ (=2\text{-}10)}$) by successively releasing GlcNAc residues from the nonreducing end of the chain must not be referred to as *exo*-chitinase. *Exo*-chitinases would follow a processive mechanism of hydrolysis and likewise release N, N'-diacetylchitobiose units, but starting at the non-reducing terminus of the substrate (Horsch et al. 1997).

The characteristics of the extensive chitinolytic enzyme system of *Hypocrea/Trichoderma* are discussed in more detail in chapters 4 and 5.

Glucanases

Chitinases and β-1, 3-glucanases are considered the main enzymes responsible for the degradation of the host cell walls by *Hypocrea/Trichoderma*, as chitin and β-1, 3-glucan are their two major cell wall components (Mahadevan and Tatum 1967).

It has been shown that β-1,3 glucanases inhibit spore germination or the growth of pathogens in synergistic cooperation with chitinases and antibiotics (Benitez et al. 2004). Many β-1, 3-glucanases have been isolated, but only a few genes have been cloned, e.g. *bgn13.1* (de la Cruz et al. 1995) and *lam1.3* (Cohen-Kupiec et al. 1999) from *H. lixii*, *glu78* from *H. atroviridis* (Donzelli et al. 2001) and *Tv-bgn1* and *Tv-bgn2* from *H. virens* (Kim et al. 2002).

However, other enzymes hydrolyzing less abundant, but structurally important components (as β-1, 6-glucan), can also contribute to the efficient disorganization and further degradation of the cell wall by *Hypocrea/Trichoderma*. Three β-1, 6 glucanases (BGN16.1-3) have been purified from *H. lixii*. BGN16.1 and BGN16.2 are secreted under conditions where chitin is present as the only carbon source (de la Cruz and Llobell 1999; Delgado-Jarana et al. 2000) and BGN16.3 is specifically secreted in the presence of fungal cell walls (Montero et al. 2005).

α-1,3-Glucanases (EC 3.2.1.59), also named mutanases, are extracellular enzymes able to degrade polymers of glucose bound by α-1,3-glycosidic links and are classified as endo-hydrolytic when two or more residues of glucose are released as reaction products, and exo-hydrolytic when glucose monomers are the final reaction products.

Two exo α-1, 3 glucanases, *agn13.1* and *agn13.2* have been cloned from *H. lixii* and *T. asperellum*, respectively, and the transcript levels of the genes as well as the enzymatic properties of the proteins were characterized and their involvement in mycoparasitism was studied (Fuglsang et al. 2000; Ait-Lahsen et al. 2001; Sanz et al. 2005).

Proteases

The study of the proteolytic system of *Hypocrea/Trichoderma* spp. and their contribution to biocontrol has been receiving increasing attention. Elad and coworkers (Kapat et al. 1998; Elad and Kapat 1999) showed that hydrolytic enzymes produced by *B. cinerea* were partially deactivated by protease activities of *H. lixii*, and demonstrated that the protease-containing culture liquid of *Hypocrea/Trichoderma* reduced germination and germ tube length of the pathogen, suggesting the involvement of proteases in biocontrol. Besides deactivation of plant

pathogens' enzymes, proteases may be important for the mycoparasitic process by degrading the protein components of the host well wall. The presence of several different extracellular proteases was detected by IEF and gel filtration chromatography methods (Antal et al. 2001; Delgado-Jarana et al. 2002; Williams et al. 2003; Suarez et al. 2004).

Several protease encoding genes have already been cloned from *Hypocrea/Trichoderma* spp. The subtilisin-like serine protease of *H. atroviridis*, Prb1 has already been characterized in more detail and was shown to be involved in mycoparasitism of *R. solani (Geremia et al. 1993; Flores et al. 1997; Cortes et al. 1998; Olmedo-Monfil et al. 2002)*. Its orthologues have also already been cloned from *H. virens* and *T. hamatum* (Pozo et al. 2004; Steyaert et al. 2004).

pra1, coding for a trypsin-like serine protease, was shown to be induced by fungal cell walls, nitrogen and carbon starvation and influenced by the pH of the media (Suarez et al. 2004) and the transcription of *papA* coding for an aspartic protease proved to be influenced by the nitrogen source and was upregulated in plate confrontation assays with *R. solani* and upon plant root attachment (Delgado-Jarana et al. 2002). Additionally, *papB*, encoding a vacuolar aspartic protease, and recently the extracellular aspartic protease P6281, which is upregulated upon growth on fungal cell wall were already described (Viterbo et al. 2004; Suarez et al. 2005).

Cellulases, Xylanases and other hydrolytic enzymes

Cellulases (β-1, 4-glucanases) comprise exoglucanases (i.e. cellobiohydrolases EC 3.2.1.91), endoglucanases (EC 3.2.1.4) and β -glucosidases (EC 3.2.1.21), which occur in various isozymic forms. Although cellulose is the major cell wall component of plant pathogenic oomycetes like *Pythium*, cellulases have not been studied in much detail for this purpose. Migheli et al (1998) overexpressed the cellulase Cel7B in *T. longibrachiatum* and obtained transformants with increased biocontrol activities. However, the cellulolytic system has been studied extensively in *H. jecorina*, which is industrially used for production of cellulases and heterologous protein expression (Mach and Zeilinger 2003; Schmoll and Kubicek 2003). Ongoing research in this field is focusing on the induction of cellulases by various carbon sources (Seiboth et al. 2004; Seiboth et al. 2005), the influence of light on this process (Schmoll et al. 2005; Schmoll and Kubicek 2005) and the impact of UPR and stress on protein secretion (Saloheimo et al. 1999; Collen et al. 2005; Pakula et al. 2005). It should also be noted that Saloheimo and coworkers (2002) cloned a gene encoding a protein with sequence

similarity to plant expansins. These are plant cell wall proteins which are thought to disrupt hydrogen bonding between cell wall polysaccharides without hydrolyzing them. The protein, named swollenin, was found to disrupt the structure of cotton fibers without detectable formation of reducing sugars.

ß-1,4-Xylans are heteropolysaccharides that have a backbone of ß-1,4-linked xylopyranosyl residues, to which side groups such as D-glucuronic acid, L-arabinose, *p*-coumaric acid, and ferulic acid are attached and which constitute 20 to 35% of the roughly 830 Gt of annually formed renewable plant biomass (Timell 1965). Enzymes capable of degrading the xylan backbone comprise endoxylanases (EC 3.2.1.8) and ß-xylosidase (EC 3.2.1.91) (Kulkarni et al. 1999). Xylanases of the ascomycete *H. jecorina* have received strong attention because of their application in the pulp and paper and feed industry (Buchert et al. 1998) but beyond this the physiological relevance of xylanases for other processes in *Hypocrea/Trichoderma* in nature has not been studied.

Likewise, studies on other hydrolytic enzymes like e.g. galactosidases, pectinases or mannosidases, are so far only conducted with *H. jecorina* due to its industrial applications. Additionally, the sequence genome of *H. jecorina* is publicly available since 03/2005 (http://gsphere.lanl.gov/trire1/trire1.home.html) which is a large advantage, especially in research fields like e.g. genomics (DNA microarrays, RaSH) or proteomics (2D-gel electrophoresis/MS/MS, LC/MS/MS) where large scale screening methods are applied.

Aims and background of the specific questions addressed in the PhD thesis - content and assembly of the following chapters

The aim of this PhD thesis was to investigate several aspects of *Hypocrea/Trichoderma* biocontrol in more detail and to address issues that have been reported as being potentially relevant for antagonism of plant pathogenic fungi.

Appressorium formation is one of the first events during the mycoparasitic attack and involves the formation of locally restricted high osmotic pressure. The turgor of the appressorium enables the penetration of the host cell wall. As polyol and trehalose pools have not been investigated in *Hypocrea/Trichoderma* spp. yet, a first step towards understanding osmotic pressure regulation in this species was made. This work is presented in **Chapter 1** of the PhD thesis. The composition of polyol and trehalose pools in *H. atroviridis* during normal growth and during osmotic stress caused by increased carbon source and salt concentrations

was assessed. The genes involved in glycerol biosynthesis and the role of the transcription factor *seb1* (stress element binding), which is an orthologue of the yeast *MSN2/4* genes were studied.

Hypocrea/Trichoderma biocontrol has so far mainly been studied with strains of *H. lixii, H. atroviridis* and *T. asperellum*, which phylogenetically are members of the *Hypocrea/Trichoderma* sections *Pachybasium* and *Trichoderma. H. jecorina*, belonging to section *Longibrachiatum* has not been tested for its biocontrol potential yet. This species is has the advantage that a number of conventional and recombinant mutants in regulatory genes, signaling cascades and hydrolytic enzymes are available, which could readily be tested for their biocontrol abilities. In the study presented in **Chapter 2** it was tested whether *H. jecorina* can be used to antagonize plant pathogenic fungi and protect plants against their attack. Available *H. jecorina* mutants were used to investigate (a) whether relieve from carbon catabolite repression via the Cre1-regulator protein would improve biocontrol, and (b) whether cellulase gene expression is necessary for biocontrol of *P. ultimum*.

The relevance of hydrolytic enzymes for mycoparasitism of *Hypocrea/Trichoderma* has already been subject of many studies and a number of genes, most of them encoding chitinases, glucanases or proteases were cloned and their impact in mycoparasitic actions was investigated. Sequencing of the *H. jecorina* genome finally made it possible to map the whole spectrum of genes encoding hydrolytic enzymes. The work presented in **Chapter 3** addressed the topic of the chitinolytic enzyme system of *Hypocrea/Trichoderma*. The whole range of chitinase encoding genes of *H. jecorina* was assessed and an extensive *in silico* analysis of the hypothetical proteins' domains and phylogenetic relationships was carried out. Additionally, 5 novel chitinase encoding genes were cloned from *H. atroviridis* and their transcript profiles were studied under different growth conditions.

Analysis of the genomic sequences of filamentous fungi showed that they possess several different genes encoding hydrolytic enzymes catalysing the same reaction. Sometimes those numbers are astoundingly high, like e.g. 18 chitinases (see Chapter 3) and 12 cellulases that can be found in the *H. jecorina* genome. Consequently the question arises, which growth conditions and carbon sources trigger their expression. It has already been shown for cellulases that their expression is induced by lactose, a carbon source which is structurally and chemically not directly linked to the educts and hydrolysis products of cellulases. This fact is

especially interesting for the industrial large scale production of such enzymes where fermentation on relatively cheap and water soluble carbon sources is desirable. While with large scale screening methods like DNA microarrays the transcription of many genes can be compared under a limited set of conditions, no tools exist to carry out a screening experiment which compares just a few genes but many different growth conditions. The work in **Chapter 4** presents such a screening system where enzymatic activities and also the transcription of single genes, using a reporter system, can be rapidly measured on 100 different carbon sources. The Biolog Phenotype Microarray (PM) system was adapted towards a high throughput system for screening of chitinase and NAGase induction by various carbon sources and the role of NAGases in spore germination and the initial phases of hyphal growth on different carbon sources studied.

Each of the following chapters consists of (1) a separate introduction to the specific topic, (2) materials and methods that were applied, (3) the obtained results and (4) a discussion of the presented results.

1

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

Abstract

Fungi counteract extracellular osmotic pressure by producing intracellular polyols to prevent loss of water. In yeast osmotic signalling involves a MAP-kinase pathway culminating at the STRE-binding transcription factors Msn2/4. We investigated the role of a putative STRE-binding orthologue of *Trichoderma atroviride*, Seb1, in osmotic stress signalling. *T. atroviride*, subjected to osmotic stress (10% glucose or glycerol, 1 M KCl or NaCl), responds by raising its intracellular glycerol level. In contrast to *Aspergillus nidulans*, no erythritol is accumulated. Accumulation of glycerol levels under osmotic stress is strongly reduced in a *seb1* deletion strain. To investigate glycerol biosynthesis in *T. atroviride*, the genes encoding glycerol dehydrogenase (*gld1*) and glycerol-3-phosphate dehydrogenase (*gfd1*) were cloned and characterized. Although both genes contain STRE-elements in their 5'-non coding regions, only *gld1* mRNA accumulates in response to osmotic stress, whereas expression of *gfd1* remains at a constitutive level. In comparison to *A. nidulans gld1* transcript levels in *T. atroviride* rise very slowly under conditions of salt stress. Deletion of *seb1* results in a delayed accumulation of the *gld1* transcript, but final levels match those in the wild-type whereas *gfd1* transcript accumulation remains unaffected. Assays for glycerol dehydrogenase and glycerol-3-phosphate dehydrogenase enzymatic activities reveal an increase of the former - whereas the latter remains mainly unaffected - in the wild-type and the Δ*seb1* strain under different kinds of osmotic stress. The data suggest that Seb1 is only involved in, but not essential for osmotic stress response which is in contrast to the yeast orthologues Msn2/4.

Seidl, V., Seiboth, B , Karaffa, L., 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(12): 1132-40.

Introduction

Free-living cells counteract extracellular osmotic pressure by producing intracellular, metabolically compatible solutes to prevent loss of water. In filamentous fungi the main up-regulated synthetic routes are those leading to the production of polyols such as glycerol, mannitol, arabinitol, erythritol and the disaccharide trehalose (Blomberg and Adler 1992). In the yeast *Saccharomyces cerevisiae* glycerol is the most predominant osmoresponsive polyol. It is synthesised from dihydroxyacetone phosphate via glycerol-3-phosphate by the sequential action of a cytosolic, NAD-dependent glycerol-3-phosphate dehydrogenase and a glycerol-3-phosphate phosphatase (Albertyn et al. 1994; Ansell et al. 1997; Pahlman et al. 2001). Two isoforms exist for both enzymes, of which only one (*GPD1* and *GPP2*) is induced under hyperosmotic conditions (Blomberg 2000). Glycerol dissimilation is mediated by a NADP-dependent glycerol dehydrogenase and a putative dihydroxyacetone kinase (Pahlman, et al., 2001).

In contrast, formation of glycerol under osmotic stress conditions in the filamentous fungus *A. nidulans* is mainly dependent on NADP-dependent glycerol dehydrogenase activity and not on glycerol-3-phosphate dehydrogenase activity, suggesting a different pathway where dihydroxyacetone phosphate is first converted to dihydroxyacetone, and only then to glycerol by a glycerol dehydrogenase (de Vries et al. 2003) (Fig.1).

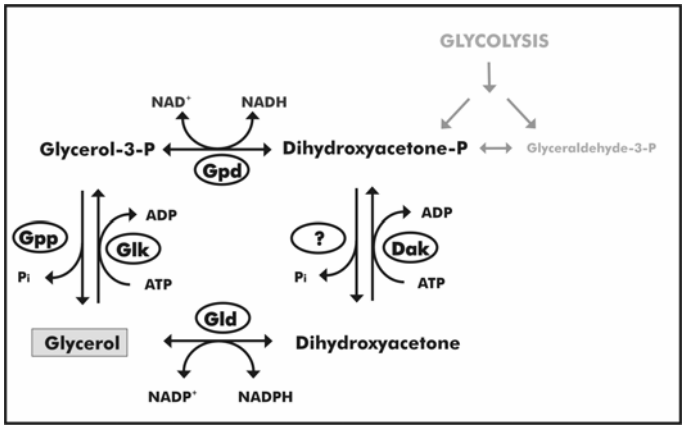

Fig 1. Pathways involved in glycerol metabolism in fungi. Gpd, glycerol-3-phosphate dehydrogenase (NAD^+); Gld, glycerol dehydrogenase ($NADP^+$); Gpp, glycerol-3-phosphate phosphatase; Glk, glycerol kinase; Dak, Dihydroxyacetone kinase.

Signal transduction during osmotic stress leading to glycerol accumulation has been studied in detail in yeast (for review see (Gustin et al. 1998): briefly, two plasma membrane localized

"osmosensors" (Sho1p and Sln1p) stimulate the osmotic response by causing the phosphorylation of Hog1p (Maeda et al. 1994; Maeda et al. 1995; Reiser et al. 1999a; Reiser et al. 1999b), which triggers its translocation into the nucleus where it phosphorylates an array of transcription factors, including Msn2 and Msn4 (Schmitt and McEntee 1996; Rep et al. 2000). The latter bind to the STRE-elements (AGGGG) found in many genes with stress-related functions (Schuller et al. 1994; Martinez-Pastor et al. 1996).

Understanding the High-Osmolarity Glycerol (HOG) pathway in filamentous fungi is only at the beginning: Han and Prade (2002) performed an *in silico* analysis of the HOG-pathway in *A. nidulans*, and found putative homologues of 13 of the 17 expected genes. The transcripts of the *in silico* reconstructed pathway genes were upregulated by salt stress in a *hogA*-dependent fashion, including the *MSN2/4* orthologue *msnA*. However, no direct evidence for the involvement of *msnA* in the osmotic response of *A. nidulans* was reported.

We have recently cloned a putative *Trichoderma atroviride* orthologue of the yeast *MSN2/4* and *A. nidulans msnA* genes, which was named *seb1* (stress element binding). Δ*seb1* strains exhibited severely reduced growth under a variety of stress conditions, including osmotic stress (Peterbauer et al. 2002b). However, as *seb1* cDNA was unable to complement an *msn2/4* mutant of *S. cerevisiae*, this raises the question whether *seb1* is indeed a functional homologue.

In the present paper, we therefore addressed the question whether *seb1* is involved in triggering glycerol biosynthesis under osmotic stress conditions in *T. atroviride.*

Materials and Methods

Strains

T. atroviride P1 (ATCC 74058) was used in this study and maintained on potato dextrose agar. The *amdS*$^+$ Δ*seb1*-mutant strain *T. atroviride* DF was kept on plates containing (in g/l): KH_2PO_4, 15; $Mg_2SO_4 \cdot 7H_2O$, 0.6; $CaCl_2 \cdot 2H_2O$, 0.6; $FeSO_4 \cdot 7H_2O$, 0.005; $ZnSO_4 \cdot 7H_2O$, 0.002; $MnSO_4 \cdot H_2O$, 0.002; acetamide, 0.6; glucose 20; Agar Noble (Difco) 20.

Escherichia coli strains ER1647 and BM25.8 (Novagen, Madison, WI) were used for genomic library screening and JM109 (Promega, Madison, WI) for plasmid propagation.

Cultivation conditions

For osmotic stress experiments *T. atroviride* was inoculated into 1 l Erlenmeyer flasks containing 250-ml of a medium (SM medium) consisting of (in g/l): KH_2PO_4, 2; $(NH_4)_2SO_4$, 1.4; $Mg_2SO_4 \cdot 7H_2O$, 0.3; $CaCl_2 \cdot 2H_2O$, 0.3; $FeSO_4 \cdot 7H_2O$, 0.005; $ZnSO_4 \cdot 7H_2O$, 0.002; $MnSO_4 \cdot H_2O$, 0.002, supplemented with 1 g/l peptone and 10 g/l either glucose or glycerol, and incubated on a rotary shaker (250 rpm) at 30°C for 30 h. The mycelia of 2 flasks were harvested by filtration, washed with sterile tap water, divided into three equal parts and transferred to three 500-ml Erlenmeyer flasks containing 200 ml of SM medium with 10 g/l of glucose or glycerol as carbon source. After 1 h of further incubation, osmotic stress was imposed by the addition of 35 ml of a 50% (w/v) solution of the corresponding carbon source or 50 ml of either 4 M KCl or NaCl in SM medium to give a final concentration of 10% (w/v) or 1 M, respectively.

Biomass determination

Mycelial dry weight was determined by withdrawing 2 x 10 ml aliquots from the cultures, suction filtration through a preweighted glass wool filter, followed by extensive washing with tap and distilled water and drying in an oven at 80°C until constant weight. The two data were averaged.

Cloning of the T. atroviride gfd1 and gld1 genes

An alignment of fungal glycerol-3-phosphate dehydrogenases from the NCBI database revealed the conserved aa-sequences EKDVQMWV and NGQKLQG to be potentially suitable for amplifying a corresponding fragment of *T. atroviride*. Consequently, the primers GPD-5' (5´-GAGAAGGATGTTCAGATGTGGGTT-3´) and GPD-3' (5´-ACCCTGCAGCTTCTGTCCGTT-3´) were deduced directly from the respective nucleotide sequence of a hypothetical protein (NCU00742.1) of the *Neurospora crassa* database (http://www-genome.wi.mit.edu/annotation/fungi/neurospora/), which shows high similarity to glycerol-3-phosphate dehydrogenases. *N. crassa* was chosen, because among the fungi for which sequence data were available it is phylogenetically closest to *Trichoderma*. A respective *gfd1* gene fragment was amplified by PCR, using 100 ng of *T. atroviride* P1 genomic DNA as template in a total volume of 50 µl containing 2.5 mM $MgCl_2$, 10 mM Tris-HCl pH 9.0, 50 mM KCl, 0.1% (v/v) Triton X-100, 0.4 µM of each primer, 0.2 mM of each dNTP and 0.5 units of *Taq*-Polymerase (Promega, Madison, WI). The amplification program consisted of: 1 min initial denaturation (94°C), 30 cycles of amplification (1 min 94°C, 1 min

54°C, 1 min 72°C), and a final extension period of 7 min at 72°C. The resulting 1 kb fragment was isolated and used to screen a genomic λ BlueSTAR library (Novagen, Madison, WI) of *T. atroviride* P1. Five positive phages were isolated and converted to plasmids according to the manufacturer's protocol. 2.2 kb of the corresponding *gfd1* gene were sequenced at MWG Biotech AG (Ebersberg, Germany), including 760 bp upstream and 60 bp downstream of the coding region.

Essentially the same procedure was carried out to clone *gld1*: NCBI Blast search with the *gldB* gene from *A. nidulans* (de Vries et al. 2003) as query identified the aa-sequences HLDCAWF and MKDTFGY to be absolutely conserved. Consequently, primers gldB5' (5´-CCTCGACTGCGCTTGGTT-3') and gldB3' (5'-AGCCAAAGGTGTCCTTCA-3') were designed according to the nucleotide sequence of a hypothetical protein (NCU04923.1) of the *N. crassa* database with highest similarity to *A. nidulans* glycerol dehydrogenase. To amplify a 750 bp fragment of the *T. atroviride gld1* gene by PCR, the same conditions as for the *gfd1* gene were used. A positive phage clone, identified with the previously amplified 750 bp *gld1*-fragment, was converted to a plasmid and 1.9 kb of the *gld1* gene including 624 bp upstream and 291 bp downstream of the coding region were sequenced.

The assembled DNA sequences were deposited in GenBank (*gfd1*: accession no. AY370658; *gld1*: no. AY484570).

Sequence analysis

Sequences were analyzed using BLAST programs (Altschul et al. 1990) and multiple sequence alignment was done by MultAlin (Corpet 1988), Clustal X 1.81 (Thompson et al. 1997) and Genedoc 2.6 (Nicholas et al. 1997). The protein sequences were analyzed with InterProScan (http://www.ebi.ac.uk/InterProScan/).

RNA isolation and hybridization

Fungal mycelia were harvested by filtration through Miracloth (Calbiochem), washed with cold tap water, squeezed between two sheets of Whatman filter paper, shock frozen and ground in liquid nitrogen. Total RNA was extracted as described previously (Chomczynski and Sacchi 1987). Standard methods (Sambrook 2001) were used for electrophoresis, blotting and hybridization of nucleic acids.

The 1 kb *gfd1* and the 750 bp *gld1* PCR fragments were used as probes for northern hybridizations, and an rDNA fragment hybridizing with the 18S and 28S rRNA was used as loading control. Relative abundance of transcripts was determined by densitometric

measurements of autoradiographs derived from different exposure times (only values with linear correlation [r>0.9] were used) with the Quantitiy One 1-D Analysis Software (Bio-Rad, Munich, Germany). The values are integrated peaks and were corrected by local background subtraction.

Preparation of cell-free extracts and enzyme assays

The mycelia were harvested by filtration through Miracloth (Calbiochem), washed with cold tap water, squeezed dry between two sheets of Whatman filter paper, and ground to a fine powder under liquid nitrogen. 1 g of the powder was then suspended in 3 ml of 0.1 M Tris-HCl, pH 7.5 (including 1 mM EDTA and 5 mM β-mercaptoethanol), and sonicated 15 times for 20 sec at 2°C. The resulting homogenate was centrifuged at 10000 × g for 20 min at 4°C, and the supernatant with a protein content between 8 and 15 mg/ml was used as a cell-free extract.

Glycerol-3-phosphate dehydrogenase activity was assayed in the presence of 50 mM triethanolamine buffer, pH 7.4, containing 0.2 mM NADH and 2 mM dihydroxyacetonephosphate (DHAP). Glycerol dehydrogenase activity was determined in the presence of 100 mM glycine buffer, pH 9.6, containing 0.5 mM $NADP^+$ and 100 mM glycerol. Activities are expressed as units (U), one unit (1 U) corresponding to the conversion of 1 µmol of substrate per min, and given as specific activities (U/mg protein).

Protein concentrations in cell extracts were determined with the Bio-Rad Protein Assay (Bio-Rad Laboratories, Munich, Germany) according to the supplier's instructions, using BSA as a standard.

Extraction and quantification of mycelial polyols

Intracellular polyols were extracted by withdrawing 2 x 5 ml aliquots from the cultures, gently filtering them through a precooled linen cloth, and resuspending the mycelia in 2 ml of distilled water. These suspensions were shock-frozen and kept for 1 h at -75°C. Thereafter, they were boiled (20 sec), and homogenized by means of a Potter-Elvehjem glass homogenizer. The homogenate was spun down at 8500 x *g* (5 min, room temperature), and the supernatant then directly assayed for the presence of polyols by HPLC using an H^+-exchange column (Merck Polyspher OA KC) at 30°C temperature with 5 mM H_2SO_4 as a mobile phase with isocratic elution (0.4 ml/min) and refractive index detection. Under these conditions, retention times of trehalose, erythritol, mannitol and glycerol were 12.4, 15.2, 16.8 and 22.4 minutes, respectively. Intracellular concentrations were calculated by assuming 2.43

ml of intracellular volume per g dry mycelial weight (Slayman 1964). Two data sets were averaged.

Results

Glycerol is the major solute accumulated under hyperosmotic conditions

As the role of polyols and trehalose as potential osmotic protectants has not yet been documented in *Trichoderma* spp., we first investigated the changes in the composition of the polyol and trehalose pools of mycelia grown on glucose (1%, w/v) after transfer to a new medium containing either 10% (w/v) glucose, or 1% (w/v) glucose plus 1 M KCl or 1 M NaCl. Fig. 2a shows that even under non stressed control conditions glycerol accounted for the major portion of the polyol pool, and rose to concentrations of 650-770 mM under conditions of osmotic stress. In contrast, such a drastic change after transfer to osmotic stress conditions could not be observed for mannitol and the disaccharide trehalose. Erythritol, which accumulates to high amounts under similar osmotic stress conditions in *A. nidulans* (Beever and Laracy 1986; de Vries et al. 2003), remained below the detection limit of our analysis (< 1 mM).

The accumulation of glycerol appeared to require a glycolytic carbon catabolism, because the intracellular concentration of glycerol was below the detection limit during growth on 1% L-arabinose or D-xylose, which are catabolized by the pentose-phosphate pathway. There was no detectable glycerol accumulation upon subjection to osmotic stress by 10% (w/v) L-arabinose or D-xylose or 1 M KCl or NaCl (L. Karaffa, unpublished data). Instead, transfer to 10% (w/v) L-arabinose or D-xylose increased the intracellular concentrations of L-arabinitol and xylitol to 388 mM and 490 mM, respectively. This accumulation required the presence of an excess of these two pentoses, because transfer of L-arabinose and D-xylose grown mycelia to 1% (w/v) of either of these carbon sources plus 1 M KCl or NaCl did not lead to a rise of either arabinitol or xylitol levels. On the other hand, glycerol was also the major solute that accumulated under hyperosmotic conditions on glycerol as carbon source (Fig. 2b); although its concentration remained lower than on glucose.

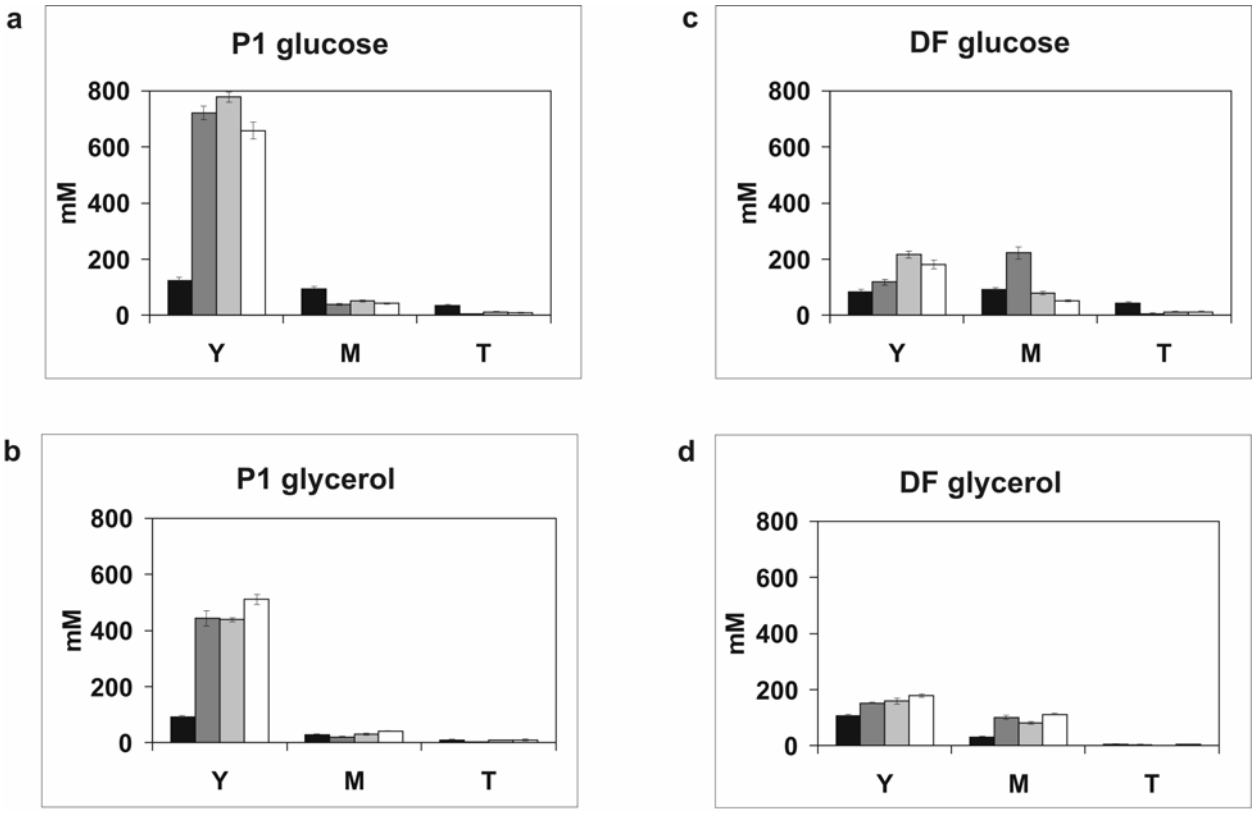

Fig. 2. Polyol concentrations of *T. atroviride* wild-type strain P1 and the Δ*seb1* strain DF under different growth conditions. Samples were taken after 4h of replacement in the corresponding media. (a) *T. atroviride* P1, glucose; (b) *T. atroviride* P1, glycerol; (c) *T. atroviride* DF, glucose; (d) *T. atroviride* DF, glycerol. ■ 1% (w/v, control), ■ 10% (w/v) carbon source, ■ 1% (w/v carbon source) + 1M KCl and □ 1% (w/v) carbon source + 1M NaCl. Y = glycerol, M = mannitol, T = trehalose. Erythritol was below the detection limit throughout and is thus not shown.

Seb1 is necessary for high level accumulation of glycerol under conditions of osmotic stress

Having confirmed that glycerol accumulates in *T. atroviride* under conditions of osmotic stress, we investigated whether the putative Msn2/4 orthologue Seb1 would be necessary for this accumulation. Therefore, the experiments described above were repeated with a *seb1*-knock out strain *T. atroviride* DF (Peterbauer et al. 2002b). The data (Fig. 2c and d) show that the accumulation of glycerol under stress conditions was indeed strongly reduced but not completely eliminated. This indicates that Seb1 is involved but not essential for glycerol accumulation in *T. atroviride*. Interestingly, the Δ*seb1* strain accumulated high concentrations of mannitol when transferred to 10% (w/v) glucose, but not during salt stress on cultures growing on 1% (w/v) glucose.

***Cloning of the* gld1 *(glycerol dehydrogenase) and* gfd1 *(glycerol-3-phosphate dehydrogenase) genes of* T. atroviride**

In order to find out whether the observed accumulation of glycerol under osmotic stress is due to a transcriptional regulation of the expression of genes involved in its metabolism, we cloned the genes encoding the glycerol dehydrogenase (*gld1*) and glycerol-3-phosphate dehydrogenase (*gfd1*). We should note that we cannot rule out at this stage that *T. atroviride* may contain multiple forms of these two genes, but only single genes are present in the genome databases of *N. crassa* and *T. reesei* (unpublished data) and we obtained only single PCR products with the primers designed.

gld1 is an intronless gene encoding a 327-aa protein with a calculated molecular mass of 36.7 kDa. InterProScan analysis identified the Gld1 as a member of the aldo-keto reductase family of NADPH-dependent oxidoreductases (Bohren et al. 1989; Bruce et al. 1994), which is also reflected in the high number of conserved amino acids with the orthologue from *N. crassa* (XP_324280; 78% aa-identity), *A. oryzae* (BAC56099; 74% aa-identity) and GldB from *A. nidulans* (CAD42649; 70% aa-identity) identified in a protein-protein BLAST search.

The coding region of *gfd1* consists of 1284 bp, interrupted by a 63 bp intron. *Gfd1* encodes a 427-aa protein with a calculated molecular mass of 46.4 kDa. Gfd1 has highest similarity to the glycerol-3-phosphate dehydrogenase from *Glomerella cingulata* (AY331190; 80% aa-identity) but significant aa-sequence identity throughout the sequence is observed with all other fungal glycerol-3-phosphate dehydrogenases. Interestingly, the fungal Gfd1 proteins contain two short aa stretches ($aa_{167\text{-}185}$ and $aa_{310\text{-}361}$ in the *T. atroviride* Gfd1), which are lacking in yeast. Whereas the aa-sequence of these two stretches is poorly conserved among fungi, the latter one is particularly rich in P, S, T and basic amino acids.

Analysis of the upstream regions of the two genes, identified 2 STRE-elements (AGGGG) in both genes, located at positions –151 and –476 in *gld1* and at –410 and –20 in *gfd1* relative to their ATG, which could possibly act as binding sites for Seb1 (Peterbauer et al. 2002b).

gld1 *but not* gfd1 *is upregulated under conditions of osmotic stress*

We now investigated whether the transcriptional regulation of the two glycerol-biosynthetic genes *gld1* and *gfd1* is influenced by osmotic stress. As shown in Fig. 3a and b, this is the case with *gld1* but not *gfd1*: whereas the latter was transcribed at a basal rate at all conditions studied, *gld1* was hardly detectable under control conditions, but responded strongly to osmotic stress induced by carbon sources or salts, irrespective of whether glucose or glycerol

was used as a carbon source. However, a notable difference between the response to high concentrations of carbon sources and salts was the late response to salt induced stress; in fact, whereas the *gld1* transcript was detectable already 10 min after transfer to media with 10% (w/v) carbon source, it was only detectable 2 hrs after transfer to 1 M NaCl and KCl. Also, a slower response of *gld1* was noted during glycerol- than glucose-induced stress, and the *gld1* transcript was less abundant when *T. atroviride* was grown on glycerol than on glucose during salt induced stress.

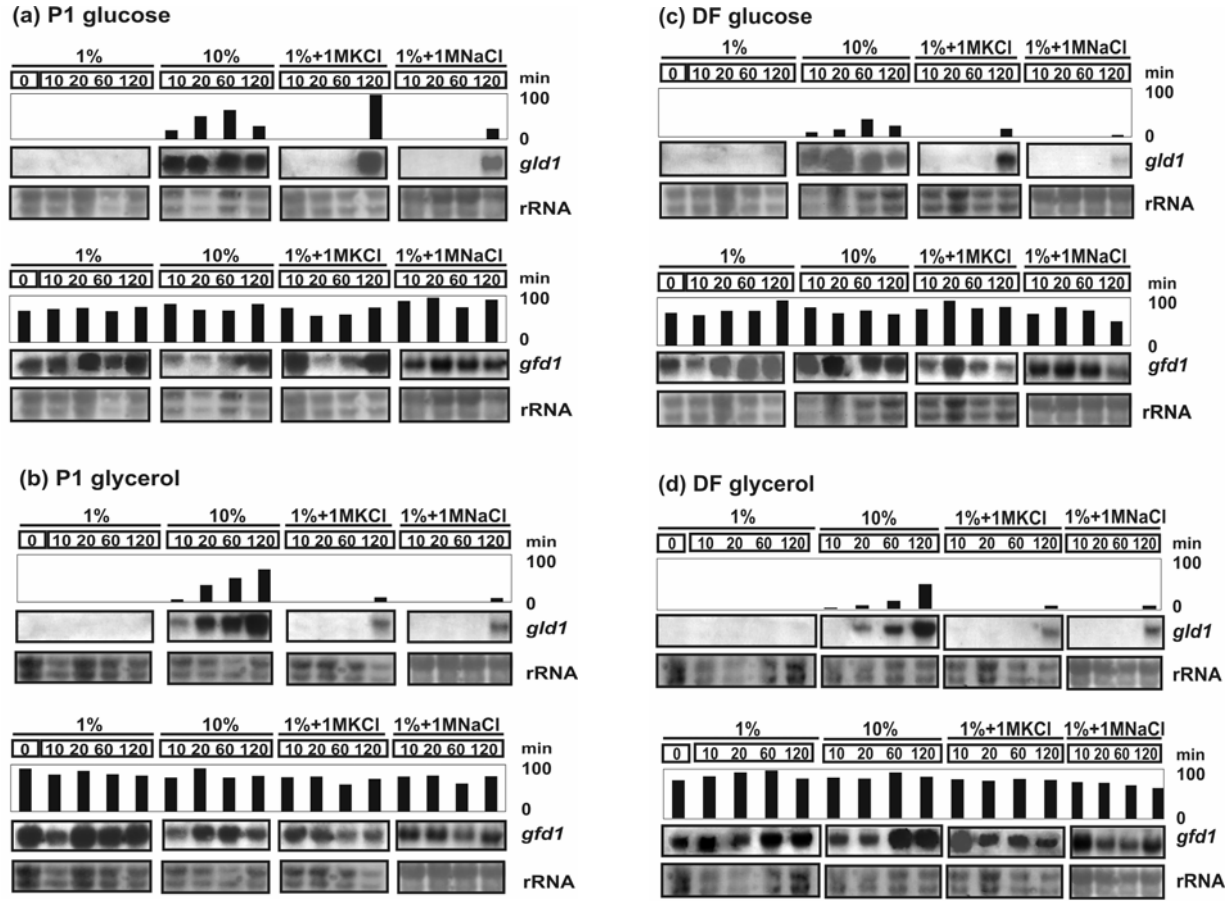

Fig 3: Northern analysis of *gld1* and *gfd1* expression in relation to osmotic stress in *T. atroviride* strains P1 and DF. Mycelia were exposed to different osmotic stress conditions (10%, w/v, glucose; 1% w/v glucose + 1M KCl, 1% w/v glucose + 1M NaCl and 1% w/v glucose as control). In panels B and D, 1% (w/v) glucose is replaced by 1% (w/v) glycerol. Samples were taken after 10, 20, 60 and 120 min, respectively after transfer of the mycelium. The 18S and 28S rRNA transcripts were used as a loading control. The bars above the RNA tracks represent the corresponding densitrometric scanning of the *gld1* and *gfd1* mRNA signal, normalized to that of the sum of both rRNAs. The values are shown relative to the highest value. (a) *T. atroviride* P1, glucose; (b) *T. atroviride* P1, glycerol; (c) *T. atroviride* DF, glucose; (d) *T. atroviride* DF, glycerol.

Seb1 partially impairs the upregulation of* gld1 *transcript

Having identified *gld1* as a glycerol biosynthetic gene responding to osmotic stress in *T. atroviride*, we studied whether this response is Seb1-dependent. Therefore, the experiments were repeated with the *T. atroviride* Δ*seb1* mutant DF (Fig. 3c and d). The results of this northern analysis showed that the general pattern of *gfd1* and *gld1* transcript accumulation in strain DF was similar to the wild-type strain. However, quantitative differences between the mutant and the wild-type were noted for *gld1*: in strain DF its transcript accumulated at a slower rate upon transfer to osmotic stress and also the abundance of the transcript appearing late upon salt stress in cultures with glucose as carbon source was lower. From this we conclude that *seb1* is involved in but not essential for the response of *gld1* gene expression to osmotic stress.

Mycelial glycerol dehydrogenase and glycerol-3-phosphate dehydrogenase activities correlate with the corresponding transcript patterns

In order to confirm that the observed patterns of expression of *gld1* and *gfd1* are also reflected in corresponding changes of the activities of the respective enzymes, activities of NADP-dependent glycerol dehydrogenase and NAD-dependent glycerol-3-phosphate dehydrogenase were assayed (Table 1). Under non-stressed conditions the activity of glycerol-3-phosphate dehydrogenase was 2 - 4 mU/mg in the wild-type and 8 - 10 mU/mg in the Δ*seb1*-mutant strain DF. Exposure to different osmotic stress conditions led to no or only a slight increase of glycerol-3-phosphate dehydrogenase activity in the wild-tpye. In strain DF also no change or even a slight decrease of glycerol-3-phosphate dehydrogenase activity could be observed under osmotic stress. A low constitutive activity was detected for glycerol dehydrogenase (3-6 mU/mg) during growth of the *T. atroviride* P1 and DF under non-stressed conditions, which was elevated 3 - 6 fold upon subjection to osmotic stress. In the mutant DF, exposure to glycerol stress resulted in even higher enhancement of glycerol dehydrogenase activity, whereas that on 1 M NaCl and KCl was somewhat decreased. Thus, although the overall changes of the enzyme activities - a strong increase of glycerol dehydrogenase activity and no significant change of glycerol-3-phosphate dehydrogenase activity under different kinds of osmotic stress - are in accordance with the results from northern analysis, the individual ratios of activities and mRNA abundances do not absolutely correlate, which is probably due to different half-lives of either the respective mRNAs or enzymes under the different conditions.

Discussion

The physiological response of fungi towards hyperosmotic stress has mainly been studied in *S. cerevisiae, Zygosaccharomyces rouxii*, *Debaryomyces hansenii* (for review see (Blomberg 2000)) and *A. nidulans* (Beever and Laracy 1986; Redkar et al. 1995; Fillinger et al. 2001; de Vries et al. 2003). These studies revealed significant differences between *A. nidulans* and yeasts, both with respect to the solutes accumulated as well as to the pathways involved in their synthesis. Since the main emphasis of the present study was to test whether the putative fungal Msn2/4 homologue Seb1 is a stress responsive transcriptional regulator, it was therefore essential to establish first whether the stress response in *T. atroviride* resembles that in *A. nidulans* or that in yeast. The results document that the overall response is similar to that of *A. nidulans*, but also points at some important differences which may indicate essential physiological differences between these two fungi.

The first of these differences was the composition of the polyol pool under normal (glucose-grown) and hyperosmotic stressed conditions: in *A. nidulans*, mannitol is the predominant polyol during normal, unstressed growth (de Vries 2003). Glycerol, erythritol and arabinitol levels are elevated during osmotic stress, with a concurrent decrease of the mannitol concentration. In contrast, in *T. atroviride* only glycerol and mannitol were detected during normal growth, and only the concentration of glycerol increased strongly upon osmotic stress. In further contrast to *A. nidulans*, *T. atroviride* accumulated slightly higher concentrations of glycerol than of mannitol even under non-stressed conditions. This may indicate a general role of glycerol in the physiology of *T. atroviride*, which is not related to osmotic stress and which is absent in *A. nidulans*. One such role may be deduced from the fact that *T. atroviride* - in contrast to *A. nidulans* - is a mycoparasite, which attacks and penetrates host fungi by means of appressoria-like structures (Inbar and Chet 1995). De Jong et al. (de Jong et al. 1997) showed that in *Magnaporthe grisea* glycerol – derived from triglyceride catabolism - generates the turgor of the appressorium. If this is also valid for *T. atroviride*, we reason that it would be advantageous for the fungus if there is already a high preformed glycerol pool which can be expanded.

In the Δ*seb1* strain, where the accumulation of glycerol was impaired, we observed increased mannitol concentrations which is probably accumulated to compensate for the loss of osmotic protection by glycerol. Mannitol has been implied to be involved in protecting conidiospores against various forms of stress in *A. niger* (Ruijter et al. 2003), but has not yet been demonstrated to play a role in osmotic protection of vegetative mycelia. Interestingly,

the highest mannitol levels in *T. atroviride* were observed in glucose grown mycelia, and thus its accumulation in the presence of high glucose concentrations could as well be a mere metabolic response only. Mannitol is an NADH-sink (its formation requires NAD-dependent mannitol-1-phosphate dehydrogenase; (Ruijter et al. 2003)) and a switch from glycerol to mannitol accumulation thus requires a significant shift in carbon fluxes from the pentose phosphate to the glycolytic pathway. This fact also explains why a glycolytic carbon source results in highest mannitol accumulation.

Another interesting difference between *T. atroviride* and *A. nidulans* is the strongly delayed response of *gld1* transcription under conditions of osmotic salt stress, irrespectively of whether NaCl or KCl were used. It is unlikely that this is due to different cultivation conditions, because the kinetics of *gld1* transcript accumulation upon glucose stress were the same as in *A. nidulans*. One explanation for this could be that the high salt sensor of *T. atroviride* is adaptive, and thus the transmission of the stimulus is delayed. Alternatively, *T. atroviride* may compensate an external high salt concentration for some time by first mobilizing its internal salt concentration stored in the vacuole. It is also possible that the different kinetics of response to osmotic stress by salts and carbon source are due to the fact that the latter also act as an energy source, thereby enabling the mycelia to respond more rapidly. Finally, high salt concentrations may be more toxic because of their potential interference with ion gradients. Although we did not aim to find an answer to that in this paper, these data nevertheless show that the kinetics of the high salt response of different fungi may be significantly different, which should be kept in mind when performing similar studies with other fungi.

This study reveals that a loss of function of the putative Msn2/4 orthologue Seb1 decreases glycerol accumulation and delays the response to osmotic stress, but is not essential for either of them. Although the accumulation of glycerol in the Δ*seb1* strain is reduced to roughly 25-30% of the concentration of the wild-type strain, accumulation of the *gld1* mRNA is only delayed and the activity of its translation product glycerol dehydrogenase is even increased on 10% carbon source. Seb1 is therefore unlikely a functional homologue of Msn2/4. Its regulatory action is apparently rather focused on different targets in *T. atroviride* and in *S. cerevisiae*. This conclusion also concurs with our earlier findings that *seb1* is unable to complement a yeast *msn2/4* mutant (Peterbauer et al. 2002b). The results from this study therefore cast doubt on a role of Seb1 in fungal osmotic stress response solely on the basis of its increased transcript accumulation in *A. nidulans* under osmotic stress conditions, as proposed by Han and Prade (2002).

This conclusion is further supported by the findings that both *gld1* as well as *gfd1* contain consensus sequences for binding of Seb1 in their 5'-upstream sequences, but only *gld1* actually responds to osmotic stress. Obviously, the mere appearance of the STRE motif in a promoter does not implicate that the corresponding gene is indeed regulated by stress and/or Seb1. In fact, we have found the STRE-consensus sequence in upstream sequences of genes involved in cellulose hydrolysis (Kubicek and Penttilä 1998) and chitin hydrolysis (Lorito et al. 1996b), of which none was upregulated under stress conditions ((Peterbauer et al. 2002b); C.P. Kubicek, unpublished data). Also, a gene encoding a protein with high similarity to Seb1 was cloned from *Haematonectria haematococca* (GenBank accession no. AAB04132), which encodes a transcription factor putatively involved in the basal regulation of a cutinase gene (Kamper et al. 1994). We therefore speculate that the fungal transcription factor Seb1 has an additive but non-essential role in the enhancement of the transcription of several genes in response to different stimuli. This assumption would be consistent with our data that Seb1-loss only partially affects glycerol accumulation under osmotic stress.

2

Antagonism of *Pythium* Blight of Zucchini by *Hypocrea jecorina* Does Not Require Cellulase Gene Expression But Is Improved by Carbon Catabolite Derepression

Abstract

Towards a better understanding of the biochemical events that lead to biocontrol of plant pathogenic fungi by *Hypocrea/Trichoderma* spp., we investigated the importance of carbon catabolite (de)repression and cellulase formation in the antagonization of *Pythium ultimum* by *Hypocrea jecorina* (*Trichoderma reesei*) on agar plates and *in planta*. *H. jecorina* QM9414 could antagonize and overgrow *Pythium ultimum* but not *Rhizoctonia solani* in plate confrontation tests, and provided significant protection of zucchini plants against *P. ultimum* blight *in planta*. A carbon catabolite derepressed *cre1* mutant of *H. jecorina* antagonized *P. ultimum* on plates more actively and increased the survival rates of *P. ultimum*-inoculated zucchini plants in comparison to strain QM9414. A *H. jecorina* mutant impaired in cellulase induction could also antagonize *P. ultimum* on plates and provided the same level of protection of zucchini plants against *P. ultimum* as strain QM9414 did. We conclude that cellulase formation is dispensable for biocontrol of *P. ultimum*, whereas carbon catabolite derepression increases the antagonistic ability by apparently acting on other target genes.

Seidl, V., Schmoll, M., Scherm, B., Balmas, V., Seiboth, B., Migheli, Q. and Kubicek, C.P. (2006). Antagonism of *Pythium* blight of zucchini by *Hypocrea jecorina* does not require cellulase gene expression but is improved by carbon catabolite derepression. FEMS Microbiol Lett. MS in press.

Introduction

Biocontrol of plant pathogens is an attractive alternative to the strong dependence of modern agriculture on fungicides, which results in environmental pollution and the selection of resistant strains. Replacement or reduction of chemical application has been achieved through use of biologically based pesticides, a concept included in the definition of biocontrol proposed by Cook and Baker (1983): "Biological control is the reduction of the amount of inoculum or disease-producing activity of a pathogen accomplished by or through one or more organisms other than man." Some mycoparasitic *Hypocrea/Trichoderma* species, primarily *H.lixii* (*T. harzianum*) and *H. atroviridis* (*T. atroviride*), have been used as biocontrol agents against several economically important plant pathogenic fungi (Harman et al. 2004). Unfortunately, the successful application of biocontrol strains is not easy to predict, and these strains are usually not economically competitive with chemical fungicides. A better understanding of the biochemical events that lead to mycoparasitism and their regulation could identify methods to improve the reliability of *Hypocrea/Trichoderma* strains as biocontrol agents (Roderick and Navajas 2003).

The majority of the molecular research on biocontrol has so far been focused on the role of hydrolytic enzymes in antagonism of plant pathogenic fungi (for reviews see Chet et al. 1998; Kubicek et al. 2001; Howell 2003; Benitez et al. 2004). However, these studies dealt only with the effect of single enzymes/genes, which - in view of the high number of hydrolytic enzymes known to be encoded in the genome of *Hypocrea jecorina* (*T. reesei*; http://gsphere.lanl.gov/trire1/trire1.home.html) and other filamentous fungi (*Magnaporthe grisea, Fusarium graminearum* etc.; http://www.broad.mit.edu/annotation/fungi/) - only leads to limited insights into the general role of these enzymes. In addition, many of these hydrolases are controlled by carbon catabolite repression. *In vitro* studies with *H. atroviridis* suggested that the onset of mycoparasitism is indeed accompanied by carbon catabolite derepression (Lorito et al. 1996b), but this has so far not been proven *in vivo*. One of the obstacles towards investigating these points is that the *Hypocrea/Trichoderma* species and strains used in biocontrol studies are difficult to manipulate genetically (Rocha-Ramirez et al. 2002; Delgado-Jarana et al. 2003). In addition, creating mutants deleted in 10 - 20 different hydrolase genes is a demanding task. In contrast, well characterized mutants defective in the formation of all cellulases (Torigoi et al. 1996; Zeilinger et al. 2000) and characterized mutants defective in carbon catabolite repression (Strauss et al. 1995; Ilmen et al. 1996) are already available for *H. jecorina*.

H. jecorina is widely used for the production of cellulolytic and hemicellulolytic enzymes and recombinant proteins (Archer and Peberdy 1997; Mach and Zeilinger 2003). While *H. jecorina* has not been evaluated for its ability to antagonize other fungi, *T. longibrachiatum,* a species closely related to *H. jecorina*, can successfully protect cucumber plants against *Pythium ultimum* (Migheli et al. 1998).

The objective of this study was (i) to test whether *H. jecorina* can be used to antagonize plant pathogenic fungi and protect plants against their attack; and (ii) to make use of available *H. jecorina* mutants to address issues that have been reported as being potentially relevant for antagonism of other fungi (Lorito et al. 1996b; Kubicek et al. 2001; Howell 2003; Roderick and Navajas 2003; Harman et al. 2004): how important is the formation of hydrolytic enzymes for antagonism of plant pathogenic fungi? And would carbon catabolite derepression be beneficial for it? Answers to these questions will expand our understanding of the mechanism of biocontrol and eventually provide new strategies towards improvement of existing biocontrol strains of *Hypocrea/Trichoderma*.

Materials and methods

Strains and culture conditions

H. jecorina QM9414 (ATCC 26921) is a moderate cellulase producing second-generation mutant of the wild-type strain QM6a; *H. jecorina* QM9978 (obtained from K. O'Donnell, US Department of Agriculture, Peoria, IL) is a mutant strain unable to produce cellulases (Zeilinger et al. 2000); *H. jecorina* RUT-C30 (ATCC 56765) is a carbon catabolite derepressed strain because of a truncation of its *cre1* gene (Ilmen et al. 1996); *H. atroviridis* P1 (ATCC 74058) is a strain used in biocontrol (Lorito et al. 1996b); *Pythium ultimum* (Woo et al. 1999) and *Rhizoctonia solani* (strain RT-10, belonging to anastomosis group AG-4, kindly provided by Dr. E. Lahoz). All strains were maintained on malt extract agar (MEX; 3% w/v).

Plate confrontation tests

Strips of 3 mm × 3 cm were cut from the growing front of *H. jecorina* and *H. atroviridis*, and *P. ultimum* and *R. solani* on MEX plates, respectively, and placed on fresh plates at a distance of 4 cm from each other. Both, MEX and minimal medium (MM; g l^{-1}: agar, 10; glucose, 3; $MgSO_4 \cdot 7H_2O$, 0.2; K_2HPO_4, 0.9; KCl, 0.2; NH_4NO_3, 1; $FeSO_4 \cdot 7H_2O$, 0.005; $ZnSO_4 \cdot 7H_2O$,

0.002; $MnSO_4 \cdot H_2O$, 0.002; and $CoCl_2 \cdot 2H_2O$, 0.002) were used; plate confrontation assays were performed in the dark at 28 °C.

RNA isolation and Northern analysis

For RNA extraction from confrontation experiments, plates were covered with a dialysis membrane (cut-off size 12 kDa, Sigma, Vienna, Austria) and mycelia in the area of interaction or close to it harvested with a spatula, immersed in liquid N_2 and ground to a fine powder. Equivalent zones were collected from control plates, inoculated with either *H. jecorina* or *P. ultimum*.

Total RNA was isolated by the guanidinium thiocyanate method (Chomczynski and Sacchi 1987). Following electrophoretic separation on a 1.2% agarose-gel containing 2.2 M formaldehyde in 1× MOPS buffer (40 mM MOPS, 1 mM EDTA, pH 7.0), RNA was blotted onto nylon membranes (Biodyne B, Pall Corporation, VWR International, Vienna, Austria) and hybridized in 50% formamide, 10% dextransulfate, 0.5% SDS, 5× Denhardt's solution and 125 μg ml^{-1} denatured fish sperm DNA at 42 °C for 20 h. Washing was performed with 2× SSC + 0.1% SDS at 42 °C (2 × 10 min).

A 1264 bp PCR fragment of *cbh1* (Shoemaker et al. 1983) was used as probe for Northern hybridizations, and a 297 bp PCR fragment of 18S rDNA (Accession number: Z48932) was used as the hybridization control.

Greenhouse experiments

Three experiments were carried out on zucchini (*Cucurbita pepo* L.) seedlings (F1 hybrid cultivar Greyzini; Zorzi Seeds, Italy). Using a sterile cork borer, plugs of 15 mm in diameter were cut from 7-day-old cultures of *P. ultimum* or *R. solani* grown at 25 °C on Schmitthenner's Agar (Atlas 1997) and potato dextrose agar (PDA, Difco, Becton Dickinson, Franklin Lakes, NJ), respectively. They were then placed in the center of plastic sowing pots (4.5 cm in diameter, 55 ml capacity, one plug per pot), and covered by a 2-cm layer of sterilized (121 °C for 60 minutes on two successive days) potting mix (Humin-Substrat N17, Neuhaus, Germany). Agar plugs of *H. jecorina* or *H. atroviridis* were cut from 7-day-old cultures grown at 25 °C on PDA. One plug of *H. jecorina* or *H. atroviridis* was paired with one plug of *P. ultimum* or *R. solani*, by placing the mycelia in direct contact. Plug pairs were incubated in the dark at 25 °C for 24 h and then transferred to the center of plastic pots and covered by sterilized substrate as described. For each treatment, five replicates (10 seeds for each replicate) were incubated on a bench in a glasshouse for 7 days before zucchini seeds

(one seed per pot) were added. Pots were watered daily and the average temperature was 25-20 °C (min 10-15 °C, max 25-35 °C). Seedling emergence was checked weekly after 7-21 days. After the last survey, the healthy plant stand was assessed.

Statistical Analysis

Data from greenhouse experiments were analyzed using the analysis of variance (ANOVA). ANOVA was conducted after transforming the original data (expressed as percentage, %) using the $arcsin\sqrt{x}$ function, where x is the relative proportion. This transformation is appropriate to percentage and useful when original data do not fall between 30 and 70% (Sokal and Rohlf 1995). Means separation was done by the Tukey-Kramer honestly significant difference (HSD; (Sokal and Rohlf 1995)) test. All the analyses were performed by using J.M.P. ver. 3.1.5 software (SAS Institute Inc. Minneapolis, MN, USA).

Results

***Plate confrontation experiments between* H. jecorina *and* P. ultimum *or* R. solani**

H. jecorina QM9414 antagonized *P. ultimum* (Fig. 1a). Although *P. ultimum* initially grew faster than *H. jecorina*, its growth stopped immediately upon physical contact with *H. jecorina*, which began to overgrow *P. ultimum* after approximately two days of incubation. Overgrowth was completed 7-9 days after inoculation and resulted in complete degradation of the host and sporulation of *H. jecorina* over the entire plate. The characteristic stages of the plate confrontation experiments were observed with fungi cultured on both, rich (MEX) and minimal medium (MM). *H. jecorina* also exhibited the characteristic coiling around the host hyphae (Fig. 2) that has been implicated in the recognition and subsequent penetration of the host (Inbar and Chet 1996).

H. jecorina did not antagonize *R. solani*, as no overgrowth occurred after physical contact between *H. jecorina* and *R. solani* (Fig. 1 b). *H. jecorina* sporulated only on its half of the plate and the characteristic brown pigments of *R. solani* appeared only in the other half.

Protection of zucchini plants against* P. ultimum *and* R. solani *blight

Although *H. jecorina* has an effective cellulase system, it causes no negative effect on the plant in the absence of pathogens (Table 1). In the presence of *P. ultimum*, *H. jecorina* QM9414 significantly antagonized the pathogen *P. ultimum*, the healthy plant stand being increased from 39 to 55% (Table 1). Plant damage caused by *R. solani* was not reduced

significantly by *H. jecorina*, and the recovery of healthy plants in the presence of *H. jecorina* was only 13% in the presence of a high level of disease pressure (healthy plant stand in the inoculated control: 6%; Table 1).

The biocontrol fungus *H. atroviridis* P1 was included as an internal control. This strain was able to effectively control both *P. ultimum* and *R. solani*, allowing a significant improvement of the healthy plant stand from 39 and 6% to 84 and 78%, respectively (Table 1).

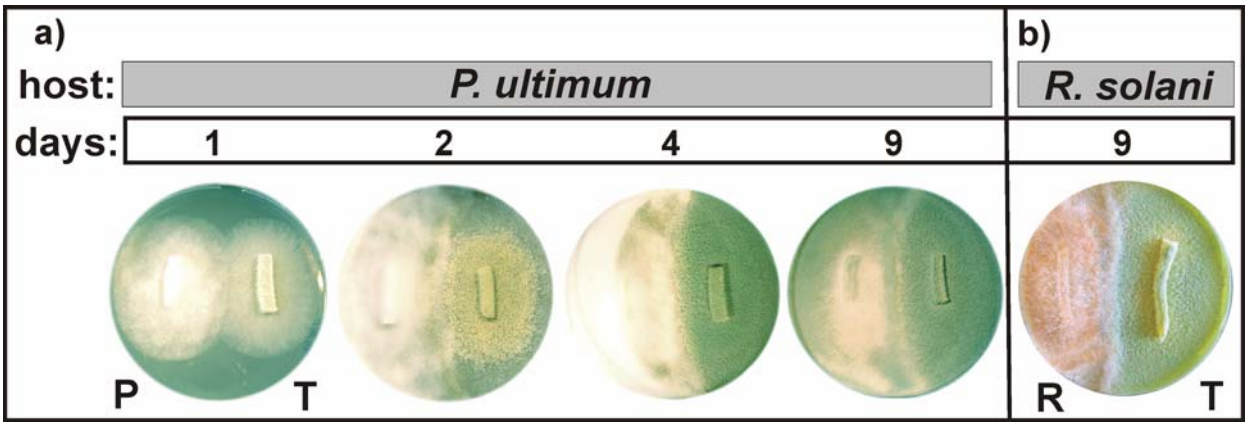

Fig 1. (a) Plate confrontation tests of *H. jecorina* QM9414 (T) against *P. ultimum* (P) performed on MEX medium. (b) Plate confrontation tests of *H. jecorina* QM9414 (T) against *R. solani* (R).

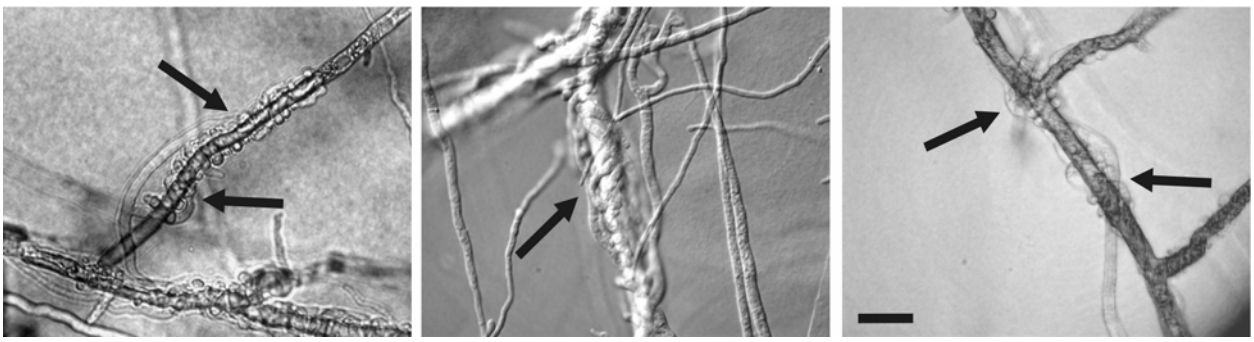

Fig 2. LM-micrographs of *H. jecorina* QM9414 coiling around *P. ultimum*. The pictures shown were taken from plate confrontation assays on MM medium after 3 days. Arrows point to interaction zones between *H. jecorina* and *P. ultimum* hyphae. The bar marker indicates 10µm (same scale on all pictures).

Table 1. Effect of *H. jecorina* QM9414, RUT-C30 and QM9978 and *H. atroviridis* P1 on the development of zucchini seedlings (cv Greyzini) sown in greenhouse in the absence or in the presence of *Pythium ultimum* or *Rhizoctonia solani*. Results are expressed as healthy plant stand (%) after 15 d from sowing in infested substrate. The data shown are mean values from three independent experiments.

Treatment	No pathogen	**+ *P. ultimum***		**+ *R. solani***	
Control	80	39	c[a]	6	b
H. jecorina RUTC-30	81	77	a	12	b
H. jecorina QM9978	68	70	ab	11	b
H. jecorina QM9414	79	55	b	13	b
H. atroviridis P1	80	84	a	78	a

[a] Values followed by the same letters do not differ significantly according to the Tukey-Kramer honestly significant difference test ($P < 0.001$).

Interaction of P. ultimum and R. solani with a cre1 mutant of H. jecorina

The *H. jecorina* mutant RUT-C30 contains a truncated copy of *cre1* (Strauss et al. 1995; Ilmen et al. 1996) and is consequently carbon catabolite derepressed. This strain was much more active in antagonizing *P. ultimum* on plates (Fig. 3) than *H. jecorina* QM9414. Upon physical contact of *P. ultimum* with RUT-C30, *P. ultimum* growth stopped completely and RUT-C30 overgrew its host much faster than QM9414 did. As with *H. jecorina* QM9414, no overgrowth occurred in confrontation experiments with *R. solani*.

The increased antagonistic ability of RUT-C30 against *P. ultimum* can also be seen in the results from the two greenhouse experiments, which show that RUT-C30 protected zucchini plants more effectively than QM9414, thereby equaling *H. atroviridis* (Table 1).

***Cellulase gene expression and biocontrol of* P. ultimum**

H. jecorina QM9978 is a mutant that is unable to induce the formation of cellulases due of a defect in cellulose to cellulase signaling (Torigoi et al. 1996; Zeilinger et al. 2000). Despite this fact, this strain also antagonized and overgrew *P. ultimum*, but not *R. solani*, on plates (Fig. 4). Protection of zucchini plants against *P. ultimum* was slightly, although statistically not significant, enhanced over that achieved with strain QM9414 (Table 1).

Strain QM9978 does not produce cellulases during growth on cellulose or sophorose (Zeilinger et al. 2000) but its lack of cellulase formation has not been proven under plate confrontation conditions. We compared therefore cellulase gene expression (using the gene encoding the major secreted cellulase Cel7A, *cbh1*) during confrontation assays of *H. jecorina* QM9414, QM9978 and RUT-C30 with *P. ultimum.* The results obtained confirm that QM9978 does not express *cbh1* (Fig. 5). Interestingly, *cbh1* expression in the cellulase-producing strain QM9414 only occurred before contact and was not detectable after contact with *P. ultimum*. In contrast, the catabolite derepressed strain RUT-C30 showed significant cellulase gene expression before and also after contact with *P. ultimum*.

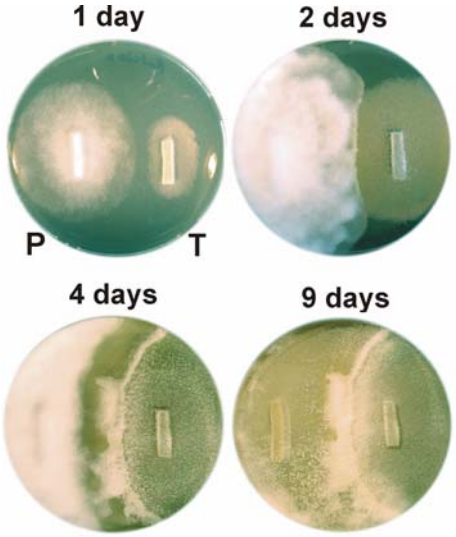

Fig. 3. Plate confrontation assays of the carbon catabolite derepressed mutant *H. jecorina* RUT-C30 (T) against *P. ultimum* (P), performed on MEX medium.

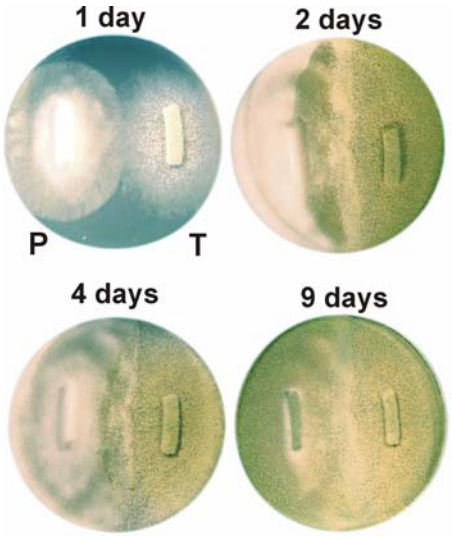

Fig. 4. Plate confrontation assays of the cellulose-negative mutant *H. jecorina* QM9978 (T) against *P. ultimum* (P), performed on MEX medium.

Discussion

We found that *H. jecorina,* a fungus used for the production of industrial enzymes, can act as an antagonist against *P. ultimum*. However, despite the strong antagonism of *H. jecorina* QM9414 in plate confrontation experiments, protection of zucchini seedlings in *in planta* assays was only moderate in comparison to the mycoparasitic strain *H. atroviridis* P1. This could be due to soil preference of *H. jecorina*: this fungus is known only from a narrow belt around the equator (Turner et al. 1996), and is particularly abundant in tropical soils

(Lieckfeldt et al. 2000b; Kubicek et al. 2003). It is possible that *H. jecorina* would display better plant protection when the respective soil characteristics of these regions are taken into account. However, a deficiency in other factors required for competence in the rhizosphere of the plant cannot be excluded at this stage. Nevertheless, the protection of zucchini plants against *Pythium* by *H. jecorina* QM9414 was significant enough to justify subsequent studies on the role of cellulolytic enzymes and carbon catabolite derepression in this process. The moderate biocontrol potential of QM9414 might even be advantageous if it enables us to identify both genes that are essential for biocontrol and those whose manipulation could improve biocontrol properties.

Production of hydrolytic enzymes has frequently been emphasized as one of the major factors contributing to the biocontrol activity of *Hypocrea/Trichoderma* spp. (Migheli et al. 1998; Zeilinger et al. 2000; Kubicek et al. 2001; Howell 2003; Roderick and Navajas 2003). Since *H. jecorina* was able to antagonize *P. ultimum*, which as an oomycete has cellulose as a major component of its cell wall, we tested the potential role of cellulases in the antagonism of *P. ultimum*. We used a mutant (QM9978) which can not hydrolyze amorphous cellulose under conditions that induce cellulases in other strains (Torigoi et al. 1996; Zeilinger et al. 2000); M. Mandels and C.P. Kubicek, unpublished results), and thus probably forms none of the 11 cellulases which are present in the *H. jecorina* genome (Foreman et al. 2003); http://gsphere.lanl.gov/trire1/trire1.home.html). The results show that the cellulase-negative mutant QM9978 can also overgrow *P. ultimum* and that this mutant protects zucchini plants against *Pythium* blight, suggesting that cellulases are not essential for the antagonism. One could argue that the cell wall of *P. ultimum* may contain still unidentified inducers of the cellulases of *H. jecorina*, whose signaling is not impaired in QM9978. To rule out this possibility, we examined the expression of cellulases by *H. jecorina* during confrontation with *P. ultimum*, using the major cellulase *cbh1* as a model gene. All studies so far showed that the expression of the various cellulase genes is coregulated (Torigoi et al. 1996; Archer and Peberdy 1997; Foreman et al. 2003; Mach and Zeilinger 2003), and we therefore assume that the expression of *cbh1* also reflects that of the other cellulases. Our data indicate that QM9978 indeed does not form cellulases during interaction with *P. ultimum* on plates. Further, the fact that QM9414 expresses some *cbh1* before contact but shuts it off during overgrowth further adds to the conclusion that cellulases are dispensable for *H. jecorina* during antagonization of *P. ultimum*. The fact that the catabolite derepressed strain RUT-C30 shows enhanced *cbh1* gene expression during confrontation and maintains this expression

during overgrowth of *P. ultimum* indicates that carbon catabolite repression is one reason for the low *cbh1* expression and its turn-off in the cellulase producer QM9414.

The finding that cellulose hydrolysis is dispensable for antagonism of *P. ultimum* by *H. jecorina* does not rule out that other hydrolytic enzymes, such as β-glucanases or proteases (Kim et al. 2002; Delgado-Jarana et al. 2003; Pozo et al. 2004; Suarez et al. 2004), may still be important for it; however, it clearly indicates that hydrolysis of the major structural polymer of the pathogens cell wall is less significant.

In the light of these findings it is unclear, why *H. jecorina* antagonized *P. ultimum* and not *R. solani*. It would be tempting to speculate that the reason could be related to a general difficulty to attack fungi with chitin as major structural cell-wall polysaccharide. However, the *H. jecorina* genome contains 18 genes encoding putative chitinases (Seidl et al. 2005), including orthologues of all of the chitinases which have so far been characterized from *H. atroviridis*, *H. lixii*, *H. virens* and *T. asperellum* (Kubicek et al. 2001; Viterbo et al. 2001) and has therefore at least the necessary genes available. The inability of *H. jecorina* to overgrow *R. solani* must therefore be more complex, and probably related to other factors such as signaling of the presence of the host or regulation of enzyme induction.

Lorito et al. (1996b) demonstrated that in *H. atroviridis* P1, binding of the Cre1 carbon catabolite repressor to the promoter of the *ech42* endochitinase is abolished during contact with the host and is replaced by a mycoparasitism-specific protein. Relief from carbon catabolite repression could thus accelerate the induction of the mycoparasitic response and improve antagonism. The results shown here with the carbon catabolite derepressed RUT-C30 demonstrated in fact improved biocontrol of *P. ultimum in planta*. While this confirmed the hypothesis, our findings that cellulases are of little relevance to antagonism of *P. ultimum* raises the question, what the targets mycoparasitism-related carbon catabolite repression are. ß-glucanases or proteases, as discussed above, could be such targets. But other physiological responses of *H. jecorina*, such as the formation of antifungal compounds (Sivasithamparam and Ghisalberti 1998), penetration structures (Goodwin and Chen 2002), or competition for fungal germination elicitors (Howell 2003) might also be subject to carbon catabolite repression and be responsible for the increased antagonistic capability of RUT-C30. In any case, our results justify a closer look at the role of carbon catabolite repression in the capabilities of agriculturally important biocontrol strains.

3

A complete survey of *Trichoderma* chitinases reveals three distinct subgroups of family 18 chitinases

Abstract

Genome wide analysis of chitinase genes in the *Hypocrea jecorina* (anamorph: *Trichoderma reesei*) genome database revealed the presence of 18 ORFs encoding putative chitinases, all of them belonging to glycoside hydrolase family 18. Eleven of these encode yet undescribed chitinases. A systematic nomenclature for the *H. jecorina* chitinases is proposed, which designates the chitinases corresponding to their glycoside hydrolase family and numbers the isoenzymes according to their pI from Chi18-1 to Chi18-18. Phylogenetic analysis of *H. jecorina* chitinases and those from other filamentous fungi, including hypothetical proteins of annotated fungal genome databases, showed that the fungal chitinases can be divided into three groups: groups A and B (corresponding to class V and III chitinases, respectively) also contained the so far identified *Trichoderma* chitinases, whereas a novel group C comprises high molecular weight chitinases that have a domain structure similar to *Kluyveromyces lactis* killer toxins. Five chitinase genes, representing members of groups A-C, were cloned from the mycoparasitic species *H. atroviridis* (anamorph: *T. atroviride*). Transcription of *chi18-10* (belonging to group C) and *chi18-13* (belonging to a novel clade in group B) was triggered upon growth on *Rhizoctonia solani* cell walls, and during plate confrontation tests with the plant pathogen *R. solani*. Therefore, group C and the novel clade in group B may contain chitinases of potential relevance for the biocontrol properties of *Trichoderma*.

Seidl, V., Huemer, B., Seiboth, B. and Kubicek C.P. (2005). A complete survey of *Trichoderma* chitinases reveals a new family 18 subgroup with potential relevance for mycoparasitism. FEBS J. 2005 Nov;272(22):5923-39.

Introduction

Next to cellulose, chitin is the second most abundant organic source in nature (Ballenweg 2005). The polymer is composed of β-(1,4)-linked units of the amino sugar *N*-acetylglucosamine. It is a renewable resource, mainly extracted from shellfish waste and can be processed into many derivatives, which are used for a number of commercial products such as medical applications (i.e. surgical thread), cosmetics, dietary supplements, agriculture and water treatment (Muzzarelli 1999; 2005; Di Martino et al. 2005).

Various organisms form chitinolytic enzymes (EC 3.2.1.14), which hydrolyze the β-1,4-glycosidic linkage (Flach et al. 1992). The chitinases known today are divided into two families (family 18 and family 19) on the basis of their amino acid sequences (Henrissat 1991). These two families do not share sequence similarity, and display different three-dimensional structures: family 18 chitinases have a catalytic $(\alpha/\beta)_8$-barrel domain (Perrakis et al. 1994; Terwisscha van Scheltinga et al. 1996; Robertus and Monzingo 1999; Hollis et al. 2000), while the family 19 enzymes have a bilobal structure and are predominantly composed of α-helices (Hart et al. 1995; Fukamizo 2000; Hahn et al. 2000). They also differ in their enzymatic mechanism: family 18 chitinases have a retaining mechanism which results in chitooligosaccharides being in the β-anomeric configuration, whereas family 19 chitinases have an inverting mechanism and consequently the products are α-anomers. Another difference is the sensitivity to allosamidin, which inhibits only family 18 chitinases (Koga et al. 1999). *N*-acetylhexosaminidases (EC 3.2.1.52), which cleave chitooligomers and also chitin progressively from the non reducing end and release only *N*-acetlyglucosamine monomers, belong to glycoside hydrolase family 20 (Kubicek et al. 2001).

Some species of the imperfect soil fungus *Trichoderma*, e.g. *T. harzianum* (teleomorph *Hypocrea lixii*), *T. virens* (teleomorph *H. virens*), *T. asperellum* and *T. atroviride* (teleomorph *H. atroviridis*), are potent mycoparasites against several plant pathogenic fungi that cause severe crop losses each year, and are therefore used in agriculture as biocontrol agents. Biocontrol is considered an attractive alternative to the strong dependence of modern agriculture on fungicides, which may cause environmental pollution and selection of resistant strains. Lysis of the host cell-wall of the plant pathogenic fungi has been demonstrated to be an important step in the mycoparasitic attack (Chet et al. 1998; Kubicek et al. 2001; Howell 2003; Benitez et al. 2004). Consequently, with chitin being a major cell-wall component of plant pathogens like i.e. *Rhizoctonia solani*, *Botriytis cinerea* and *Sclerotinia sclerotiorum*, several chitinase genes have been cloned from *Trichoderma* spp (Carsolio et al. 1994; Garcia et al. 1994; Hayes et al. 1994; Draborg et al. 1995; Peterbauer et al. 1996; Viterbo et al. 2001;

Kim et al. 2002; Viterbo et al. 2002), and for some of them also the encoded protein has been characterized (de la Cruz et al. 1992; Boer et al. 2004). Most recently the chitinase Ech30 from *H. atroviridis* was overexpressed in *E. coli* and characterized (Hoell et al. 2005), but neither its expression pattern nor biological relevance were studied. The possible roles of the endochitinases Ech42 and Chit33 and the *N*-acetylglucosaminidase Nag1 in mycoparasitism have been investigated (Cortes et al. 1998; Baek et al. 1999; Woo et al. 1999; Kullnig et al. 2000; de las Mercedes Dana et al. 2001; Brunner et al. 2003).

In order to obtain a comprehensive insight into the chitinolytic potential of *Trichoderma*, we screened the recently published genome sequence of *H. jecorina* (anamorph: *T. reesei*) for chitinase-encoding genes. In this study, we present a supposedly complete list of chitinases of *Trichoderma,* and demonstrate their evolutionary relationships to each other and to those from other fungi. The chitinases were characterized *in silico* and we propose a unifying nomenclature for the large number of chitinase-encoding genes that can be found in the *H. jecorina* genome. Finally, five selected chitinase genes were cloned from the mycoparasitic species *H. atroviridis* and their transcription studied under conditions relevant for chitinase formation and mycoparasitism. A member of a new, in filamentous fungi so far unidentified, group of high molecular weight chitinases (*chi18-10*) thereby shows a transcription profile which suggests that it may be relevant for biocontrol.

Materials and Methods

Strains

H. atroviridis P1 (ATCC 74058) was used in this study and maintained on PDA (Difco, Franklin Lakes, NJ). *Escherichia coli* strains ER1647 and BM25.8 (Novagen, Madison, WI) were used for genomic library screening and JM109 (Promega, Madison, WI) for plasmid propagation.

Cultivation conditions and preparation of special carbon sources

Shake flasks cultures were prepared with the medium described by Seidl et al. (2004) and incubated on a rotary shaker (250 rpm) at 28°C. Cultures were pregrown for 28 h on 1 % (w/v) glucose and then harvested by filtering through Miracloth (Calbiochem, Darmstadt, Germany), washed with medium without nitrogen or carbon source and transferred to a new flask containing 1 % (w/v) glucose for 2 h or 1 mM NAG for 30 min, respectively. Starvation was induced by replacing on either i) 0.1 % (w/v) glucose (carbon limitation), ii) 1 % (w/v)

glucose and 0.14 g/l $(NH_4)_2SO_4$ (nitrogen limitation) or iii) 0.1 % glucose and 0.14 g/l $(NH_4)_2SO_4$ for 15 h (carbon and nitrogen starvation). Cultivations on 1 % (dry weight) colloidal chitin or *Rhizoctonia solani* CW were directly grown for 48 h. Mycelia were harvested by filtration through Miracloth, washed with cold tap water, squeezed between two sheets of Whatman filter paper, immersed in liquid N_2 and stored at -80°C.

Colloidal chitin was prepared essentially as described by Roberts et al. (Roberts and Selitrennikoff 1988). Briefly 20 g crab shell chitin (Sigma, Vienna, Austria) were suspended in 400 ml conc. HCl, stirred overnight at 4°C and filtered through glass wool. The filtrate was precipitated with 2 l ethanol and washed with distilled water at 4°C until a pH of 5.0 was reached. *R. solani* CW were prepared by growing *R. solani* on PDA plates covered with cellophane, grinding the mycelium under liquid nitrogen and suspending it in distilled water containing 0.1 % (w/v) SDS (30 ml/g CW). The suspension was further homogenized in a Potter-Elvehjem pistill homogenisator, centrifuged for 15 min at 18000 *g*, 4°C and the pellet washed with distilled water to remove attached proteins (the flow through was checked by measuring OD at 280 nm).

For plate confrontation assays, stripes of 30 × 3 mm were cut out from the growing front of *H. atroviridis* and *R. solani*, respectively, and placed on PDA fresh plates (d = 9 cm) covered with cellophane at a distance of 4 cm from each other. The mycelia were harvested at 3 different time points: i) before contact (BC), when the mycelia were at a distance of approximately 10 mm, ii) contact (C), when the mycelia were just touching and iii) after contact (AC) when *H. atroviridis* had overgrown *R. solani* by ca. 5-10 mm. Mycelium from the growing front (ca 7 mm) was harvested with a spatula, frozen in liquid nitrogen and stored at -80°C. Equivalent zones were collected from control plates, inoculated with *H. atroviridis* or *R. solani* only.

Biomining of the H. jecorina genome

The *H. jecorina* genome (http://gsphere.lanl.gov/trire1/trire1.home.html) was screened for chitinases by using the tblastn (protein vs translated nucleotide) program. First we used the protein sequences of the published chitinase sequences of other *Trichoderma* spp. (listed in Table 1) as query to search the *H. jecorina* genome. Then all chitinases including the newly identified from *H. jecorina* were used to identify further proteins with similar domains and finally all hypothetical proteins encoding chitinases from the annotated genomes of the Broad Institute (http://www.broad.mit.edu/) including *Emericella nidulans (Aspergillus nidulans)*, *Neurospora crassa*, *Giberella zeae (Fusarium graminearum)* and *Magnaporthe griseae* were

used. The loci of the *H. jecorina* chitinases in the *H. jecorina* genome database are listed in Table 3.

Cloning of chitinase genes from H. atroviridis

Novel chitinase-encoding genes from *H. atroviridis* were cloned by using PCR fragments from *H. jecorina* chitinases as probes. The primers listed in Table 4a were used to amplify the respective fragments from *H. jecorina* by PCR, which were then isolated and used to screen a genomic λ BlueSTAR library (Novagen, Madison, WI) of *H. atroviridis* P1. Isolated phages were converted to plasmids and sequenced at MWG Biotech AG (Ebersberg, Germany).

The assembled DNA sequences were deposited in GenBank (acc. no. DQ068748-DQ68752)

Sequence analysis

Sequences were analysed using BLAST programs (http://www.ncbi.nlm.nih.gov/BLAST/). The MEME Motif Discovery and Search tool (http://meme.nbcr.net, (Bailey and Elkan 1994)) was used for analysis of the 5'-non-coding regions of the cloned *H. atroviridis* chitinase genes. Theoretical pI and M_r of the proteins were calculated with the pI/MW tool (http://us.expasy.org/tools/pi_tool.html, (Gasteiger et al. 2005). Analysis of theoretical subcellular localization and prediction of signal peptide cleavage sites was carried out with PSORT II (http://psort.ims.u-tokyo.ac.jp/form2.html, Nakai and Horton 1999), TargetP (http://www.cbs.dtu.dk/services/TargetP/, Emanuelsson et al. 2000) and SignalP (http://www.cbs.dtu.dk/services/SignalP/, Nielsen et al. 1997) Conserved protein domains were analyzed with InterProScan (http://www.ebi.ac.uk/InterProScan/, Zdobnov and Apweiler 2001).

Phylogenetic analysis

Protein sequences were aligned first with ClustalX 1.8 (Thompson et al. 1997) and then visually adjusted using Genedoc 2.6 (Nicholas and McClain 1987). Phylogenetic analyses were performed in MEGA 2.1 using Neighbour Joining, a distance algorithmic method. Stability of clades was evaluated by 1000 bootstrap rearrangements. Bootstrap values lower than 50% are not displayed in the cladogram.

PCR-aided methods

PCR reactions were carried out in a total volume of 50 µl containing 2.5 mM $MgCl_2$, 10 mM Tris- HCl pH 9.0, 50 mM KCl, 0.1 % (v/v) Triton X-100, 0.4 µM of each primer, 0.2 mM of each dNTP and 0.5 units of *Taq*-Polymerase (Promega, Madison, WI). The amplification program consisted of: 1 min initial denaturation (94°C), 30 cycles of amplification (1 min 94°C, 1 min primer specific annealing temperature (see Table 4), 1 min 72°C), and a final extension period of 7 min at 72°C. For RACE-PCR amplification cycles were increased to 35 and RT-PCR was carried out with 25 or 35 cycles.

RNA isolation

Total RNA was extracted as described previously (Chomczynski and Sacchi 1987).

Rapid amplification of cDNA ends (RACE)

cDNA was synthesized with the Creator SMART cDNA library construction kit (BD Biosciences, Palo Alto, CA) from RNA from *H. atroviridis* cultures grown on glucose. The primers used for RACE-PCR are listed in Table 4b. Amplification of 5'- and 3'- cDNA ends was carried out with the 5'PCR and CDSIII primers from the cDNA kit and gene specific primers followed by a second PCR using the 5'PCR and CDSIII primers and nested gene specific primers.

The resulting fragments were cloned into pGEMT-Easy (Promega, Mannheim, Germany) and sequenced at MWG Biotech (Ebersberg, Germany).

RT-PCR

RNA obtained from various cultivations was treated with Deoxyribonuclease I (Fermentas, St. Leon-Rot, Germany) and purified with the RNeasy MinElute Cleanup Kit (Qiagen, Hilden, Germany). 5 µg RNA/reaction were reverse transcribed using the RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany) and the oligo$(dT)_{18}$ primer.
The cDNA was used for PCR with sequence specific primers listed in Table 4c to assess the exon/intron boundaries. For transcript analysis (RTQ-Primers, Table 4d) annealing temperature, RNA-concentration and the number of amplification cycles were optimized and finally 5 µg RNA/reaction, 25 cycles (unless otherwise stated) and the temperatures listed in Table 4d were used. 40 µl of each PCR reaction were separated on a 1.5% agarose gel containing 0.5 µg/ml ethidium bromide.

The following controls were carried out in parallel with each RT-PCR experiment. To ensure the absence of genomic DNA, RNA was treated with Deoxyribonuclease I, purified and subjected to the reverse transcription procedure as described above, but no reverse transcriptase was added during this step. This RNA was subsequently used for PCR under the same conditions that were used for RT-PCR with 35 cycles. Additionally, PCR reactions without template were set up to rule out contamination of other PCR components. In none of the controls a PCR product could be detected when they were visualzed by agarose gel electrophoresis.

Table 1. Properties of *H. jecorina* chitinases. The theoretical pI, M_r, subcellular localization of the *H. jecorina* chitinases and the number of ESTs found in the *H. jecorina* genome database for the respective genes are given. Novel chitinases are written in bold letters. Orthologues already cloned from other *Trichoderma* spp. and the orthologues from the mycoparasitic strain *H. atroviridis*, cloned in this study, are listed. The affiliation to the phylogenetic group as determined in this study is also given.

H. jecorina chitinase	pI	M_r [kDa]	subcellular localization	ESTs	Previously cloned orthologues in other *Trichoderma* spp.	Cloned from *H. atroviridis* in this study	Phylogenetic group
Chi18-1	3.97	146.5	EC	-	-		C
Chi18-2	4.05	44.5	cytoplasmic	-	-	Chi18-2	A
Chi18-3	4.15	38.7	mitochondrial	-	-	Chi18-3	A
Chi18-4	4.16	44.2	ER-targeted	-	-	Chi18-4	A
Chi18-5	4.39	46.0	EC	32	Ech42, Chit42, Tv-ech1,… var. *Trichoderma* spp. (see Fig 2)	-	A
Chi18-6	4.64	54.2	EC	-	Tv-ech3 (*H. virens,* AAL78812)	-	A
Chi18-7	4.68	44.6	cytoplasmic	38	Tv-ech2 (*H. virens,* AAL78814)	-	A
Chi18-8	4.80	139.1	EC	-	-	-	C
Chi18-9	4.81	163.2	EC	-	-	-	C
Chi18-10	4.96	136.1	EC	-	-	Chi18-10	C

Chi18-11	5.18	41.5	EC	-	-	-	A
Chi18-12	5.18	35.0	EC	2	Chit33 (*H. lixii*, CAA56315) Tv-Cht1 (*H. virens,* AAL78810)	-	B
Chi18-13	5.36	41.0	EC	4	Ech30 (*H. atroviridis*, AAP81811)	Chi18-13	B
Chi18-14	5.44	42.6	EC	4	-	-	B
Chi18-15	5.84	36.2	EC	-	Chit36 (*H. lixii*, AY028421) Chit36y (*T. asperellum*, AAL01372)	-	-
Chi18-16	6.31	41.9	EC	-	-	-	B
Chi18-17	6.41	41.4	EC	-	Tv-Cht2 (*H. virens,* AAL78811)	-	B
Chi18-18	9.69	104.2	EC/cell wall bound (?)	9	-	-	A

Table 2. Transcription products of the new *H. atroviridis* chitinase-encoding genes. The 5'- and 3'-UTRs and coding regions were determined with RACE and RT-PCR.

H. atroviridis chitinase gene	5'-UTR [bp]	coding region [bp]	3'-UTR [bp]
chi18-2	84	1491	66
chi18-3	152	1077	466
chi18-4	196	1179	292
chi18-10	60	3978	163
chi18-13	56	930	215

Table 3. *H. jecorina* chitinase-genes. The scaffolds and nucleotide regions for the *H. jecorina* chitinase-genes in the *H. jecorina* genome database (http://gsphere.lanl.gov/trire1/trire1.home.html) are given.

H. jecorina chitinase	scaffold	region [bp]
chi18-1	1	1713711 - 1718094
chi18-2	4	688779 - 690161
chi18-3	71	778292 - 779755
chi18-4	72	327190 - 328487
chi18-5	23	536259 - 537734
chi18-6	26	13457 - 15071
chi18-7	30	46966 - 48343
chi18-8	21	495790 - 500197
chi18-9	25	22081 - 27013
chi18-10	35	28381 - 32975
chi18-11	49	28108 - 29480
chi18-12	1	1208675 - 1209856
chi18-13	22	377267 - 378539
chi18-14	40	50144 - 51724
chi18-15	58	53755 - 54786
chi18-16	28	121366 - 122635
chi18-17	19	605284 - 606626
chi18-18	15	419611 - 422850

Table 4. Primers used in this study.

a) Primers for amplification of *H. jecorina* genomic DNA fragments for phage library screening

primer for phage library screening	sequence 5' → 3'	fragment from *H. jecorina* chitinase gene	annealing temp. [°C]	fragment length [bp]
5' chi18-2TR	GATGGCTCACTTCGGGTATGATG	*chi18-2*	60.1	900
3' chi18-2TR	CGGCACGTCAAACGTCAGATAG			
5' chi18-3TR	TCTCAAGCAGAGGCACCCTCAC	*chi18-3*	60.0	868
3' chi18-3TR	CTTCACCTTCACCGTCTCGTGG			
5' chi18-4TR	GTCCGATGTGTTCAATGTGGACG	*chi18-4*	59.5	865
3' chi18-4TR	TCCCAGTATCCGTAGCTTCCGTC			
5' chi18-10TR	ACGAGGACTACTCCGTCAATATCG	*chi18-10*	58.7	615
3' chi18-10TR	CACCGACGGTGATCATGTTAGAC			
5' chi18-13TR	TGATGCCGCCAATGTTGGG	*chi18-13*	61.5	815
3' chi18-13TR	AACGTCTGCGCCGACTCTTC			

b) RACE-PCR primers

primer for RACE-PCR	sequence 5' → 3'	fragment from *H. atroviridis* chitinase gene	annealing temp. [°C]
5' PCR Primer	AAGCAGTGGTATCAACGCAGAGT		
CDSIII	ATTCTAGAGGCCGAGGCGGCCGAC ATG-d$(T)_{30}N_{-1}N$		
5Race-2	GAAGATGTGCGTAATATTAGC	*chi18-2*	51.3
5Race-2nest	GTCTTGTCTTTATACACCAGCC		55.4
3Race-2	GGGAAATGGACTACTACGAG		55.0
3Race-2nest	AGCCTGGTACGTAGATGCA		54.7
5Race-3	ATTGAGCATTCCCGGCGA	*chi18-3*	55.5
5Race-3nest	TTCTGCTGCTAGGGAAATAG		52.9
3Race-3	GACTCTCGAGATCAAGCAC		54.7
3Race-3nest	TCTGATTGCGGCTGGTTTC		54.7
5Race-4	GCAATTGAGAGCAGTTTCG	*chi18-4*	52.6
5Race-4nest	TTGAAGAAGGAGCACGAATGCC		57.2
3Race-4	AAGAGAAGAGATGGTGGTCC		55.0
3Race-4nest	CTCTCACCATCAAAGCCAAAG		55.2

5Race-10	TCATGTCTAAGAGCATAGGC	*chi18-10*	52.9
5Race-10nest	TGTCCAGTTGCCCGAGTTGA		57.0
3Race-10	CGGGCTATCTGATCCTCA		54.5
3Race-10nest	CACCTCGTTCACTCATATCA		55.3
5Race-13	GTGTCGAGGAAGGCAAGA	*chi18-13*	55.5
5Race-13nest	CCATAAGAACTGTCTGAACAC		53.2
3Race-13	GCCAAGCTCTATATCGGTGC		57.0
3Race-13nest	GATGGCGATCAGGGCTTTG		56.9

c) RT-PCR primers for identification of coding regions and introns

primer for RT-PCR	sequence 5' → 3'	fragment from *H. atroviridis* chitinase gene	annealing temp. [°C]	fragment length [bp]
2TA-RT-fw	CTCGCGGCTATATGAACGG	*chi18-2*	56.7	438
2TA-RT-rv	TGCGGCACTCTTGGAGAAG			
3TA-RT-fw	CCAATGCAGTCTATTTCCCTAG	*chi18-3*	56.8	989
3TA-RT-rv	AGCCGCAATCAGACTTCG			
4TA-RT-fw	CGTCAACAGTCGCCTTCAGG	*chi18-4*	57.7	745
4TA-RT-rv	GCCGATGGCATTGACATTG			
10TA-5RT-fw	TACCGCACAACAAAAGGGA	*chi18-10*	52.6	1206
10TA-5RT-rv	TCTTTTAGTTCCAGGAACCTG			
10TA-RTm-fw	AAGAAGACCTGGGGCTGGA		51.0	893
10TA-RTm-rv	ATGTAGATGATGTAGTCGAC			
10TA-3RT-fw	GTATCTCAAGGGATTCCCCA		53.5	1242
10TA-3RT-rv	GAATTCTTCTATCAACGAGAGG			
13TA-RT-fw	CATCGGCAAAGCCCTGATC	*chi18-13*	57.7	704
13TA-RT-rv	AGCAGAAGACGATTCAACGACG			

d) RT-PCR primers for transcript analysis under different growth conditions

primer for RT-PCR (transcript analysis)	sequence 5' → 3'	fragment from *H. atroviridis* gene	annealing temp. [°C]	fragment length [bp]
RTQ2-fw	GACGGCGTCGAATATATCTTG	*chi18-2*	57.8	316
RTQ2-rv	TGCCCGTCCAGTAGAACAG			
RTQ3-fw	GCTTCCTCAAGGCCAATG	*chi18-3*	59.5	284
RTQ3-rv	AGCCGCAATCAGACTTCG			

RTQ4-fw	GACGGCGTCGAATATATCTTG	*chi18-4*	59.5	324
RTQ4-rv	TGCCCGTCCAGTAGAACAG			
RTQ10-fw	CCATCTGTCTGCGTTCTTG	*chi18-10*	57.0	
RTQ10-rv	ATAATCGACGGGTTGTTGTAG			
RTQ13-fw	TTTGGAGACATTAAGCTTGACG	*chi18-13*	57.8	287
RTQ13-rv	TTGCCAATACCGCTGCTC			
RTQ42-fw	CATGCCCATCTACGGACGAG	*ech42 (chi18-5)*	61.7	272
RTQ42-rv	CTTCCCAGAACATGCTGCCTC			
tef-fw	GGTACTGGTGAGTTCGAGGCTG	*tef1*	60.8	351
tef-rv	GGGCTCAATGGCGTCAATG			

Results

Biomining the H. jecorina *genome for chitinase genes*

Chitinase genes, present in the *H. jecorina* genome sequence, were identified by using an iterative strategy of BLAST searches with fungal chitinases as described in the materials and methods section. We could identify 18 ORFs encoding putative chitinases (Table 1), including orthologues of all chitinases described from *Trichoderma* so far (*ech42, Tv-ech2, Tv-ech3, chit33, Tv-cht2, ech36, ech30*). In addition to these 7 known chitinases there are 11 novel, yet undescribed/unknown chitinase-encoding genes present in the *H. jecorina* genome. InterProScan predicted all of them to encode a family 18 chitinase.

To identify potential chitinases of glycoside hydrolase family 19, a chitinase from *Hordeum vulgare* (GenBank acc.no. P11955) and a chitinase from *Encephalitozoon cuniculi* (GenBank acc. no. Q8STP5) were used for a tblastn search. This strategy was unable to produce any hits, however.

Tblastn search of the *H. jecorina* genome database with Nag1 (*N*-acetylglucosaminidase) of *H. atroviridis* (Peterbauer et al. 1996), which is a member of glycoside hydrolase family 20 (Henrissat 1991), produced two hits that corresponded to the two *N*-acetylglucosaminidase encoding genes previously cloned from *H. lixii* (Draborg et al. 1995) and *T. asperellum* (Ramot et al. 2004). Using the same iterative BLAST strategy as for the family 18 chitinases we were unable to identify further members of the glycoside hydrolase family 20 in *H. jecorina*.

Having presumably identified the whole chitinase spectrum of *H. jecorina* we used the following nomenclature, which is based on the proposal of Henrissat (1999) to name chitinases according to their glycoside hydrolase family, and on the IUB nomenclature for numbering isoenzymes, which starts with the protein having the lowest pI (Liébecq 1992).

Therefore, the *H. jecorina* family 18 chitinases are named *chi18-1* to *chi18-18*. Numbers were used instead of letters to follow the nomenclature for genes from pyrenomycetes. Table 1 shows a list of all chitinase-encoding genes of *H. jecorina* including the pI and M_r of the hypothetical proteins. Also given are the hitherto existing names of chitinases that are already known in other *Trichoderma* spp., the number of *H. jecorina* ESTs (Chambergo et al. 2002; Foreman et al. 2003; Diener et al. 2004) that have been sequenced for the respective genes (roughly giving an estimate of their level of expression).

Properties of the* H. jecorina *chitinase proteins

We used InterProScan to predict the domain structure of the identified chitinase sequences and the presence of potential target sequences for cellular traffic and location (Fig. 1). The high M_r (>136kDa) chitinases - Chi18-1, Chi18-8, Chi18-9 and Chi18-10 (Table 1) - are predicted to contain two LysM domains (InterPro acc. no. IPR002482) that are suggested to bind to peptidoglycan-like structures (Bateman and Bycroft 2000) and a chitin-binding domain 1 (InterPro acc. no. IPR001002, Butler et al. 1991; Wright et al. 1991). This type of chitin binding domains corresponds to CBM (carbohydrate binding module) 18 in the CAZy classification (Carbohydrate-Active Enzymes database, http://afmb.cnrs-mrs.fr/CAZY/, Coutinho and Henrissat 1999). In addition, Chi18-10 also displays an EGF-1 like domain known to be involved in protein-protein interactions (InterPro acc. no. IPR001336, Wouters et al. 2005). For the four chitinases Chi18-1, Chi18-8, Chi18-9 and Chi18-10, considerable similarity (e^{-100}, about about 55 % functionally identical amino acids on approx. 50% of the length of the *Hypocrea* proteins) was obtained with the α- and β-subunits of the *Kluyveromyces lactis*-type killer toxins of yeasts (*K. lactis*, *Pichia etchellsii, P. acaciae,P. inositovora, Debaromyces robertsiae* and *D. hansenii*). These toxins consist of three subunits (α, β, γ) with α and β encoded by one ORF and the γ subunit by a separate ORF. The α subunit has chitinase activity that is required for the toxin to act on susceptible yeast cells. The β subunit may - together with α - play a role in binding and translocation of the toxin, allowing the γ subunit to enter the cell which leads to cell cycle arrest (Magliani et al. 1997).

Chi18-14, Chi18-16 and Chi18-17 contain a cellulose-binding domain (InterPro acc. no. IPR000254, Tomme et al. 1995; Linder and Teeri 1996), CBM 1 in the CAZy classification) and Chi18-14 has additionally a subtilisin-like serine protease domain (InterPro acc. no. IPR000209, Siezen and Leunissen 1997).

All except of three chitinases (Chi18-2, Chi18-3 and Chi18-7) show the presence of a typical signal peptide and often also a dibasic or basic-acid Kex2-like cleavage site (Julius et

al. 1984; Goller et al. 1998) and are therefore likely to be secreted proteins. Chi18-3 is predicted to be located in the mitochondrion, whereas the highest subcellular localization probability for Chi18-2 and Chi18-7 is the cytoplasm. Interestingly, the putative mitochondrial location of Chi18-3 is also predicted for its orthologues from other fungi (cf Fig. 2). This protein has also two S-globulin domains (InterPro acc. no. IPR000677, (Shewry and Halford 2002)) which are frequently reported in association with glycoside hydrolase family 18 domains. Chi18-4 contains an endoplasmic reticulum (ER) retention signal (KDEL) which causes a re-localization of the posttranslationally modified protein in the ER (Pelham 1990).

Chi18-18 consists of 2 domains, one of them being the glycoside family 18 domain, the other of unknown function, which are linked through a large unstructured region of about 40 kDa that could possibly be a cell wall anchor (Jaques et al. 2003). This region consists of only the four aa-residues K, A, S and T. The large number of Ks is also responsible for the unusually high theoretical pI of 9.69 of Chi18-18.

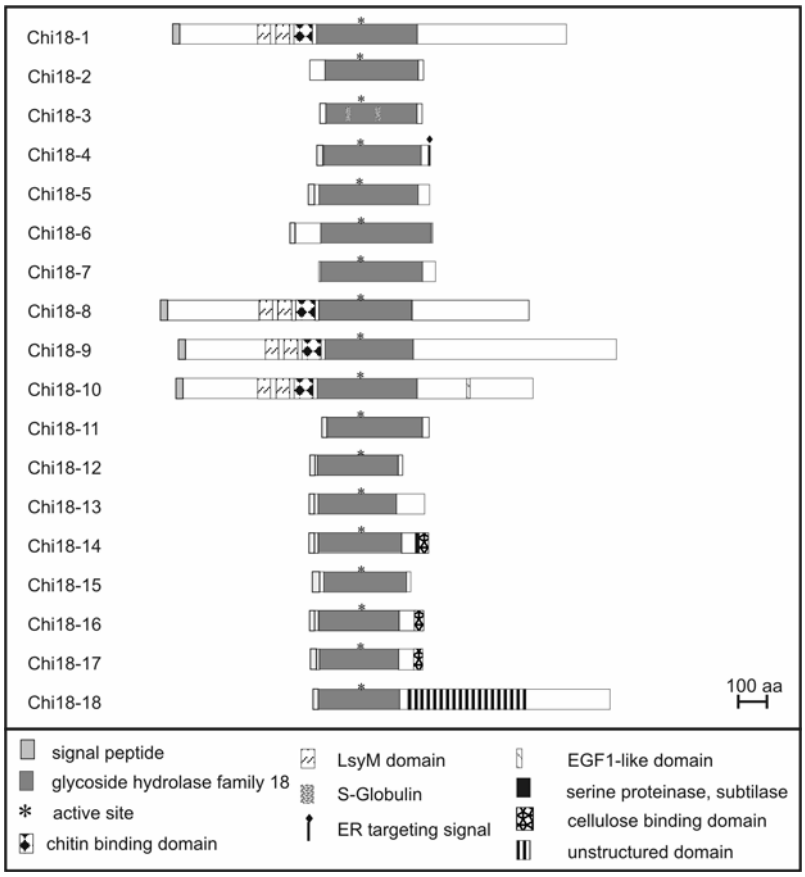

Fig. 1. Domain structure of *H. jecorina* chitinases. Protein domains as identified with InterProScan are shown. Blank parts of the proteins indicate no match with yet characterized protein domains could be found. The bar marker in the bottom right corner represents the length of 100 aa.

Phylogenetic relationship of the* H. jecorina *chitinases

The 18 chitinases were aligned with putative ortho- and paralogues present in the databases from *N. crassa*, *G. zeae*, *M. grisea*, and *A. nidulans*, and from other filamentous fungi found in GenBank. Also, the deduced protein sequences of five chitinases from *H. atroviridis*, which were cloned in this study, are included. A reliable alignment of all these protein sequences together was not possible due to insufficient similarity between some members, and consequently three separate alignments were made. Group A contains proteins showing similarity to Ech42, group B consists of chitinases similar to Chit33 and group C comprises several so far unknown chitinase proteins. These groups were subjected to neighbour joining analysis using MEGA2.1. Corresponding phylogenetic trees are shown in Fig. 2 - 4. The phylogenetic relationship of the fungal chitinases (Fig 2 – 4) is also represented by characteristic amino acid exchanges in the consensus motifs of these family 18 chitinases (Renkema et al. 1998; Robertus and Monzingo 1999). However, the E residue in motif 2 that has been shown to be essential for catalytic activity is conserved in all chitinases (Watanabe et al. 1993). Chi18-15 is not included in any of the trees since it did not show any similarity to fungal chitinases except to its orthologues from different *Trichoderma* spp. and to one chitinase from *Cordyceps bassiana* (GenBank acc. no. AAN41259; e^{-157} and 88 % functionally identical aa; 100% of the aa- sequence of *H. jecorina* Chi18-15 were used for the significant alignment). It should be noted that the only other proteins with high similarity to Chi18-15 were chitinases from the gram-positive bacterium *Streptomyces* (GenBank acc. no. CAB61702 and BAC67710; e^{-151} and 87 % functionally identical aa; 100% of the aa - sequence of *H. jecorina* Chi18-15 were used for the significant alignment).

The group A tree (Fig. 2) contained eight of the *H. jecorina* chitinases of which three are already known in other *Trichoderma* spp. (Chi18-5 (=Ech42), Chi18-6 and Chi18-7) and five are new including the intracellular Chi18-2, mitochondrial Chi18-3, ER-targeted Chi18-4 and extracellular Chi18-11 and Chi18-18. The latter occurred in a basal position (clade A-I) and only had an orthologue in *Gibberella zeae* (EAA72615). The remainder of the tree displayed five strongly supported clades: A-III, consisting of Chi18-4 and Chi18-11 as sister clades; A-IV containing the two intracellular chitinases Chi18-2 and Chi18-3; and A-V, which also bifurcated into two sister clades, one containing Chi18-6, and the other one containing both Chi18-5 (Ech-42) as well as the intracellular Chi18-7 in a terminal branch. The topology of the group A tree suggests that none of the *H. jecorina* chitinases are the products of gene duplication events, although such cases are seen for *M. grisea* and *G. zeae* (e.g. in the Chi18-6-branch of clade A-V).

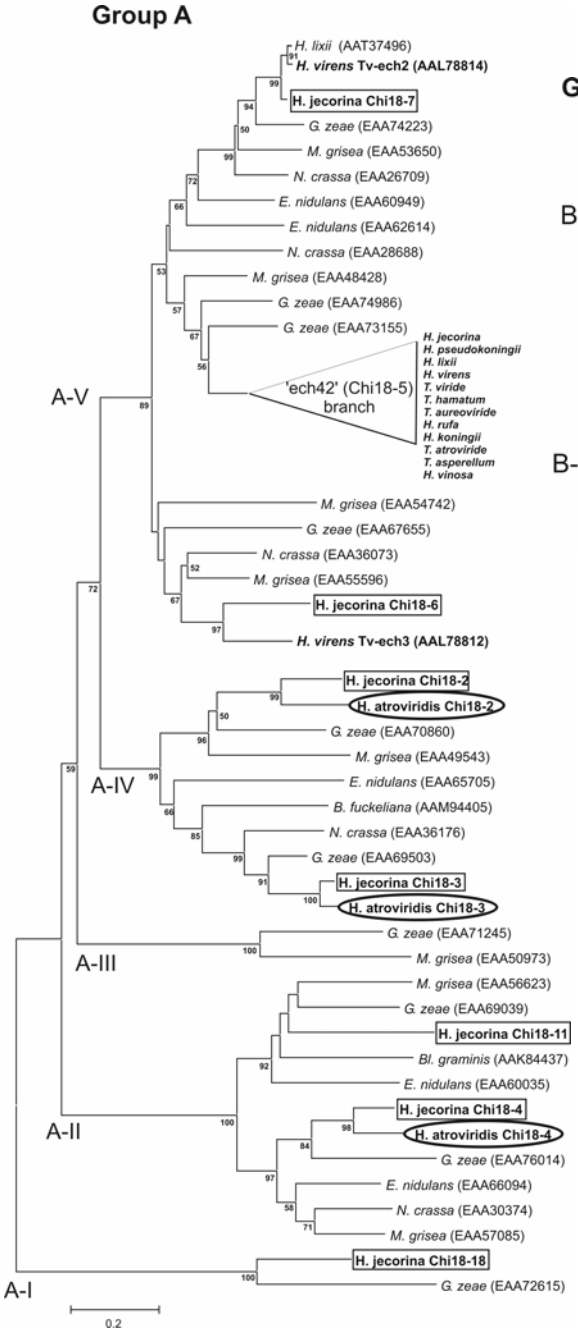

Fig. 2. Phylogeny of fungal family 18 chitinases, group A. Phylogenetic analyses were performed using Neighbour Joining. Numbers below nodes indicate the bootstrap value. The bar marker indicates the genetic distance which is proportional to the number of aa substitutions. GenBank accession numbers are given in brackets. Chitinases which had been published earlier are indicated in bold. Chitinases of *H. jecorina* and *H. atroviridis* are framed with rectangles and ovals, respectively. *Bl.: Blumeria*, *B.: Botrytinia*.

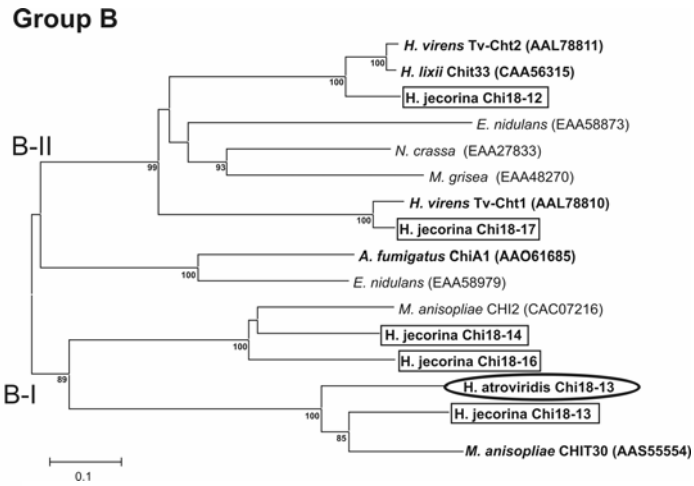

Fig. 3. Phylogeny of fungal family 18 chitinases, group B. Symbols and formatting were used as in Fig. 2. *M.: Metarhizium*.

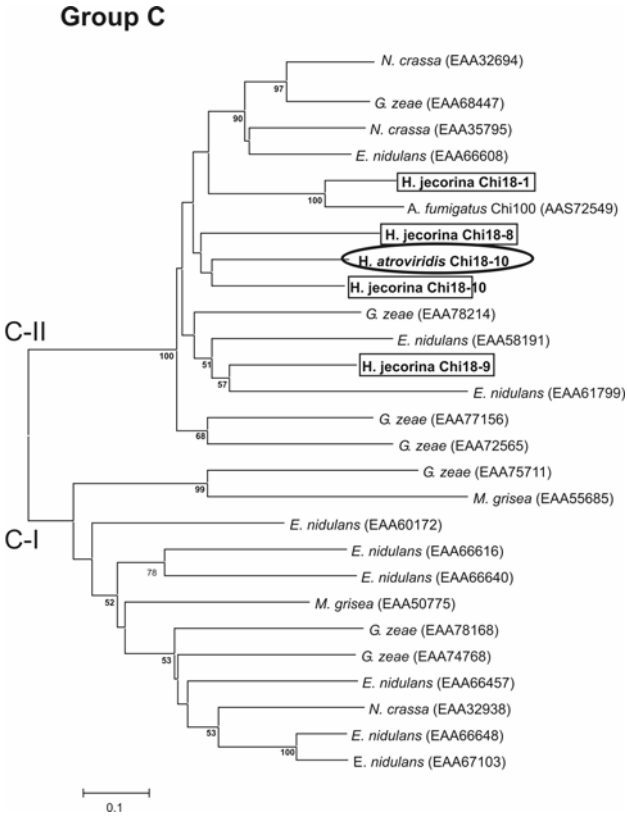

Fig. 4. Phylogeny tree of fungal family chitinases, group C. Symbols and formatting were used as in Fig. 2.

The group B tree (Fig. 3) contained five chitinases of which three (Chi18-13, Chi18-14 and Chi18-16) were new. All of the cellulose-binding domain containing chitinases occur in this tree which splits into two major clades: B-I branching into two subclades, each containing also chitinases from *Metarhizium anisopliae*, which have orthologues in *H. jecorina*. Chit18-13 is the orthologue of Ech30, for which enzymatic properties were recently described (Hoell et al. 2005). The other branch contains Chi18-16 and Chi18-14, the latter apparently having arisen by gene duplication. Clade B-II bifurcates into two subclades containing the orthologues of the previously cloned *H. virens* Tv-cht1 and Tv-cht2 (Kim et al. 2002), Chi18-12 and Chi18-17.

The tree of group C (Fig. 4) contains one major supported clade (C-II), which separates from a poorly resolved clade (C-I) containing several putative chitinases from *E. nidulans, G. zeae* and *M. grisea*. All group C *H. jecorina* chitinases (Chi18-1, Chi18-8, Chi18-9, and Chi18-10) - which contain class I chitin-binding domains - are located in C-II, but the branches are mostly poorly supported, and it is thus unclear whether Chi18-8 and Chi18-10 are also a consequence of gene duplication.

Cloning and characterization of five novel chitinases from H. atroviridis

H. atroviridis P1 is a powerful biocontrol agent. To investigate whether some of the new genes would eventually be relevant for biocontrol, we cloned five representatives of those phylogenetic clusters which contained yet uncharacterised chitinase-encoding genes: *chi18-2*, *chi18-3*, *chi18-4, chi18-10* and *chi18-13*. The coding regions and 5'- and 3'-UTRs of the five chitinases were determined by RT-PCR and RACE (for details see Table 2).

The domain structure of the novel *H. atroviridis* chitinases is similar to their *H. jecorina* orthologues which are shown in Fig 1. *H. atroviridis* Chi18-10 has an additional gamma-crystallin like element (aa 77 to 117) which can also be found in yeast killer toxins, antifungal and antimicrobial proteins (InterPro acc. no. IPR011024, Graw 1997). In all three phylogenetic trees (Fig. 2 – 4), the five cloned chitinases from *H. atroviridis* clustered immediately beneath the corresponding *H. jecorina* protein, proving that they are true orthologues of them.

Sequence analysis of the 5'-noncoding regions of the novel *H. atroviridis* chitinases identified numerous consensus binding sites for fungal transcription factors that have previously been associated with the regulation of chitinases or other polysaccharide degrading enzymes (Fig 5). Consensus sites for the transcription factors AbaA (5'-CATTAY-3'; Andrianopoulos and Timberlake 1994) and BrlA (5'-MRGAGGGR-3'; Chang and Timberlake 1993), AceI (5'-AGGCA-3'; Aro et al. 2003), AreA (5'-WGATAR-3', Kudla et

al. 1990), Cre1 (5'-SYRGGRG-3', Strauss et al. 1995; Ilmen et al. 1996), PacC (5'-GCCARG-3', Denison 2000) and STRE-elements (5'-AGGGG-3', Schuller et al. 1994; Martinez-Pastor et al. 1996; Seidl et al. 2004) are present in the 5'-noncoding regions of the novel *H. atroviridis* chitinase genes. The putative *Trichoderma*-mycoparasitism related consensus sites MYC1-3 (Cortes et al. 1998) were also detected in some of the 5'-noncoding regions. We used the MEME motif discovery tool (Bailey and Elkan 1994) to identify additional motifs in the upstream regions of the cloned *H. atroviridis* chitinases. However, the only highly conserved regions that were detected were 'chitinase consensus region 1' (CCR1) 5'-GAGACGTGCTAC-3' which is present upstream of *chi18-3* and *chi18-13* and 'chitinase consensus region 2' (CCR2) 5'-CACTCTCAGATC-3' which was found in the 5'-noncoding regions of *chi18-3* and *chi18-10* (Fig 5).

The length of the 5'- and 3'- UTRs of the new chitinases was very variable ranging from 52 bp to 196 bp for the 5'-UTRs and 66 bp to 466 bp for 3'-UTRs (Table 2). Interestingly the 3'-UTR of *chi18-13* contains the motif 5'-UGUANAUA-3' which has been shown to be involved in post-transcriptional regulation. In *Saccharomyces cerevisiae* binding of the RNA-binding protein Puf3p results in rapid deadenylation and decay of the respective mRNA (Olivas and Parker 2000; Jackson et al. 2004).

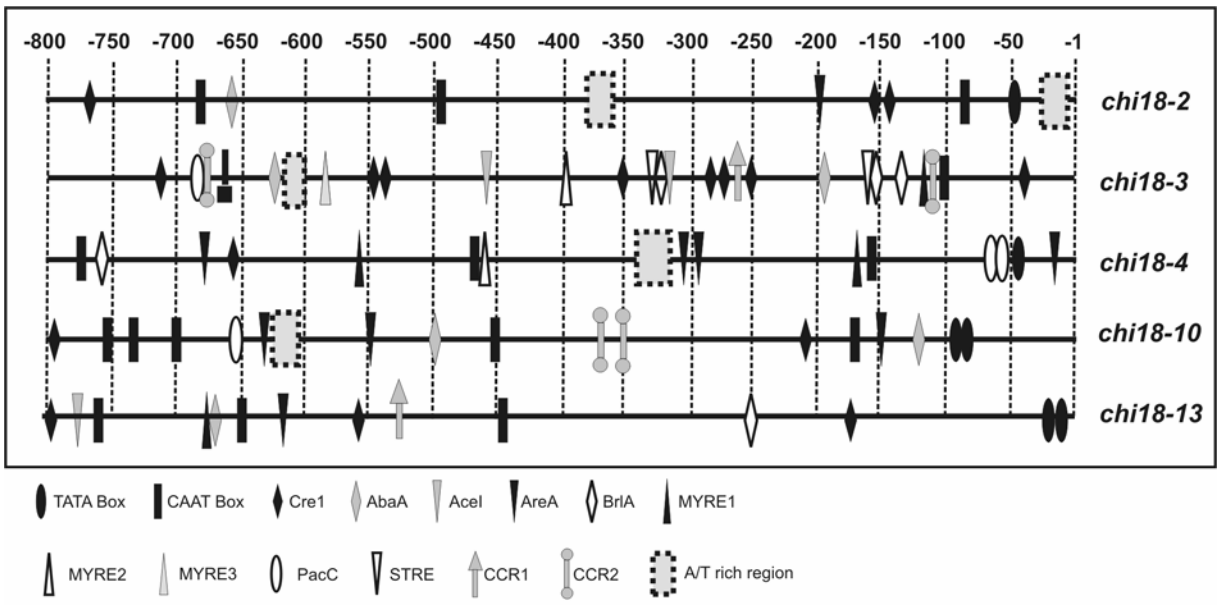

Fig. 5. Presence of consensus binding sites for known fungal transcription factors in the upstream noncoding regions of the new *H. atroviridis* chitinases. Numbers indicate the nt-positions upstream of the translation start codon (ATG; A being +1).

***Transcription profiles of five new chitinases from* H. atroviridis**

We examined the transcription of the new *H. atroviridis* chitinases under several conditions relevant for chitinase induction and biocontrol/mycoparasitism: various stages of plate confrontation assays with the fungal host *R. solani*, growth on chitin and *R. solani* cell walls, presence of the putative inducer *N*-acetylglucosamine, and starvation for carbon and/or nitrogen. *Chi18-5* (=*ech42*), whose transcription profile had been studied in this regard before (Carsolio et al. 1994; Mach et al. 1999a; Zeilinger et al. 1999; Donzelli and Harman 2001) and the constitutively expressed translation elongation factor 1-alpha (*tef1*) (Nakari et al. 1993) were used as controls. A preliminary analysis showed that most of the transcripts were of too low abundance to be detected by Northern analysis, therefore we used RT-PCR instead (Fig. 6). The results show that *H. atroviridis chi18-10* and *chi18-13* strongly respond to mycoparasitic conditions: both are upregulated during growth on fungal cell walls and before contact with the host, respectively, *chi18-10* also after contact. Neither chitin, *N*-acetylglucosamine nor starvation for carbon or nitrogen triggered the transcription of these two genes. This is in contrast to *chi18-5*, which showed a constitutive basal transcription level and induction by chitin, *R. solani* cell walls and carbon starvation, but was only moderately transcribed in confrontation assays. Transcription of *chi18-5* was even stronger when *H. atroviridis* grew on plates in the absence of its host than during confrontations. Similarly, *chi18-4*, whose translation product is ER-targeted, was transcribed constitutively and – although its transcription varied under the different conditions to some degree – no clear triggering by any of the conditions tested was found. The two putatively intracellular chitinases *chi18-2* and *chi18-3* were also constitutively transcribed.

During this study, we observed that *chi18-3* and *chi18-13* produced two cDNA bands of different size. Sequencing showed that the larger products still contained introns. Tests for contamination with genomic DNA were negative therefore implying the presence of indeed two mRNA species. Interestingly, for *chi18-13* only the unspliced mRNA could be detected when the mycelium was grown on glucose, whereas under other conditions, i.e. when the *H. atroviridis* was grown on plates, the spliced transcript was predominantly present (Fig. 6). This suggests post-transcriptional regulation mechanisms for *chi18-13*. The presence of different levels of spliced and unspliced mRNAs has also already been reported from other organisms (Clark et al. 2002; Ebbole et al. 2004; Salati et al. 2004). Similarly, for *chi18-3* the ratio of spliced to unspliced transcript and their abundance seemed to depend on growth conditions. RT-PCR-products of the other chitinase genes did not contain introns and the possibility of differential mRNA splicing could therefore not be investigated. Some of them

contained introns at the 5' ends of the coding regions, but primers for transcript analysis were placed close to the 3' end of the coding region to rule out differences in RT-PCR due to inefficient reverse transcription.

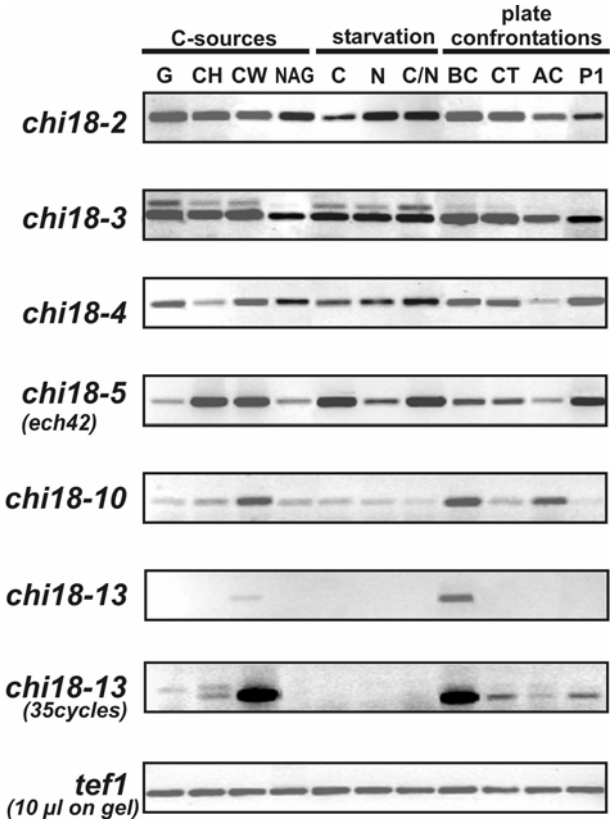

Fig. 6. Analysis of transcript formation of the *H. atroviridis* chitinases *chi18-2*, *chi18-3*, *chi18-4*, *chi18-10* and *chi18-13*. Cultivation conditions used were: growth on glucose (G), colloidal chitin (CH), *R. solani* cell walls (CW), *N*-acetylglucosamine (NAG); incubation under conditions of carbon (C)-, nitrogen (N)- and carbon as well as nitrogen (C/N)-starvation; and different stages of plate confrontation assays with the plant pathogen *R. solani*: BC, before contact; CT, contact; AC, after contact; and *H. atroviridis* alone on plates (control, P1). The *tef1* gene encoding translation elongation factor 1-alpha was used as control and the already characterized *chi18-5* (= *ech42*) was included for comparison. RT-PCR was carried out with 25 cycles (for *chi18-13* also with 35 cycles as indicated in the figure) and same volumes (40 µl) of each PCR were loaded on the gel (only 10 µl were loaded for *tef1* due to its high transcript abundance).

Discussion

In this study we identified eighteen genes encoding proteins belonging to glycoside hydrolase family 18 and two members of family 20 in the *H. jecorina* genome, whereas no members of family 19, primarily found in plants, were detected. Previously most authors named *Trichoderma* chitinases according to the putative M_r, thereby frequently also attaching an abbreviation of the species from which it was cloned (cf. Kim et al. 2002; Viterbo et al. 2002;

Ramot et al. 2004). However, the high number of chitinases in *H. jecorina* presented in this study and the clear presence of orthologues in other filamentous fungi makes a more systematic nomenclature for these proteins necessary. In this paper we have therefore applied the rules of the IUPAC-IUB Commission on Biochemical Nomenclature (CBN) to the *Trichoderma* chitinases, and numbered the isoenzymes starting with the protein having the lowest theoretical pI (Liébecq 1992). Since we assume that we have assessed the complete chitinase spectrum of *H. jecorina*, we propose that the names of *Trichoderma* chitinases should be based on their *H. jecorina* orthologue and then be numbered accordingly. In addition, we follow the proposal of Henrissat (1999) to include the glycoside hydrolase family identification number after the three letter code for the gene (*chi*). *Chi* was chosen because it is already the most commonly used name for chitinases from other organisms.

Seventeen of the *H. jecorina* family 18 chitinases members could be classified into three phylogenetic groups containing also several chitinases from other filamentous fungi, whereas Chi18-15 could not be aligned with any of them. Chi18-15 was previously cloned and characterized from *T. asperellum* as Chit36 by Viterbo et al. (Viterbo et al. 2001; Viterbo et al. 2002). The only orthologues that could be found in other organisms are a chitinase from the entomopathogen *C. bassiana*, which has been demonstrated to be involved in the attack of the fungus on insects (Fang et al. 2005) and two chitinases from *Streptomyces* spp.. These data suggest that the occurrence of *chi18-15* in the genome of *H. jecorina*, *H. atroviridis* and *C. bassiana* is due to horizontal transfer, which - because *C. bassiana* and *Trichoderma* both being members of the *Hypocreaceae* - has apparently taken place rather recently (110 - 150 mya ago; cf. Berbee and Taylor 1992).

All other family 18 chitinases have orthologues in filamentous fungi, including phylogenetically diverse ascomycetes *A. nidulans, N. crassa* and *G. zeae*. This indicates that the ancestors of these genes/proteins were formed very early in evolution of the ascomycetes and their gene products therefore very likely fulfill vital functions in the fungal life cycle and/or ecology.

Particularly for chitinases of group A orthologues were found in almost all other filamentous fungi. The closest neighbors to *Trichoderma* chitinases were mostly the *G. zeae* orthologues, indicating that evolution of these genes parallels the evolution of these species. In fact, one of these genes, *chi18-5* (*ech42*), is used as a locus for phylogenetic analysis of the genus *Trichoderma* (Lieckfeldt et al. 2000a; Kullnig-Gradinger C. M. et al. 2002). Chi18-5 is a chitinase which is well conserved throughout the ascomycetes, and is therefore likely to have a vital function in them. This is supported by the finding that for *H. jecorina chi18-5* and

the closely related *chi18-7*, encoding a putatively intracellular chitinase, a large number of ESTs can be found in the *H. jecorina* genome database, whereas only 2 to 4 or no ESTs were sequenced from other chitinases. It is intriguing that this gene has also been frequently investigated with respect to its involvement in mycoparasitism and biocontrol by *H. atroviridis, H. lixii* and *H. virens* (Baek et al. 1999; Carsolio et al. 1999; Woo et al. 1999; Zeilinger et al. 1999; Kullnig et al. 2000). Knock-outs of this gene resulted in some, albeit small, reduction in biocontrol of the corresponding strains (Baek et al. 1999; Woo et al. 1999), consistent with the interpretation that *chi18-5* rather has a different function in *Trichoderma*. Since transcription of *chi18-5* is triggered by carbon starvation, Brunner et al. (2003) speculated that its main function may be associated with mycelial autolysis.

In contrast, group B, which contains chitinases with similarity to Chi18-12 (Chit33) seems to contain proteins with more species-specific functions. One striking feature of this cluster is that we could not detect any orthologue of these proteins in *G. zeae*, indicating that this group of chitinases is dispensable for a plant pathogenic fungus and therefore most likely not essential. With the exception of Chi18-12, all members of this cluster have a fungal cellulose-binding domain (CBD) (InterPro acc. no. IPR000254), consisting of four strictly conserved aromatic amino acid residues that are implicated in the interaction with cellulose and four strictly conserved cysteine residues that are predicted to form two disulfide bonds (Kraulis et al. 1989). CBDs occur not only as domains of cellulose-degrading enzymes, but have also been identified in other polysaccharide-degrading enzymes (listed as CBM 1 entries in the CAZy database, http://afmb.cnrs-mrs.fr/CAZY/, Coutinho and Henrissat 1999). Limon et al. (2001) demonstrated that addition of a CBD to *H. lixii* Chit42 (Chi18-5) increased its activity towards high molecular mass insoluble chitin substrates, such as those found in fungal cell walls. It is therefore likely that the presence of CBDs in this cluster of family 18 chitinases may support them in chitin degradation during the mycoparasitic attack.

Interestingly, Kim et al. (2002) reported that the CBD with highest similarity to Chi18-17 (Tv-cht1) was found in an endochitinase from the entomopathogenic fungus *M. anisopliae* var. *acridum* (CHI2, GenBank acc. no. CAC07216). While this was true for the limited sample of chitinases available for the study, we found three chitinases from *H. jecorina* which are phylogenetically more close to CHI2, and indeed - together with a second chitinase from *M. anisopliae* (CHIT30; GenBank acc no. AAS55554) - form a separate clade within group B. The absence of orthologous members of this clade from all other ascomycetous genomes makes it highly likely that these proteins have a special function in chitin degradation by mycoparasitic fungi (like *Trichoderma*) and entomopathogens like

Metarhizium. Consistent with this assumption, we showed that one member of this cluster (*chi18-13*) is strongly upregulated in *H. atroviridis* in the presence of *R. solani* cell walls and in plate confrontations before contact. Thus, *chi18-13*, and likely also *chi18-14* and *chi18-16* are genes which are potentially involved in mycoparasitism and biocontrol.

It should be noted that groups A and B in the phylogenetic analysis correspond to the family 18 chitinase subgroups class V and III, respectively. Together with the chitinase classes I, II and IV, which contains members of glycoside hydrolase family 19, this classification was used for plant chitinases prior to the glycoside hydrolase family classification (Hamel et al. 1997; Fukamizo 2000). This prompted authors to use names like fungal/plant (class III) and fungal/bacterial (class V) chitinases for these subclasses due to similarities to either plant chitinases or bacterial chitinases (Takaya et al. 1998; Jaques et al. 2003). Since we detected a third subgroup of glycoside hydrolase family 18 chitinases, but our phylogenetic analysis was restricted to filamentous fungi, we simply called the subgroups (according to the clusters in Fig 2 - 4) group A (which is consistent with class V, also called fungal/bacterial chitinases), group B (consistent with class III and fungal/plant chitinases) and group C (a novel group of family 18 chitinases).

This third cluster (group C) of chitinases probably contains the most intriguing members of family 18. First, none of these proteins has as yet been characterized from any filamentous fungus, the cluster comprising - with the exception of *A. fumigatus* Chi100, for which however only a GenBank entry is available - only putative proteins from other fungal genome databases. Second, all of its members have a domain structure consisting of a class I chitin-binding domain (InterPro acc. no. IPR001002, CBM 18 according to the CAZy classification, Coutinho and Henrissat 1999), comprising 8 disulfide linked cysteines (Wright et al. 1991) accompanied by two LysM domains and then followed by the glycoside family 18 domain. Although the occurrence of orthologues of these proteins in other non-mycoparasitic ascomycetes indicates that these proteins have not specifically evolved for antagonism of other fungi by *Trichoderma*, it is intriguing to note that these high molecular weight chitinases have high similarity to the killer toxins of certain yeasts (Magliani et al. 1997) and *chi18-10* of *H. atroviridis* is only expressed during growth on fungal cell walls and during plate confrontation assays, and not upon carbon starvation or growth on chitin. No protein with similarity to the γ-subunit of the yeast killer toxins - which is the actual toxicity factor - can be found in the *H. jecorina* genome. However, since the γ-subunit causes cell cycle arrest in yeast, it is probably dispensable for the antagonization of multicellular fungi. Rather, we speculate that *Trichoderma* uses a killer-toxin like mechanism to enable the penetration of

antifungal molecules into its host. For this reason, we also consider this group of chitinases potentially interesting candidates for proteins that are connected with the biocontrol properties of *Trichoderma*.

Transcription analysis of the novel *H. atroviridis* chitinases *chit18-2*, *chi18-3*, *chi18-4*, *chi18-10* and *chi18-13* showed that, although transcript levels were generally rather low as they could not be detected by northern analysis and one has to be careful with interpreting the RT-PCR data quantitatively, a clear influence of different growth conditions and carbon sources could be detected. This indicates the functional diversity of the *Trichoderma* chitinases and that they are not just substitutes for each other, but that they have indeed specific roles in the organism. Especially the transcript patterns of *chi18-10* and *chi18-13* were explicitly linked to the presence of components apparently present in the cell wall of *R. solani*. No striking similarities in the upstream regions of *chi18-10* and *chi18-13* could be detected. The extensive *in silico* analysis of the novel *H. atroviridis* chitinase genes (Fig. 5) gives some hints as to which regulation mechanisms might be important for the respective chitinase genes, but detailed promotor studies are certainly necessary to elucidate any common consensus sites and transcription factors responsible for the regulation of *Trichoderma* chitinases.

In this study we showed for the first time that posttranscriptional regulation is involved in chitinase expression. We could demonstrate that at least for *chi18-3* and *chi18-13* different mRNA species were present and that their occurrence was influenced by the growth conditions. Additionally we found a Puf-binding site in the 3'-UTR of *chi18-13*. It should be noted that proteins with Puf-RNA binding domains (InterPro acc. no. IPR001313) are indeed present in the *H. jecorina* genome. The aspect of posttranscriptional regulation has not been much studied in filamentous fungi yet. It comprises interesting insights into the actual protein levels that can be observed *in vivo* and could contribute to a more accurate understanding of enzyme-mediated events such as mycoparasitism.

4

A screening system for carbon sources enhancing β-*N*-acetylglucosaminidase formation in *Hypocrea atroviridis* (*Trichoderma atroviride*)

Abstract

To identify carbon sources that trigger β-*N*-acetylglucosaminidase formation in *Hypocrea atroviridis* (anamorph *Trichoderma atroviride*), we have designed a screening system that consists of a combination of Biolog Phenotype MicroArray plates, which contain 95 different carbon sources, and specific enzyme activity measurements using a chromogenic substrate. The results revealed growth dependent kinetics of β-*N*-acetylglucosaminidase formation and we could show that β-*N*-acetylglucosaminidases were enhanced on carbon sources sharing certain structural properties, especially on α-glucans (e.g. glycogen, dextrin and maltotriose) and oligosaccharides containing galactose. β-*N*-acetylglucosaminidase activities were assessed in the wild-type and a *H. atroviridis* Δ*nag1* strain to study the influence of the two β-*N*-acetylglucosaminidases, Nag1 and Nag2, on total β-*N*-acetylglucosaminidase activities. Reduction of β-*N*-acetylglucosaminidase levels was strongly carbon source and growth phase dependent, indicating distinct physiological roles of those genes. Transcript abundance of *nag1* and *nag2* was increased on carbon sources with elevated β-*N*-acetylglucosaminidase activities indicating transcriptional regulation of those genes.

The screening method for the identification of carbon sources that induce enzymes or a gene of interest presented in this paper can be adapted for other purposes if appropriate enzyme- or reporter-assays are available.

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*). MS submitted.

Introduction

Some species of the soil fungus *Hypocrea* (anamorph *Trichoderma*), e.g. *H. atroviridis* (*T. atroviride*), *H. lixii* (*T. harzianum*), *H. virens* (*T. virens*) and *T. asperellum*, are potent mycoparasites against several plant pathogenic fungi and lysis of the host cell-wall has been demonstrated to be an important step in the mycoparasitic attack (Chet et al. 1998; Kubicek et al. 2001; Howell 2003; Benitez et al. 2004). Consequently, with chitin being a major cell-wall component of plant pathogens like i.e. *Rhizoctonia solani*, *Botrytis cinerea* and *Sclerotinia sclerotiorum*, several chitinolytic genes, encoding chitinases (EC 3.2.1.14) and β-*N*-acetylglucosaminidases (NAGases, EC 3.2.1.52), have been cloned from *Hypocrea/Trichoderma* spp. (Carsolio et al. 1994; Garcia et al. 1994; Hayes et al. 1994; Draborg et al. 1995; Peterbauer et al. 1996; Viterbo et al. 2001; Kim et al. 2002; Viterbo et al. 2002) and for some of them also the encoded protein has been characterized (de la Cruz et al. 1992; Boer et al. 2004; Hoell et al. 2005). The regulation of expression of NAGases and chitinases in *Hypocrea/Trichoderma* has so far, besides *Trichoderma*-host interaction assays, only been studied with respect to their upregulation during growth on colloidal chitin, chitin degradation products and fungal cell walls (Carsolio et al. 1994; Mach et al. 1999b; de las Mercedes Dana et al. 2001; Kim et al. 2002; Ramot et al. 2004). Detailed studies of the *H. jecorina* (*T. reesei*) genome revealed that *H. jecorina* has 18 different genes encoding glycoside family 18 chitinases, but interestingly only two genes encoding NAGases (glycoside family 20) (Seidl et al. 2005). Similar numbers can be expected for other *Hypocrea/Trichoderma* spp. and the corresponding two genes encoding NAGases have already been cloned from mycoparasitic *Hypocrea/Trichoderma* spp., namely *nag1* from *H. atroviridis*, *tv-nag1* and *tv-nag2* from *H. virens*, *exc1* and *exc2* from *H. lixii* and *exc1y* and *exc2y* from *T. asperellum*. It has been shown that transcription of *H. atroviridis nag1* is induced by fungal cell walls and low molecular weight chitooligosaccharides (Mach et al. 1999b). Brunner et al. (2003) reported that *nag1* is essential for triggering chitinase gene expression.

Although some of the host cell walls (e.g. from asco- and basidiomycetes) contain chitin, it is not readily available for *Hypocrea/Trichoderma* because it is linked to proteins and other polymers (Mahadevan and Tatum 1967; Schoffelmeer et al. 1999; De Groot et al. 2005). This raises the question as to which types of carbon sources, derived from fungal cell walls, possibly also trigger NAGase and chitinase expression and act as inducers for the formation of chitinolytic enzymes in *Hypocrea/Trichoderma*.

To investigate this, we have extended the Biolog Phenotype MicroArray (PM) system (Bochner et al. 2001; Bochner 2003) towards a high throughput system for screening of carbon sources for their ability to induce NAGases. This system consists of 96-well microtiter plates containing 95 different carbon sources, and has recently been adapted to investigate carbon source utilization by filamentous fungi as a means of strain characterization (Tanzer et al. 2003; Druzhinina et al. 2006). We used a combination of the PMs with specific enzyme activity measurements with a chromogenic substrate to identify carbon sources that trigger NAGase formation in *H. atroviridis* and compared those data with the transcript patterns of *nag1* and *nag2* obtained with Real-Time RT-PCR. To study the influence of Nag1 and Nag2 on total NAGase activities, enzyme activities were assessed in the wild-type and a *H. atroviridis* Δ*nag1* strain.

Methods

Strains and cultivation conditions

H. atroviridis P1 (ATCC 74058), referred to as wild-type, was maintained on PDA (Difco). The *amdS*$^+$ Δ*nag1* strain *H. atroviridis* P1ND1 (Brunner et al. 2003) was kept on a minimal medium containing acetamide as the sole nitrogen source (Seidl et al. 2004).

The medium described by Seidl et al. (2005) containing 50 mM MES (pH 6·6) and 1 % (w/v) carbon source was used throughout the experiments not involving PMs. Agar plates (1·5 % w/v) were covered with cellophane, inoculated with 6×10^6 spores and incubated in constant darkness at 25 °C. Mycelia were harvested after 24, 30, 40, and 48 h with a spatula, immersed in liquid N_2 and stored at -80 °C.

Biolog Phenotype Microarrays

Carbon utilization patterns were investigated using Biolog FF MicroPlates™ (Biolog). The FF MicroPlate test panel comprises 95 wells with different carbon-containing compounds and one well with water. Nutrients and test reagents are prefilled and dried into the 96 wells of the microplate.

Inoculum was extracted after conidial maturation (5 - 8 days) from *Trichoderma* strains by rolling a sterile, wetted cotton swab over sporulating areas. Conidia were suspended in 16 ml of sterile phytagel solution (0·25 % (w/v) phytagel, 0·03 % (v/v) Tween 40) in disposable borosilicate test tubes (20 × 150 mm). The suspension was agitated in a vortex mixer for about 5 sec, and additional inoculum added as required to adjust the OD of the

suspension to 75 (± 2) % transmission at 590 nm wavelength. 60 µl of conidial suspension were dispensed into each of the wells of a Biolog FF MicroPlate. Inoculated microplates were incubated in the darkness at 25 °C, and absorbance determined after 12, 18, 24, 36, 42, 48, 66 and 72 h. In the current study, optical density readings were taken at 750 nm (OD_{750}), which measures turbidity reflecting mycelial production on the tested substrate using a microplate reader (Biolog). Analyses were repeated at least three times for each strain. Joining cluster analysis - complete linkage rule and Euclidean distance measure as described in (Druzhinina et al. 2006) - was employed to differentiate carbon sources depending on their utilization by *H. atroviridis* P1.

Enzyme activity measurements in Biolog PMs

NAGase activities were measured by a modification of the method of Yagi et al. (1989), which is based on the release of *p*-nitrophenol from the respective aryl-chitosides. After incubation of the microplates at 25 °C in constant darkness for 30 h and 48 h, 20 µl of 50 mM potassium phosphate buffer, pH 6·7, containing 300 µg ml^{-1} 4-nitrophenyl *N*-acetyl-β-D-glucosaminide were added to each well. Microplates were incubated at 30 °C with gentle agitation. After 10 min, the reactions were terminated by the addition of 20 µl 0·4 M Na_2CO_3 to each well. The plates were then put on ice for 5 min with gentle agitation to ensure complete mixing of the stop solution in the wells. Thereafter, the OD_{400} was determined in a microplate reader (MR7000, Dynex). The formation of product was linear with time during the observation interval (optimization data not shown). Control measurements of enzyme activities were performed by omitting the substrate from the phosphate buffer. Preliminary experiments proved that this yielded more reliable results than adding the Na_2CO_3 solution at t = 0. Two independent assays with a minimum of three separate plates for each reaction were carried out.

Two sets of mean values were calculated from the OD_{400} values obtained in reactions with the substrate and from incubations without the substrate, respectively. For each carbon source the mean value of the control was then subtracted from the mean value of the enzymatic measurement. In this way calculated enzymatic activities, divided by the amount of biomass (expressed as OD_{750}) formed at the corresponding time point, result in specific enzymatic activities (S.A.), given as arbitrary units (A.U.). Outliers of enzyme activities were defined as values which were higher/lower than the average of the residual values +/- 2-fold standard deviation. Basic statistical evaluations of data were performed using the STATISTICA 6.1 (StatSoft) software package.

RNA isolation

Total RNA was extracted as described previously (Chomczynski and Sacchi 1987), but the mycelia were disrupted using a bead mill homogenization method described by Griffiths et al. (2000) with the Fastprep F120 (Qbiogene).

***Cloning and sequencing of a* nag2 *orthologue from* H. atroviridis**

The primers nag2-fw (5'-GCACGCTCTTCATTGACCAG-3') and nag2-rv (5'-CACAGTCATGCACATCAACCTG-3') were designed from conserved regions of *H. lixii exc2* (GenBank accession number S80070) to clone a 1·8 kb fragment of *H. atroviridis nag2*. The resulting sequence was submitted to GenBank (accession number DQ364461).

Transcript analysis of* nag1 *and* nag2 *by Real-Time RT-PCR

RNA was treated with Deoxyribonuclease I (Fermentas), purified with the RNeasy MinElute Cleanup Kit (Qiagen) and reverse transcribed using the RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas) and the oligo$(dT)_{18}$ primer.

For Real-Time RT-PCR experiments a 130 bp fragment of *nag1* (GenBank accession number S83231) was amplified with the primers nag1RT-fw (5'-GAACTGGAGGCTCATCTAC-3') and nag1RT-rv (5'-GATGATGTTGTCCATGTTG-3'), and a 146 bp fragment of *nag2* with the primers nag2RT-fw (5'-TGCGACCCGACCAAGAACTG-3') and nag2RT-rv (5'-CAGATGATGGTGTCGAGGCTG-3'). *tef1* (encoding elongation factor 1-alpha, GenBank accession number AF456892) was used as reference gene and a 100 bp fragment was amplified with the primers tefRe-fw (5'-TACTGGTGAGTTCGAGGCTG-3') and tefRe-rv (5'-GATGGCAACGATGAGCTG-3').

Real-time PCR amplification was carried out with the iQ 5 Real-Time PCR detection system (Bio-Rad) in a 25 µl reaction containing 12·5 µl iQ SYBR Green Supermix (Bio-Rad), each primer at a concentration of 250 nM and sample corresponding to an initial concentration of 0·5 µl total RNA. Amplification was carried out with the following PCR program: initial denaturation for 3 min at 95 °C, followed by 40 cycles consisting of 95 °C for 15 s, 52·0 °C (*nag1*), 58·7°C (*nag2*) and 54·0 °C (*tef1*), respectively, for 20 s, and 72 °C for 20 s. Successful amplification was verified by determination of the melting temperature and by agarose gel electrophoresis. For each gene a series of dilutions was performed with two

different samples to assess the efficiency of the PCR. Two independent experiments were carried out and PCR reactions were performed in triplicates.

. To ensure the absence of genomic DNA, control samples were subjected to the same procedure as described above, but no reverse transcriptase was added and PCR reactions without template were set up to rule out contamination of other PCR components.

The results of the Real-Time RT-PCR were analysed with the iQ 5 optical system software (Bio-Rad). Using the PCR base line subtracted mode, the threshold cycle was calculated for all samples and the amplification efficiency for each gene was determined. To compare different samples, the threshold cycles for *nag1* and *nag2*, respectively, were corrected with a factor for the *tef1* amplification, as described by Reithner et al. (2005). The transcript value on glucose (24 h) was arbitrarily set to 1 and all other values given as multiples (-fold induction) of it.

Results

Carbon source utilization profile of H. atroviridis

Prior to enzymatic assays we examined the growth of *H. atroviridis* wild-type on 95 carbon sources under conditions of Biolog PMs. Detailed analysis of all growth curves (data not shown) led us to conclude that the time points 36, 42 and 48 hours correspond to the phase of linear (active) growth on the majority of carbon sources. This observation is consistent with previous results on *H. jecorina* (Druzhinina et al. 2006). We applied joining cluster analysis to OD_{750} values from these time points only, to detect possible groupings of carbon sources depending on the respective growth kinetics. Data for previous (germination) and subsequent (growth saturation and sporulation) phases were used as a reference when needed. The general carbon source utilization profiles in *H. atroviridis* are represented by four distinct clusters (Fig. 1): cluster I contained best utilizable carbon sources for this species, which lead to the fastest growth and in most cases resulted in termination already after 48 h. It comprised mainly monosaccharides and polyols and also γ-amino-butyric acid, which is reported to be the best carbon source for *H. jecorina* (Druzhinina et al. 2006). Additionally, it was conspicuous that *N*-acetyl-D-glucosamine belonged to cluster I, while neither other hexosamines nor D-glucosamine promoted fast growth for *H. atroviridis*. Cluster II contained again mostly monosaccharides, but also some oligosaccharides and aryl-glucosides. On those carbon sources *H. atroviridis* exhibited a slower increase in biomass formation compared to cluster I, which was constant during the whole time-course of the experiment (72 h). Cluster

III comprised carbon sources, on which biomass formation started with a considerable delay (between 42 and 48 h) and contained predominantly di- and oligosaccharides, aryl-glucosides and L-amino acids. Cluster IV contained several L-amino acids, peptides, biogene and heterocyclic amines, some TCA-cycle intermediates, and aliphatic organic acids, which promoted only very slow growth at 48 h. Weak and delayed biomass formation was detectable on some of those carbon sources, but the majority of them lead to no growth at all.

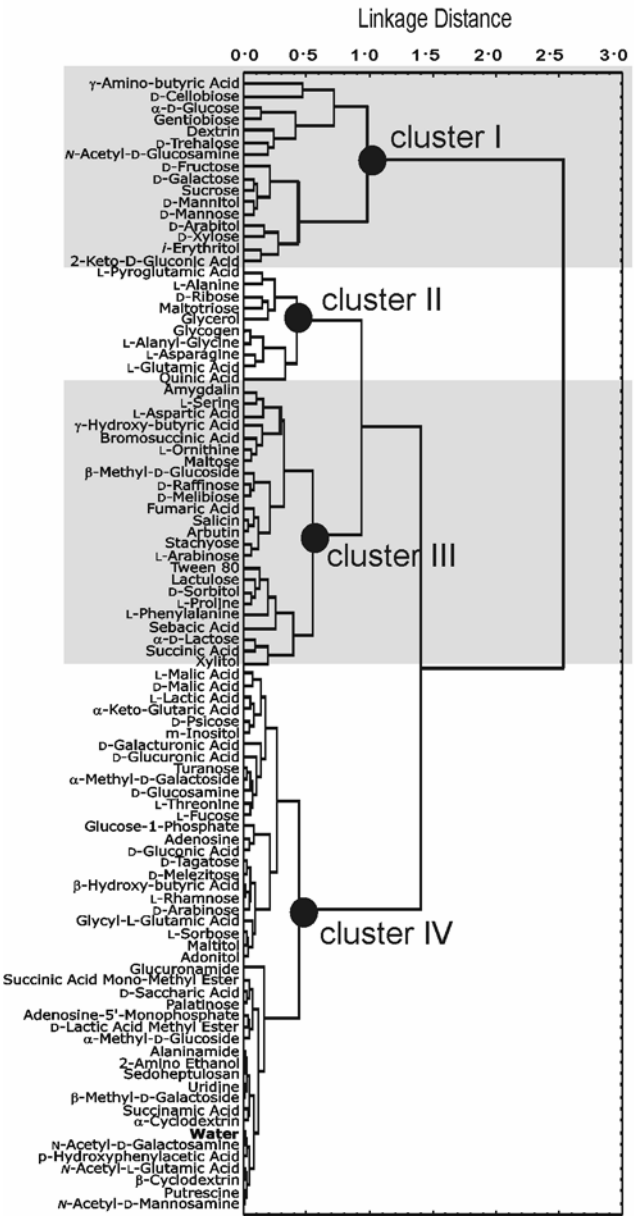

Fig. 1. Utilization of carbon sources by *H. atroviridis* P1. Joining cluster analysis was applied to mycelial growth values (OD_{750}) at 36, 42 and 48 h, what corresponds to the linear growth on the majority of carbon sources. ● indicate branching points of clusters.

Carbon sources inducing β-N-acetylglucosaminidases activity

We examined the formation of NAGase activity by *H. atroviridis* after 30 h and 48 h directly in the Biolog PMs, which has the advantage that the measurement includes both the enzyme secreted into the medium as well as that bound to the fungal cell-wall. Results of the NAGase activity measurements after 30 h are shown in Fig. 2a. The obtained values displayed low variance, indicating reproducible enzyme activity measurements.

The results showed a statistically significant correlation between NAGase activity and biomass formation ($r = 0{\cdot}60$, $p < 0{\cdot}05$; Fig. 2a) after 30 h. The growth rate influenced the level of NAGase S.As, with higher growth rates leading to statistically significant increased NAGase S.As ($r = 0{\cdot}42$, $p < 0{\cdot}05$; Fig. 2b). NAGases were formed on most carbon sources, but only a minor number yielded elevated S.As (see below).

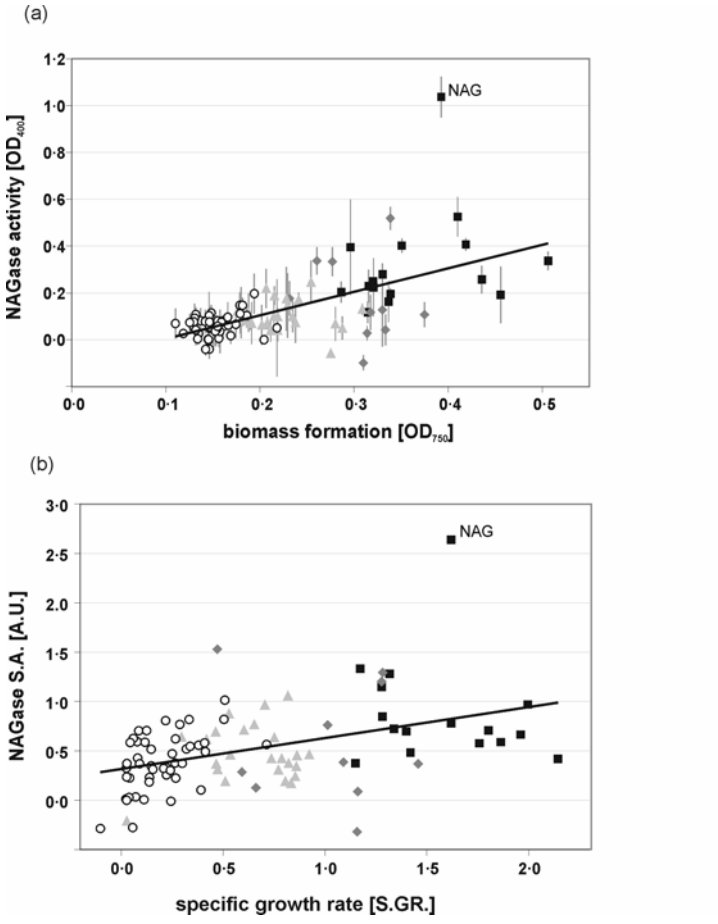

Fig. 2. (a) Correlation of NAGase activities with biomass after 30 h of growth. Vertical bars indicate the SDs for the NAGase activity measurements. (b) Influence of the specific growth rate (the growth rate between 24 and 36 h divided by the biomass at 24 h, [S.GR.]) on NAGase S.As after 30 h of growth. ■ cluster I, ◆ cluster II, ▲ cluster III, O cluster IV, (———) trendline. NAG…*N*-acetyl-D-glucosamine.

We defined two grades of elevated enzyme activities: weakly inducing carbon sources, which produced an increase in S.A. of 150 – 200 % of the calculated average of all carbon sources for a given time point and moderately inducing carbon sources, which produced S.A. higher than 200 % of the average. *N*-acetyl-D-glucosamine was the only carbon source that caused strong NAGase induction (cf Fig. 2a and b) and therefore it was omitted from the calculations of average NAGase values.

After 30 h of growth, increased NAGase S.As were found on carbon sources, which mainly belonged to the clusters I and II based on the respective growth kinetics. After 48 h of growth there was a marked shift of the affiliation of carbon sources causing increased NAGase S.As from clusters I/II to cluster II/III (Fig. 3). This correlates well with the fact that cluster III contains those carbon sources where active growth and biomass formation starts at 48h.

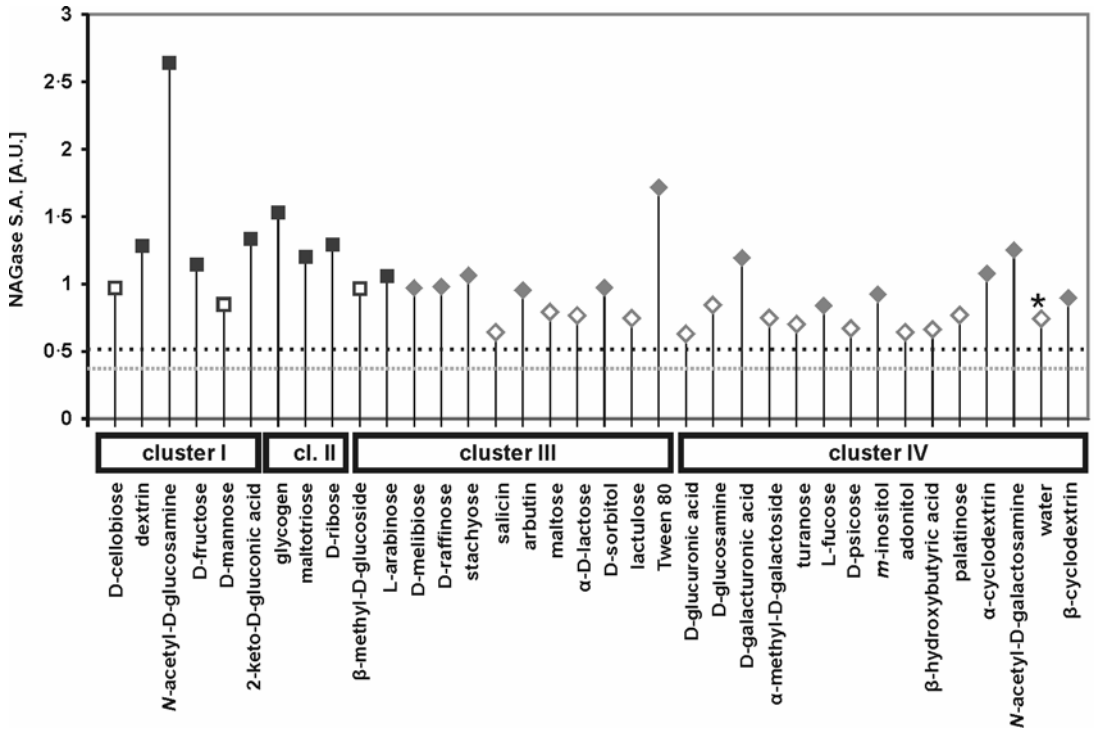

Fig. 3. NAGase specific activity (S.A.) in *H. atroviridis* on carbon sources from different growth clusters. Values above 200 % of the average are indicated by ■ (30 h) and ◆ (48 h) and values between 150 and 200 % are indicated by □ (30 h) and ◇ (48 h). (· · · ·) shows the average for all carbon sources at 30h and (····) at 48 h.

In addition to this strong inducer *N*-acetyl-D-glucosamine, which is known to induce NAGase formation already at concentrations as low as 1 mM (Mach et al. 1999b), the following carbon sources resulted also in elevated NAGase activities: the α1→4 linked glucans/glucosides glycogen, dextrin, and maltotriose, α- and β- cyclodextrin and maltose,

the ß-glucosides cellobiose and ß-methyl-glucoside, the α-glucosides palatinose, turanose, salicin and arbutin, the sugar acids 2-keto-D-gluconate and D-glucuronate, the monosaccharides D-ribose, L-and D-arabinose, D-mannose, D-fructose, D-sorbitol (which is also a constituent of Tween 80), psicose, adonitol, *m*-inositol, and Tween 80 (polyoxyethylensorbitan monooleate) and β-hydroxybutyric acid. Additionally, it was conspicuous that NAGase activity was enhanced on the D-galactose containing carbohydrates D-melibiose, D-raffinose and stachyose, lactulose, α-D-lactose, *N*-acetyl-D-galactosamine and α-methyl galactoside and also the D-galactose-derivates fucose and D-galacturonic acid. The NAGase S.As that could be found on the well solely containing water can be explained by the fact that the phytagel spore carrier is a heteropolysaccharide composed of glucuronic acid, rhamnose (6-deoxy mannose) and glucose.

Carbon sources inducing β-*N*-acetylglucosaminidases activities in a* Δnag1 *strain

For the above described results we measured total NAGase S.A., which in fact is a mixture of the activity of the two NAGases, Nag1 and Nag2. To identify whether the Nag1 and the remaining NAGase activity are coordinately or differentially regulated by inducing substances, enzyme activity measurements were carried out with a *H. atroviridis* Δ*nag1* strain (Brunner et al. 2003). We did not find significant differences when the phenotpye profile of the Δ*nag1* and the wild-type strain were compared. With respect to enzyme activities, the Δ*nag1* showed a strong reduction of NAGase activity on most carbon sources compared to the wild type. This demonstrated that Nag1 was mainly responsible for the total NAGase S.As in the wild-type. However, the effect was still strongly carbon source dependent as can be seen in Fig. 4. The main role of Nag1 during hyphal growth became apparent when the reduction of NAGase activity was compared for different growth clusters. After 30 h, clusters I and II, promoting fast growth and high biomass yield, had an average reduction of 65 % of NAGase activity, while the reduction was only 38 % in cluster III and 16 % in cluster IV.

The reduction of NAGase activities was even more pronounced after 48 h of growth: an average reduction of 64 % of NAGase activity for carbon sources of cluster I, 56 % for cluster II, 76 % for cluster III and 55 % for cluster IV.

***Transcript analysis by Real-Time RT-PCR of* nag1 *and* nag2**

In order to test whether the data obtained by enzyme measurements actually reflect the expression of the *nag1* and *nag2* genes, we have scaled up the incubation experiments to obtain enough mycelia for the extraction of RNA. Preliminary experiments with submerged

cultivations (shake flask cultures) showed that biomass formation was accompanied by early sporulation on carbon sources that provided slow growth of *H. atroviridis*, whereas on agar plates the fungus was growing slowly, but did not sporlulate during growth. Consequently, cultivations on agar plates containing the respective carbon sources and covered with cellophane, were chosen to obtain mycelial biomass for Real-Time RT-PCR analysis of *nag1* and *nag2* transcript formation. A representative set of carbon sources that displayed elevated NAGase S.As, namely dextrin, glycogen, maltotriose, D-melibiose, D-raffinose, β-methyl glucoside and *m*-inositol, were chosen for these experiments.

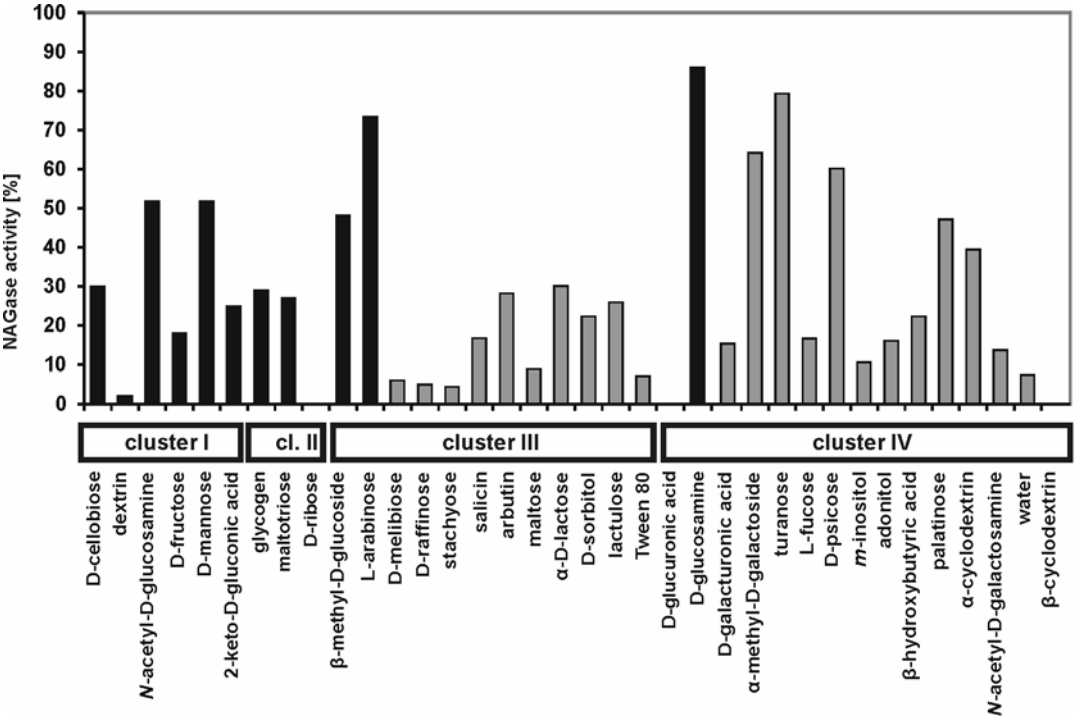

Fig. 4. Reduction of NAGase S.As in *H. atroviridis* Δ*nag1*. Values are given as % in relation to NAGase S.As in the *H. atroviridis* wild-type (Fig. 3). ■ 30h, ◆ 48h.

The results (Fig. 5 a and b) demonstrate that the NAGase activities described above are in good accordance with the respective transcript abundance of *nag1* and *nag2*. Growth on carbon sources which caused elevated NAGase activity resulted in higher transcript formation than the 'negative' controls glucose and glycerol, indicating that *nag1* and *nag2* are regulated on the transcriptional level. Increased *nag1* transcript formation was statistically significant at 24 h and 30 h (one-way ANOVA, $F(1, 18) = 6{\cdot}97$, $p = 0{\cdot}02$). The abundance of the *nag2* transcript (Fig. 5 b) essentially reflected the relative abundances of the *nag1* transcript. Thus, *nag1* and *nag2* transcription increased 2 - 4 fold on 'inducing' carbon sources in comparison

with glucose and glycerol, but the data also suggest that *nag1* is stronger regulated than *nag2* on better utilizable carbon sources.

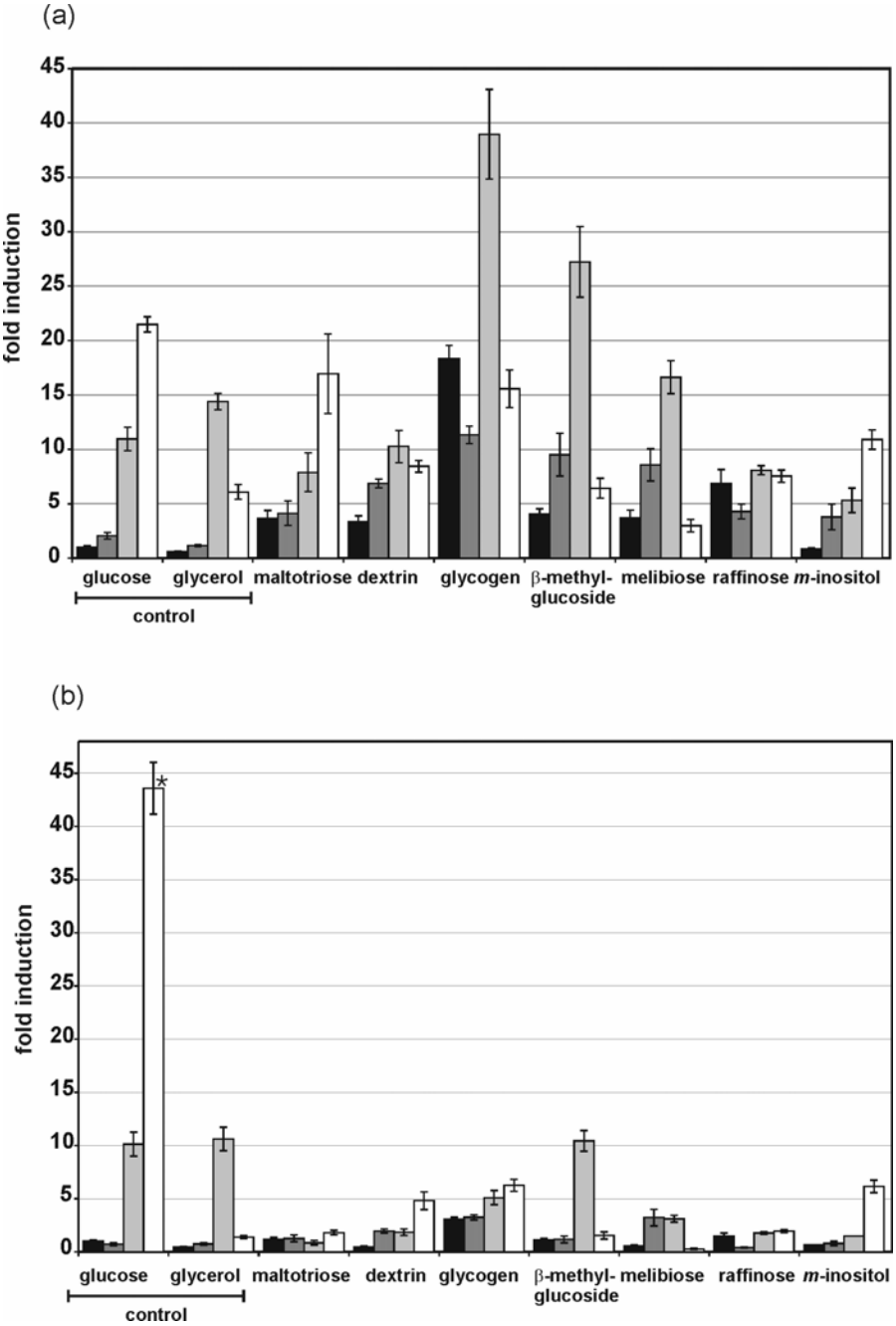

Fig. 5. Results of transcript analysis of *nag1* and *nag2* after growth for 24, 30, 40 and 48 h on selected carbon sources. Values given are ratios of (a) *nag1* and (b) *nag2* transcript levels, normalized to *tef1* as determined by Real-Time RT-PCR and are shown as 'fold induction' in relation to the respective values for glucose, 24h, which was set 1. ■ 24 h, ■ 30 h, ■ 40 h, □ 48 h. * indicates samples where conidiation could be observed.

Discussion

In this study we have analysed the stimulation of NAGase formation in *H. atroviridis*, influenced by various carbon sources. A number of carbon sources with clearly enhanced NAGase activity were detected. They comprised α-glucans like glycogen, dextrin and maltotriose, and several oligosaccharides, particularly such containing D-galactose. Polysaccharides with the same type of glycosidic linkage are constituents of the cell wall of the majority ascomycetes (Schoffelmeer et al. 1999; Latge et al. 2005; Tomazett et al. 2005) including plant pathogens (Wolski et al. 2005). Moreover, the formation of an α 1,3-glucanase has been shown to be part of the mycoparasitic response of *H. lixii* (Sanz et al. 2005). The stimulation of NAGase activity by α-glucans and D-galactose containing oligosaccharides may thus be part of a mechanism by which *H. atroviridis* senses the presence of a host cell wall containing chitin. In fact, chitin is deeply imbedded within the fungal cell wall (Mahadevan and Tatum 1967) and not readily accessible without attack of the outer glucaneous layer. The availability of the respective oligosaccharides may signal that a cell-wall degradation process has just been started.

As expected, *N*-acetyl-D-glucosamine, which has already been reported to induce *nag1* expression in *H. atroviridis* and other *Hypocrea/Trichoderma* spp. (Peterbauer et al. 1996; Mach et al. 1999b; Peterbauer et al. 2002a; Brunner et al. 2003) was also the strongest soluble inducer of NAGase activity among all tested carbon sources in our experiments.

It is an important finding that NAGase activities were not enhanced at low growth rates. This indicates that the stimulatory effect of various carbon sources detected in this study is not caused by carbon catabolite derepression at decreased growth rates (Ilyes et al. 2004). In fact, to date there is no evidence that either *nag1* or *nag2* would be subject to carbon catabolite repression at all.

Comparison of NAGase formation in the *H. atroviridis* wild-type strain and the Δ*nag1* mutant showed that the reduction of NAGase activities varied strongly among the different carbon sources and furthermore, this ratio was not constant but dependent on the growth phase. NAGase activity induced by D-glucosamine was almost completely maintained in the *H. atroviridis* Δ*nag1* mutant, indicating that D-glucosamine mainly induces *nag2*. In the same strain, induction by *N*-acetyl-D-glucosamine was reduced to about 50 %. Therefore, *nag1* is mainly induced by *N*-acetyl-D-glucosamine whereas *nag2* is induced by both *N*-acetyl-D-glucosamine and D-glucosamine. Interestingly, these two carbon sources are assimilated by *H. atroviridis* at different rates: *N*-acetyl-D-glucosamine is utilized fast, whereas D-glucosamine

provides only slow growth. This suggests that *nag2* is likely subject to a receptor-mediated induction mechanism which deserves further investigation.

D-glucosamine was reported to cause stronger induction of residual NAGases in a strain deleted in the *nag2*-orthologue in *T. asperellum* than *N*-acetyl-D-glucosamine (Ramot et al. 2004). Unfortunately, no comparison to the wild-type was given in that paper, therefore the proportion of *nag2* of total NAGase activities cannot be deduced. Although D-glucosamine caused also elevated NAGase activities in our experiments, the induction was only moderate in comparison with other carbon sources. This difference could be explicable by the fact that Ramot *et al*. used shake flask cultures in their study while we tested for NAGase activities in solid media. Influence of the cultivation method under otherwise similar conditions on gene expression has recently been the subject of several studies (Holker et al. 2004; te Biesebeke et al. 2005a; te Biesebeke et al. 2005b). However, it should be noted that we also did not get high NAGase activities when *H. atroviridis* was grown directly on D-glucosamine in shake flask cultures (data not shown) and therefore, we consider it likely that the different inducibility of NAGases by *N*-acetyl-D-glucosamine and D-glucosamine could be due to the interspecific variability between *H. atroviridis* and *T. asperellum*.

Disproportionately high levels of NAGase activity remained in the Δ*nag1* mutant when it was grown on some compounds such as L-arabinose, turanose and D-psicose, indicating prefered induction of *nag2* by these compounds. These findings show that Nag1 and Nag2 are not redundant but probably have different, specific functions in *H. atroviridis* metabolism. Separate analysis of *nag1* and *nag2* transcription on selected carbon sources generally confirmed the induction deduced from measurement of enzyme activities, although the relative abundance of the *nag2* transcript varied less strongly than was deduced from the differences in NAGase activity between the wild-type and the Δ*nag1* mutant. Brunner et al. (2003) have shown that the presence of Nag1 is necessary for full induction of chitinase activity in *H. atroviridis*, and it is possible that it also influences the induction of *nag2*. However, other factors such as stability of the enzyme and proteolytic degradation may influence this process.

Multiple genes encoding NAGases are also present in all fungi whose genome sequenceas are available today (e.g. *Phanerochaete chrysosporium*, *Neurospora crassa*, *Magnaporthe grisea*, *Fusarium graminearum*, *Botrytis cinerea*, *Aspergillus fumigatus*, *A. nidulans*, *A. oryzae*), and which are no mycoparasites. This implies that the physiological role of these enzymes is not exclusively connected with mycoparasitism. The positive correlation between NAGase activities and the growth rate in *H. atroviridis*, as found in this work, and its

occurrence in the cell-wall (Brunner et al. 2003; Ramot et al. 2004) suggests an involvement of these enzymes in cell wall turnover. This is consistent with previous results (Brunner et al. 2003) that the Δ*nag1* strain has a reduced rate of autolysis.

The screening system developed in this paper was based on a combination of the PMs and an enzymatic assay using a chromogenic substrate. It is a fast and reliable screening method to measure enzymatic activities on a large set of carbon sources. Also, it can be adapted for enzyme activity measurements of a variety of extracellular and cell wall bound enzymes. By using appropriate promoter-fusion reporter systems, this system can be further used to monitor the expression of specific genes, even coding for intracellular enzymes. In fact, we have already tested one such system using the secreted *Aspergillus niger* glucose oxidase *goxA* gene fused to the *nag1*-promotor (Mach et al. 1999b), and the data obtained (V. Seidl, unpublished data) were generally concordant with those reported in this study. However, other highly sensitive and secretion independent reporter systems such as the green fluorescent protein (Larrainzar et al. 2005) or luciferase (Morgan et al. 2003) may prove to be even more effective in combination with the PM system. The rapidly growing number of fungal genome sequence databases leads to an increase in the identification of genes for which orthologs in even closely related species do not exist (O'Brian et al. 2003; Dogra and Breuil 2004; Schmoll et al. 2004). Such findings direct the attention of researchers to novel, yet uncharacterized enzymes with unknown substrate specificities and physiological functions. Even for proteins with defined enzymatic activities knowledge about their physiological roles is often restricted to transcript analysis for a limited set of growth conditions. Having an array-type system available to screen carbon sources and/or growth conditions under which a novel gene is actually expressed would facilitate assigning functions to newly found genes and greatly increase the knowledge about their metabolic functions. In fact, the differences in regulation between *nag1* and *nag2* as shown in this work would probably have gone undetected without this tool.

Concluding remarks

The different topics that were investigated during this work are a good example to illustrate the variety of mechanisms and strategies of *Hypocrea/Trichoderma* to survive in its environment, and which may thus be considered to be used to improve its competitivity and action as a biocontrol agent:

The studies presented in chapter 1 were a first step towards understanding the polyol pools of *H. atroviridis* during normal growth and different kinds of osmotic stress. *H. atroviridis*, subjected to osmotic stress, responded by raising its intracellular glycerol level. Biomolecular investigations of the basis of this process revealed that upon osmotic stress glycerol biosynthesis occurred via the glycerol dehydrogenase (Gld1) but, despite a fast response of *gld1* transcript levels under high carbon source concentrations, *gld1* transcript levels rose only very slowly under conditions of salt stress. It was conspicuous that *H. atroviridis* accumulated - in contrast to *A. nidulans* - predominantly glycerol during unstressed growth and osmotic stress. An interesting topic for follow-up studies would be the investigation of polyol pools of appressoria during mycoparasitism and the pathways involved in the biosynthesis of the respective polyols. The step of appressorium formation of the mycoparasitic attack has so far not been studied in *Hypocrea/Trichoderma* spp.

With respect to the mycoparasitic attack, despite the knowledge about the involvement of numerous enzymes, secondary metabolites and the influence of other physiological factors, it is unknown, which group of enzymes and possibly also other mechanisms are key-components of mycoparasitism. In the study presented in chapter 2, *H. jecorina* and available mutants of this species were used to investigate new aspects of this process. First it was evaluated whether *H. jecorina* can antagonize plant pathogenic fungi and protect plants against them. *H. jecorina* displayed against *Pythium ultimum* but not against *Rhizoctonia solani* good antagonism in plate confrontation assays and also statistically significant, albeit rather weak, biocontrol action in greenhouse experiments. Interestingly, although *P. ultimum* has cellulose as its main cell wall component, a cellulase negative *H. jecorina* mutant showed a similar range of antagonism in plates and plant protection. This questions the importance of chitinases during mycoparasitism of e.g. *H. atroviridis* of *R. solani* and emphasizes the role of other lytic enzymes and mechanisms during this process. Carbon catabolite (de)repression was also an important component for the biocontrol efficiency of *H. jecorina*. A carbon catabolite derepressed *H. jecorina* mutant antagonized *P. ultimum* on plates more actively and also increased the survival rates of *P. ultimum*-inoculated zucchini plants. The fact that a

number of *H. jecorina* gene knock-out mutants are available and the rather weak biocontrol properties of *H. jecorina* make it an interesting target to study positive and negative influences of selected genes on the biocontrol potential of *H. jecorina*.

Despite the clear ability of *H. jecorina* to antagonize *P. ultimum*, its inability to antagonize *R. solani* suggests that *H. atroviridis* possesses additional, better developed, sensing and attack mechanisms and has obviously better biocontrol abilities. Many biocontrol studies with *H. atroviridis*, *H. lixii*, *H. virens* and *T. asperellum* focused on the chitinolytic enzyme system of *Trichoderma*. While the range of importance of single chitinases for the mycoparasitic attack is not known, it has been demonstrated for several of them that they are at least involved in mycoparasitism. In the work presented in chapter 3, the *H. jecorina* genome database - the first sequenced *Hypocrea/Trichoderma* species - was used to obtain a comprehensive insight into the chitinolytic potential of *Hypocrea/Trichoderma*. The *H. jecorina* genome contains 18 ORFs encoding putative chitinases, all of them belonging to glycoside hydrolase family 18. Eleven of them encode yet undescribed chitinases. Five novel chitinase genes were subsequently cloned from *H. atroviridis*. Transcription of *chi18-10* (belonging to group C, a novel phylogenetic subgroup of chitinases with similarity to *Kluyveromyces lactis* killer toxins) and *chi18-13* (belonging to a novel clade in group B) was triggered upon growth on *Rhizoctonia solani* cell walls, and during plate confrontation tests with the plant pathogen *R. solani*. Detailed studies about the properties of these two chitinases, their carbohydrate binding domains, substrate specificities, patterns and eventually localization of their expression and the evaluation of the importance of the novel group of killer-toxin similar chitinases and their mode of action provides certainly a very interesting basis for further studies on this topic.

Transcription of the novel *H. atroviridis* chitinases *chi18-10* and *chi18-13* was specifically triggered by components of the *R. solani* cell wall that were not the chitin itself (as their transcription was not upregulated upon growth on chitin). This raises the question which types of carbon sources, derived from fungal cell walls, can possibly trigger β-*N*-acetylglucosaminidase and chitinase expression and act as inducers for the formation of chitinolytic enzymes in *Hypocrea/Trichoderma*. In the work presented in chapter 4, β-*N*-acetylglucosaminidase activities were analysed on a set of 95 different carbon sources. The screening system used for this purpose was a combination of the Biolog phenotype microarrays and an enzyme activity measurement method with a chromogenic substrate. It can be used to study the expression of extracellular or cell wall bound enzymes, but also the expression of specific genes using reporter gene assays. In this study, the influence of 95

carbon sources on β-*N*-acetylglucosaminidase activities was investigated. The results showed that the expression of β-*N*-acetylglucosaminidases is not restricted to mycoparasitism-related growth conditions but that their expression is dependent on the growth rate. However, the growth-rate dependent expression is superimposed by a carbon source specific effect on β-*N*-acetylglucosaminidase expression which was specifically found on glucans, glucosides and oligosaccharides containing a galactose moiety. The question arises whether the elevated β-*N*-acetylglucosaminidase activities were due to the involvement of β-*N*-acetylglucosaminidases in hyphal growth and cell wall turnover or whether those carbon sources elicit an increased expression of enzymes that are responsible for mycoparasitic host sensing mechanisms.

The results from this work thus point out eventual directions for future *Hypocrea/Trichoderma* research projects and raised new questions about the investigated topics. The versatile abilities of *Hypocrea/Trichoderma* spp. to react on environmental stimuli and the complex interplay of different factors and mechanisms that influence this reaction make it both, difficult and an interesting and fascinating organism to study. Quite unnoticed from the general public *Hypocrea/Trichoderma* spp. are among the most ubiquitously occurring fungi that can be found in very diverse habitats. *Hypocrea/Trichoderma* research was in the past mainly divided into those people who studied the cellulolytic enzyme system of *H. jecorina* and others who investigated the biocontrol potential of *Hypocrea/Trichoderma* spp. in more detail. Only in the 1990ies phylogenetic studies on the genus *Hypocrea/Trichoderma* were increasingly conducted and resolved mycoparasitic *Hypocrea/Trichoderma* spp. into *H. lixii*, *H. atroviridis*, *H. virens* and *T. asperellum* that were mainly used for biocontrol experiments. With gradually more research groups working with *Hypocrea/Trichoderma* spp., also the diversity of covered topics is rapidly increasing. In the past years the topics of *Hypocrea/Trichoderma* spp research extended to the fields of signal transduction, protein secretion and stress, light responses, monosaccharide catabolism, mushroom diseases, human pathogenicity and others. Biocontrol research was split up into mycoparasitic and plant defense response studies and went more into the details of the specific roles of hydrolytic enzymes and secondary metabolites in those processes.

Two aspects can be observed in emerging interest and knowledge about *Hypocrea/Trichoderma* spp: on one hand understanding of e.g. signal transduction pathways can be valuable for different topics such as cellulase induction and mycoparasitism and the increasing number of studies certainly helps to form an overall picture of the physiology of *Hypocrea/Trichoderma*. On the other hand, studies like EST sequencing projects or strain characterization with Biolog phenotype microarrays of different *Hypocrea/Trichoderma* spp.

demonstrate impressively the huge variability within the genus *Hypocrea/Trichoderma* which is often underestimated or overlooked by researchers who are mainly focussed on molecular biology. The fact that up to now approximately 116 *Hypocrea/Trichoderma* spp. are already characterized should make clear that the variability within this genus is certainly not negligible and great caution should be paid when findings and conclusions are applied to different species.

The mechanisms of biological control turn out to be far more complex than previously expected. Already only at the level of enzymes involved in this process, a huge consortium of chitinases, glucanases and proteases has been shown to participate in *Hypocrea/Trichoderma*-host fungus interactions. A number of previous studies tried to elucidate the role of certain chitinases in this process, but the total number of 18 chitinase encoding genes that can be found in the *H. jecorina* genome database - and a similar number can be expected for other *Hypocrea/Trichoderma* spp. - sheds a different light on this story. Although certainly not all chitinases will be involved in mycoparasitic responses, it is still obvious that it will probably not be possible to abolish mycoparasitic propterties with single gene deletion mutants. With respect to studying the expression of specific genes during mycoparasitism, the fact that the mycoparasitic attack and lysis of the host cell wall seems to be a very localized process and a means to *penetrate* the cell wall and not to use it as a carbon source, brings up the question how significant the upregulation is that could be expected for genes essential for this process. However, more emphasis on detailed microscopic studies with e.g. GFP-fusions of potentially interesting proteins during *Hypocrea/Trichoderma*-host fungus interactions could eventually greatly increase the knowledge about mycoparasitic mechanisms.

Despite the interesting aspects of mycoparasitism it should not be disregarded that biocontrol also involves *Hypocrea/Trichoderma*-plant interactions and biocontrol research has recently also focussed more on the abilities of certain *Hypocrea/Trichoderma* spp. to induce localized and systemic resistance responses in plants. Studies about the root colonization potential of *Hypocrea/Trichoderma* spp. and proteins and secondary metabolites involved in this process are presently a very active field of research.

The currently ongoing sequencing project of *H. atroviridis* by the Joint Genome Research Institute of the US Department of Energy emphasises the increasing interest of research and industry in the above mentioned topics. The genome database of this mycoparasitic species will certainly significantly increase the understanding of biocontrol and boost research of this interesting topic to a new level.

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