Glycobiology vol. 21 no. 1 pp. 122–133, 2011
doi:10.1093/glycob/cwq142
Advance Access Publication on September 14, 2010

Analysis of subgroup C of fungal chitinases containing chitin-binding and LysM modules in the mycoparasite *Trichoderma atroviride*

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Received on May 24, 2010; revised on September 6, 2010; accepted on September 6, 2010

Fungi have a plethora of chitinases, which can be phylogenetically divided into three subgroups (A, B and C). Subgroup C (sgC) chitinases are especially interesting due to their multiple carbohydrate-binding modules, but they have not been investigated in detail yet. In this study, we analyzed sgC chitinases in the mycoparasites *Trichoderma atroviride* and *Trichoderma virens*. The expression of sgC chitinase genes in *T. atroviride* was induced during mycoparasitism of the fungal prey *Botrytis cinerea*, but not *Rhizoctonia solani* and correspondingly only by fungal cell walls of the former. Interestingly, only few sgC chitinase genes were inducible by chitin, suggesting that non-chitinous cell wall components can act as inducers. In contrast, the transcriptional profile of the most abundantly expressed sgC chitinase gene *tac6* indicated a role of the protein in hyphal network formation. This shows that sgC chitinases have diverse functions and are not only involved in the mycoparasitic attack. However, sequence analysis and 3D modelling revealed that TAC6 and also its ortholog in *T. virens* have potentially detrimental deletions in the substrate-binding site and are thus probably not catalytically active enzymes. Genomic analysis showed that the genes neighboring sgC chitinases often encode proteins that are solely composed of multiple LysM modules, which were induced by similar stimuli as their neighboring sgC chitinase genes. This study provides first insights into fungal sgC chitinases and their associated LysM proteins.

Keywords: cell walls / chitinases / LysM / mycoparasitism / *Trichoderma*

Introduction

Chitin is an abundant, insoluble biopolymer composed of β-1,4-linked subunits of the acetylated amino sugar *N*-acetylglucosamine. It is the main compound of invertebrate exoskeletons and an essential component of the fibrillar structural core of the cell walls of filamentous fungi. Cell walls protect fungi against environmental stress factors, e.g. desiccation or high osmotic pressure, and also against mechanical injuries and hostile compounds such as toxins and lytic enzymes (Latgé 2007). Filamentous fungi have a large machinery of chitin-degrading enzymes and have on average 15 different chitinases (Seidl 2008). In the carbohydrate active enzymes (CAZy) classification (http://www.cazy.org; Cantarel et al. 2009) fungal chitinases belong to glycoside hydrolase (GH) family 18 and catalyze the hydrolysis of the β-1,4 linkages in chitin and chitooligomers, resulting in the release of short-chain chitooligosaccharides. The potential biological functions of fungal chitinases cover a plethora of different aspects including cell wall remodeling during the fungal life cycle and the degradation of exogenous chitin as nutrient source. Fungi with a more aggressive lifestyle such as mycoparasitic, entomopathogenic and nematode-trapping fungi use chitinases to directly attack the prey. In mycoparasitic *Trichoderma* spp., e.g. *Trichoderma atroviride* and *Trichoderma virens*, chitinolytic enzymes have been shown to be involved in cell wall hydrolysis of the host during the mycoparasitic attack. Genome surveys revealed that fungal chitinases are highly variable with respect to their molecular masses and modular structures and some of them have multiple carbohydrate-binding modules (CBMs) (Seidl et al. 2005; Karlsson and Stenlid 2008). The substrate-binding clefts of chitinases can have different architectures, where (processive) exochitinases often have a tunnel-shaped substrate-binding cleft, whereas endochitinases tend to have a more shallow and open substrate-binding region (Horn, Sikorski et al. 2006; Horn, Sorbotten et al. 2006). Some GH18 proteins show amino acid (aa) alterations in their actives sites and lack the

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essential catalytic acid and are thus likely to act rather as lectins than as enzymes (Funkhouser and Aronson 2007). Based on a genomic survey and a phylogenetic analysis of chitinases in *Trichoderma reesei*, we previously proposed the clustering of fungal chitinases in three subgroups A, B and C (Seidl et al. 2005). This classification has recently been applied in a phylogenetic survey of chitinases from different fungi and was shown to overall reflect the variability of fungal chitinases (Karlsson and Stenlid 2008). In addition to their phylogenetic relationships based on the aa sequences of the GH18 modules, chitinases from the three subgroups also have different modular structures. Subgroup A chitinases do mostly not contain CBMs, whereas many subgroup B chitinases have CBMs at their C-terminal ends. The CBMs of subgroup B chitinases from *Trichoderma* spp. belong exclusively to the CBM1 family (as classified in the CAZy database), a family of CBMs that have been shown to possess cellulose and chitin-binding properties. A strong expansion of subgroup B chitinases with family 1 CBMs was found in the genomes of mycoparasitic *Trichoderma* spp. (Karlsson and Stenlid 2009). Subgroup C (sgC) chitinases have particularly interesting features. They contain family 18 CBMs (chitin-binding) and LysM modules (LysMs) and their predicted open-reading frames (ORFs) encode proteins with a molecular mass of up to 200 kDa. In comparison, the average molecular mass of subgroups A and B chitinases is 35–45 kDa.

LysMs, which are now also classified in the CAZy database as CBM family 50, were first studied in enzymes degrading bacterial cell walls and are thought to have peptidoglycan-binding functions in these proteins (Buist et al. 2008). Later, LysMs were also found in various other prokaryotic enzymes, e.g. peptidases and esterases (Buist et al. 2008). Recent genome analysis of an increasing number of eukaryotic species revealed that LysMs occur also frequently in eukaryotic proteins (Zhang et al. 2007, 2009; Lohmann et al. 2010). In plants, LysMs are found in receptor-like kinases (LysM-RLKs), which are composed of one to three extracellular LysMs, a single-pass transmembrane domain and an intracellular kinase. In legume plants, LysM-RLKs act as receptors for nodulation (Nod) factors, which are lipochitinoligosaccharide-signaling molecules that are secreted by symbiotic nitrogen-fixing *Rhizobium* bacteria to mediate symbiosis with their hosts (Limpens et al. 2003). However, LysM-RLK genes are also found in the genomes of non-legume plants, indicating their involvement in processes other than establishing symbiosis (Shiu et al. 2004; Zhang et al. 2007). In *Arabidopsis*, LysM-RLKs were shown to be involved in defense signaling against fungal attacks (Miya et al. 2007; Wan et al. 2008). Additionally, LysMs are present in plant chitinases and have been implied to have chitin-binding and antifungal activities in the fern *Pteris rykuyuensis* (Ohnuma et al. 2008; Onaga and Taira 2008). Binding to chitin oligosaccharides was demonstrated for plant LysM proteins (Ohnuma et al. 2008), and it was shown that they have a high affinity for longer oligosaccharides (Iizasa et al. 2010). In fungi, we reported the occurrence of multiple LysMs in sgC chitinases in *T. reesei* and *T. atroviride* (Seidl et al. 2005), and in the plant pathogenic fungus *Cladosporium fulvum*, the protein Ecp6, a virulence factor with LysMs, was recently shown to bind chitin oligosaccharides and prevent elicitation of host plant immunity (Bolton et al. 2008; de Jonge et al. 2010). A survey of fungal genome sequences showed that secreted LysM-containing proteins are widespread in the fungal kingdom and are not only found in pathogenic, but also saprobic fungi (de Jonge and Thomma 2009). The functions of these proteins in saprobic fungi, as well as their carbohydrate-binding specificities, are still unknown. de Jonge and Thomma (2009) proposed that in plant pathogenic fungi LysM effectors—secreted proteins solely consisting of (multiple) LysMs—might have a role in the sequestration of chitin oligosaccharides to dampen host defense responses, i.e. secretion of chitinases, which was confirmed for Ecp6 in their recent study (de Jonge et al. 2010).

In the *T. reesei* genome, four genes encoding sgC chitinases are present and all of them contain two LysMs (Seidl et al. 2005). Analysis of the predicted ORFs revealed considerable sequence differences at the N- and C-terminal ends of these proteins. *T. reesei* has only weak mycoparasitic activities (Seidl et al. 2006; Druzhinina et al. 2010) and so far no information was available about the numbers and variability of sgC chitinases in fungi with a more aggressive, chitinolytic lifestyle, e.g. mycoparasites from the genus *Trichoderma*. The release of the genome sequences of the mycoparasites *T. atroviride* and *T. virens* by the JGI (Joint Genome Institute, US Department of Energy) enabled us to assess the complete spectrum of their chitinolytic enzymes and gain new insights into the proposed roles of chitinases. Genome analysis revealed that *T. atroviride* and *T. virens*, with their respective 29 and 36 chitinases, have an even higher diversity of chitinases than that found in *T. reesei*. Furthermore, *T. virens* contains the highest number of chitinases at all found in fungal genomes so far (Kubicek et al., in preparation). This emphasizes the link between a mycoparasitic lifestyle and the presence of chitinases in the mycoparasitic fungi.

This study presents a detailed analysis of sgC chitinases from *Trichoderma*. Owing to their divergent sequences, automatic annotation of sgC chitinases turned out to be rarely correct. We therefore assessed the complete ORFs for several genes encoding sgC chitinases in *T. atroviride* and *T. virens* to be able to construct more reliable protein models. Further, we analyzed the modular structures and phylogenetic relationships of the resulting proteins and constructed 3D models of representative sgC chitinase catalytic modules. A survey of the genomic loci revealed that the genes next to sgC chitinases often contain multiple LysMs but no apparent catalytic modules, which renders them pure carbohydrate-binding proteins without hydrolytic activities. Further, we analyzed the transcript patterns of sgC chitinase and LysM genes under a variety of different growth conditions to gain insights into the potential biological functions of the respective proteins. The results showed that sgC chitinase genes are not coregulated and indicated that they do not have a common, general role in, for example, mycoparasitism, but have diverse functions in the fungal life cycle.

Results

Mycoparasitic sgC chitinases display two types of modular structures

The release of genome sequences of *T. atroviride* and *T. virens* (http://genome.jgi-psf.org/pages/fungi/home.jsf) enabled us to

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study the complete set of chitinases in these mycoparasitic fungi. First data on sgC chitinases from *T. reesei* already indicated that this subgroup of chitinases contains proteins particularly variable in their aa sequences, which have almost no similarities outside of the GH18 modules and CBMs (Seidl et al. 2005). We were therefore interested how different sgC chitinases were with respect to their numbers, protein sequences and modular structures in the mycoparasites *T. atroviride* and *T. virens* in comparison to *T. reesei*, which has only weak mycoparasitic activities (Seidl et al. 2006; Druzhinina et al. 2010). Upon analysis of the genome databases of *T. atroviride* and *T. virens*, we found a strong expansion of sgC chitinases in the two mycoparasites, i.e. 9 in *T. atroviride* and even 15 in *T. virens* versus 4 in *T. reesei*. An in-depth bioinformatic study was carried out to deduce the correct protein models of sgC chitinases in the two mycoparasites. Owing to low similarities to available protein sequences and among each other, particularly at their C-terminal ends, ORFs automatically predicted by the genome annotation software were often incorrect in the sequence stretches neighboring the GH18 modules. Our ORF analysis therefore involved nucleotide and aa sequence alignments as well as cDNA sequencing, and the resulting protein sequences were used to update and correct the protein models in the gene catalog of the JGI genome databases of *T. atroviride* and *T. virens*. The sgC chitinases from *T. atroviride* and *T. virens*, which we named TAC1-TAC9 and TVC1-TVC15, respectively, their protein IDs in the JGI database, length (aa) and theoretical molecular masses are listed in Table I. Analysis of the protein sequences in both species revealed that some sgC chitinases have clear orthologs in the other species (aa sequence identities of almost 100%; e.g. TAC6/TVC6 and TAC7/TVC7), whereas other sgC chitinases have unique N- and C-terminal ends and appear to have no clear ortholog. For a few sgC chitinases, such as TAC9, TVC11 and TVC15, only one of the 10 algorithms used in the JGI genome database suggested a protein model. Factors further complicating the assessment of the ORFs were low mRNA expression levels and the presence of unspliced mRNAs (Gruber and Seidl-Seiboth, data not shown). Because of the large size of the sgC chitinase-encoding genes, it was therefore not possible to find a suitable protein model for TAC9, TVC11 and TVC15. However, the remaining 21 genes were successfully annotated and the ORFs and predicted protein sequences can be found in their corrected form in the JGI genome database, v2.0 of the *T. atroviride* and *T. virens* genomes (Table I).

Next, an analysis of the modular structure of the sgC chitinases was performed. The results for *T. atroviride*, *T. virens* and *T. reesei* are shown in Figure 1A, B and C, respectively, and indicate that sgC chitinases have two general types of architecture. In the first type, the chitinases have their GH18 module approximately located in the middle of the protein and contain N-terminally of the GH18 module one CBM18 and two LysMs. All sgC chitinases from *T. reesei* exhibited this kind of structure, but only two from *T. atroviride* and eight from *T. virens* shared it. The other sgC chitinases in the two mycoparasites have only CBMs of family 18 but no LysMs and the CBMs as well as the GH18 module are all located in the N-terminal part of the proteins. No functional

Table I. SgC chitinases in *T. atroviride* and *T. virens*

Protein name	Protein ID[a]	Size (kDa)	Length (aa)
TAC1	348130	145.7	1311
TAC2	348134	125.4	1120
TAC3[b]	10286	141.7	1327
TAC4	348132	195.9	1774
TAC5	348128	179.3	1645
TAC6	348129	174.8	1575
TAC7	247300	139.6	1289
TAC8	282750	123.1	1142
TAC9	93784	—[c]	—[c]
TVC1	194859	145.0	1364
TVC2	29598	120.5	1079
TVC3	49587	154.3	1465
TVC4	81573	199.3	1804
TVC5	112098	183.5	1682
TVC6	53627	165.0	1492
TVC7	53606	139.6	1285
TVC8	201336	112.4	1057
TVC9	43963	139.2	1286
TVC10	112097	164.1	1523
TVC11	61563	—[c]	—[c]
TVC12	70999	98.2	883
TVC13	52866	156.6	1444
TVC14	66617	139.4	1273
TVC15	206731	—[c]	—[c]

[a]The protein IDs refer to the JGI *T. atroviride* and *T. virens* genome databases, v2.0.
[b]The name CHI18-10 was used for this protein in a previous study (Seidl et al. 2005).
[c]These parameters were not assessed because the protein models are incomplete.

domains could be annotated in the C-terminal parts of sgC chitinases. A phylogenetic analysis of sgC chitinases was conducted using exclusively the sequences of the GH18 modules in order to exclude a bias by the CBMs. The phylogenetic tree (Figure 1D) revealed two major clades: sgC chitinases with LysMs grouped exclusively in clade I of the tree, whereas that the majority of sgC chitinases without LysMs grouped in clade II. This shows that the differences in the modular architecture of sgC chitinases are also evolutionarily reflected in the aa sequences of the GH18 modules and thus not the result of a recent recombination event.

Structure modelling indicates exo-enzyme activity and alterations in the substrate-binding sites of some sgC chitinases

In order to obtain information about the architecture of the substrate-binding sites, a 3D model of the TAC2 sequence was generated using the human chitotriosidase structure as a template (PDB ID: 1GUV (Fusetti et al. 2002), 25% sequence identity to TAC2). The TAC2 sequence was chosen for modeling as it had the fewest insertions/deletions (indels) of the *T. atroviride* sequences compared with the template sequence. Human chitotriosidase is a chitinolytic enzyme expressed in maturing macrophages, which suggests that it plays a part in antimicrobial defense and is considered to be an exochitinase (Fusetti et al. 2002). An alignment of the human chitotriosidase and TAC2 is shown in Supplementary data, Figure S1A and an alignment of the GH18 modules of sgC chitinases

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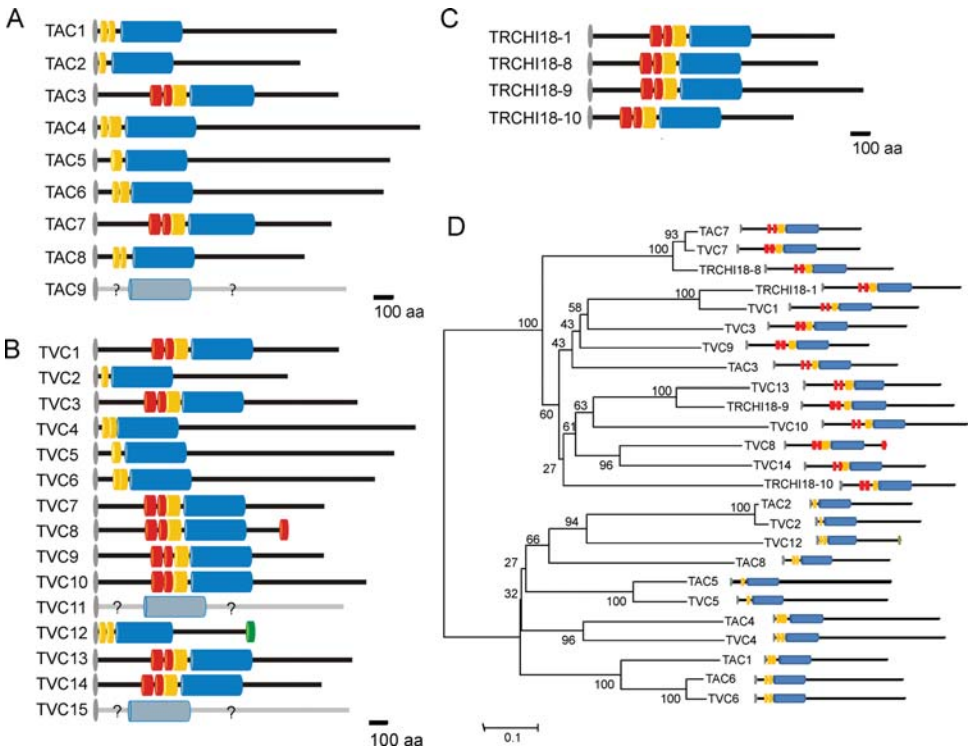

Fig. 1. Modular structure and phylogenetic relationships of *Trichoderma* sgC chitinases. The models are drawn to scale, GH18 modules (IPR001223) are indicated with blue barrels, LysMs (IPR002482) are indicated in red, CBM18s (IPR001002) in yellow and signal peptides, predicted with SignalP (Emanuelsson et al. 2007), in gray. (**A**) *T. atroviride* sgC chitinases, (**B**) *T. virens* sgC chitinases, (**C**) *T. reesei* sgC chitinases (Seidl et al. 2005), (**D**) Phylogenetic tree of *Trichoderma* sgC chitinases. It should be noted that only bootstrap coefficients above 50 indicate stable clades.

from *Trichoderma* spp. is shown in Supplementary data, Figure S1B. All sgC chitinases share the typical [SA]XGGW motif, in which tryptophane has been shown to be important for chitinase processivity and efficiency on crystalline chitin (Horn, Sikorski et al. 2006; Horn, Sorbotten et al. 2006; Zakariassen et al. 2009). Further, the α + β domain that is known to constitute one of the "walls" of the substrate-binding cleft, and thereby deepening the groove (van Aalten et al. 2000), is also present in all *T. atroviride* GH family 18 chitinases (Supplementary data, Figures S1B and S2). The presence of this subdomain is usually indicative of a processive exo-enzyme activity, while endo-enzymes usually have a shallow substrate-binding groove (Davies and Henrissat 1995; von Ossowski et al. 2003; Horn, Sikorski et al. 2006; Horn, Sorbotten et al. 2006). All chitinase sequences contained the catalytic glutamic acid in the diagnostic DXXDXDXE motif (Supplementary data, Figure S1B), which is essential for catalysis by family GH 18 chitinases (Watanabe et al. 1994; van Aalten et al. 2001; Bokma et al. 2002). However, TAC1 and TAC6/TVC6 have potentially inactivating indels in this essential succession of residues. TAC1 has a three aa insert after the first aspartate in the motif and a glutamate as the second aspartate in the motif, which is unusual for family GH 18 chitinases and potentially detrimental for the enzyme activity as it may distort the delicate arrangement of the important acidic aa in the DXXDXDXE motif. On the other hand, TAC6 is missing the third and fourth aa in the DXXDXDXE motif (Supplementary data, Figure S1B), which includes the second aspartate, a residue that has been shown to be crucial for efficient catalysis in GH18 chitinases by indirectly keeping the catalytic acid protonated in the final stage of the catalytic cycle (Watanabe et al. 1994; Kolstad et al. 2002; Synstad et al. 2004).

The substrate-binding clefts of *T. atroviride* sgC chitinases seem to be open in both ends, whereas some other chitinases are blocked in one end, e.g. ChiB from *Serratia marcescens* (van Aalten et al. 2000). Aromatic aa are lining the whole length of the groove of *T. atroviride* sgC chitinases (Figure 2). It should be noted that some of these residues are not completely conserved, e.g. TAC5 has a leucine instead of an aromatic aa in position 343 (residue 256 in TAC2; Figure 2) and TAC8 has a cysteine in position 194 (residue 97 in TAC2; Figure 2).

SgC chitinase gene expression is induced in T. atroviride *by the prey* Botrytis cinerea *but not by* Rhizoctonia solani

In order to investigate the potential roles of the *T. atroviride* sgC chitinases, gene expression profiles were determined under various growth conditions. Since the number of sgC chitinases is specifically expanded in the mycoparasites, gene expression profiles were first analyzed under simulated mycoparasitic conditions using agar plate confrontation assays. As preys the ascomycete *B. cinerea* and the basidiomycete *R. solani* were used, which can be well parasitized by *T. atroviride.* As control *T. atroviride* was confronted with itself. It is common for confrontation assays to overlay agar

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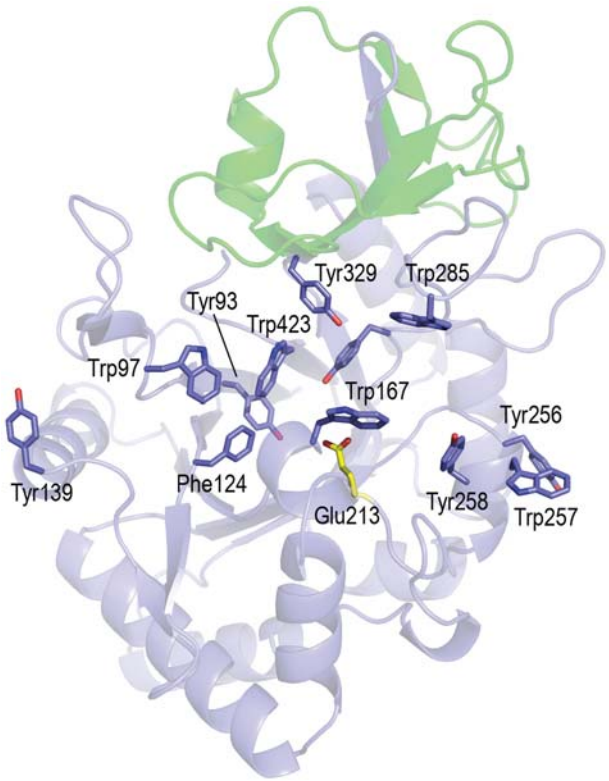

Fig. 2. The 3D model of the TAC2 catalytic GH18 module. The structure model is shown in blue-colored cartoon representation. The region representing the α + β domain that constitutes one of the "walls" of the substrate-binding cleft is colored green. Solvent-exposed aromatic amino acids in substrate-binding cleft are shown in stick representation with carbon, oxygen and nitrogen atoms colored light blue, red and dark blue, respectively. The catalytic glutamate is shown in stick representation with carbon, oxygen and nitrogen atoms colored yellow, red and dark blue, respectively. Note that the orientation of the side chains shown are predicted by the protein structure modeling software (Modeller v9.4) and may therefore not be completely accurate.

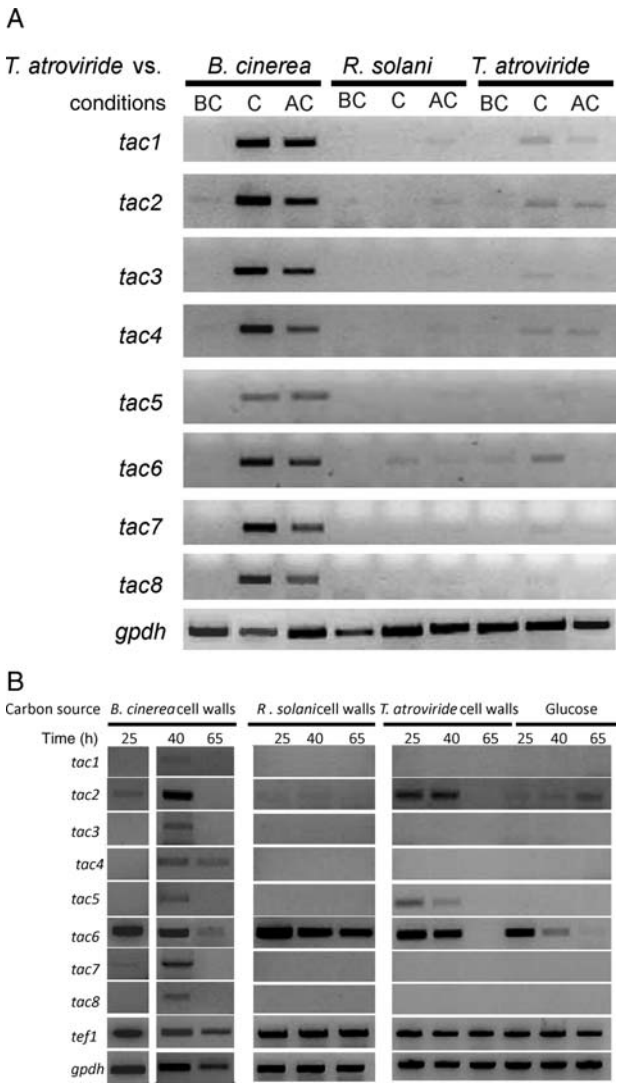

Fig. 3. Transcript patterns of sgC chitinases genes *in T. atroviride*. (**A**) Simulated mycoparasitism in agar plate confrontation assays. As control *T. atroviride* was confronted with itself. Mycelia were harvested before contact (BC), at contact of the mycelia (C) and after contact (AC). (**B**) Growth on fungal cell walls (0.5% w/v) in liquid standing cultures. The *tef1* gene, encoding translation elongation factor 1-alpha, and the gene *gpdh*, encoding glyceraldehyde-3-phosphate-dehydrogenase, were used as control genes.

plates with cellophane membranes to facilitate harvesting of the mycelia. However, cellophane consists of regenerated cellulose which is structurally similar to chitin and in order to exclude the potential influence of cellophane on chitinase gene expression, an optimized RNA extraction method from agar was developed (see Materials and methods for details). The gene expression results (Figure 3A) showed a clear induction of all sgC chitinase genes during mycoparasitism at contact and after contact with *B. cinerea*, but interestingly not when *R. solani* was used as prey or when *T. atroviride* was confronted with itself. Next, *T. atroviride* was grown on purified cell walls of *B. cinerea*, *R. solani* and *T. atroviride*. In order to enable proper attachment of *T. atroviride* to these insoluble carbon sources, these cultivations were carried out in liquid standing cultures (see Materials and methods for details). Transcript patterns were in agreement with the findings from the confrontation experiments (Figure 3B). All sgC chitinase genes were induced during growth on *B. cinerea* cell walls, whereas only *tac6* was found on *R. solani* cell walls. Cultivation on *T. atroviride* cell walls resulted in a strong expression of *tac6* and weaker expression of *tac2* and *tac5*. Interestingly, in controls using glucose as carbon source (Figure 3B), we also detected a strong *tac6* expression and a weak band for *tac2* at early time points. None of the sgC chitinase genes of *T. atroviride* were expressed during growth on glucose at late cultivation stages where biomass formation had ceased due to nutrient limitation and autolysis had set in. Further, the sgC chitinase genes of *T. atroviride* were not induced during nitrogen starvation (data not shown).

Transcript patterns of tac2 *and* tac6 *indicate a role of the respective proteins in hyphal network formation*

The sgC chitinase gene *tac6* was expressed under all cultivation conditions except during confrontation with *R. solani* and

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T. atroviride (Figure 3) and *tac2* was also detected under most of the tested growth conditions. The main difference between the confrontation assays and growth on cell walls was that in the former the mycelial colony was formed on a solid medium starting from a piece of agar and only the peripheral hyphal zone was harvested. In contrast, liquid standing cultures were inoculated with a spore suspension (6×10^5 spores/mL) and a hyphal network was formed from the germinating spores. We therefore hypothesized that *tac6* and *tac2* were induced during hyphal network formation. To test this, we "inversed" the growth conditions and inoculated agar plates with a spore suspension and harvested the mycelia at different time points. Further, we inoculated liquid standing cultures with a piece of agar from a sporulated culture and harvested the peripheral hyphal zone. The expression patterns of *tac2* and *tac6* from these samples showed clearly that these genes were expressed during hyphal network formation, but not in leading hyphae of a fungal colony, irrespective of solid or liquid media used (Figure 4). It should be noted that the expression of these genes was detected in mycoparasitism assays with *B. cinerea* where only the peripheral hyphal zone of the fungal colony that growth toward the prey that is harvested for transcript analysis, showing that they can also be induced by other stimuli.

Only few sgC chitinase genes are expressed on chitin

Chitin is, even in its pretreated colloidal form, a poor carbon source for *T. atroviride* (Mach et al. 1999), but we recently found that growth on chitin is improved when solid media are used instead of shake flask cultivations (Lopez-Mondejar et al. 2009), possibly due to the enhanced contact of the fungus and its enzymes with the insoluble substrate. To analyse the transcriptional profiles of sgC chitinase genes during growth on chitin and to generate results comparable to our experiments using fungal cell walls as carbon sources, we implemented the liquid standing cultures method described for growth on fungal cell walls. For gene expression analysis, we used untreated chitin powder from shrimp shells as well as

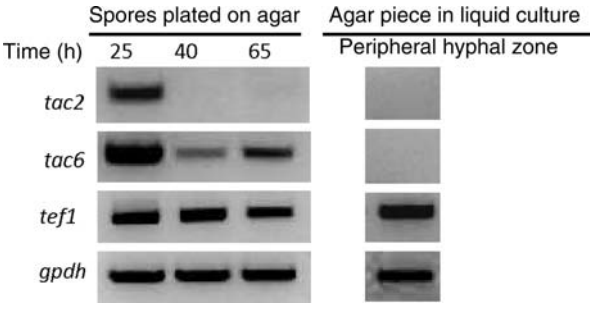

Fig. 4. Expression analysis of *tac2* and *tac6* during hyphal network and colony formation in *T. atroviride*. To simulate hyphal network formation of liquid standing cultures on a solid medium, spores of *T. atroviride* were inoculated on minimal medium agar plates complemented with 1% (w/v) glucose and 1.5% (w/v) agar. Correspondingly, in analogy to colony formation on agar plates, liquid standing cultures were inoculated with a piece of agar. The *tef1* gene, encoding translation elongation factor 1-alpha, and *gpdh*, encoding glyceraldehyde-3-phosphate-dehydrogenase were used as reference genes.

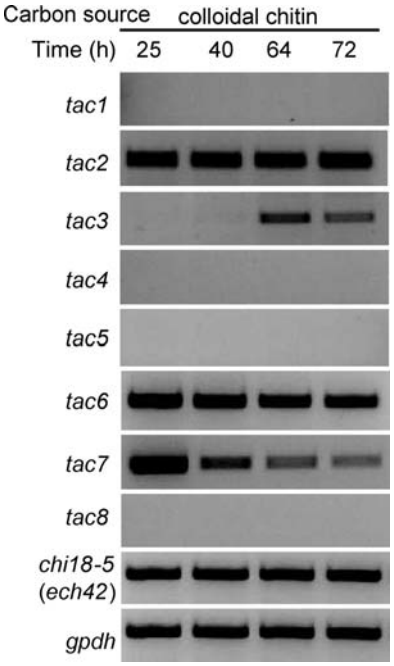

Fig. 5. SgC chitinase gene expression in *T. atroviride* during growth on colloidal chitin. The chitinase gene *chi18–5* (*ech42*) was used as expression control for the growth condition.

colloidal chitin, and the same genes were expressed in both of these experiments, but expression was stronger with colloidal chitin (Figure 5). On this carbon source, *tac2*, *tac6* and *tac7* were expressed and *tac3* was detected at late time points of growth, but *tac1*, *tac4* and *tac8*, which were induced by *B. cinerea* cell walls (Figure 3B), were not detected.

Proteins with multiple LysMs and sgC chitinases show genomic clustering of their genes

With respect to the genomic organization of sgC chitinases, we found that several were in syntenic clusters of three to four genes (Supplementary data, Figure S2) that were also present in the other *Trichoderma* spp. Some of these syntenic clusters were strongly conserved between *T. atroviride* and *T. virens* but not present in *T. reesei*, e.g. the TAC2/TVC2 cluster, whereas others were found in all three *Trichoderma* species, e.g. the TAC7/TVC7/TRCHI18-8 cluster (Figure 1D and Supplementary data, Figures S1 and S2).

Investigation of genes adjacent to the sgC chitinase genes strikingly revealed ORFs frequently encoding proteins containing a signal peptide and two to six LysMs (IPR: 002482, HMMSmart). However, no catalytic domains were indentified within these ORFs, suggesting they may be secreted proteins solely consisting of CBMs. de Jonge and Thomma (2009) suggested calling these proteins LysM effectors due to their potential roles in the infection process in plant pathogenic fungi. However, in the context of this study, we will refer to them as LysM proteins because *T. atroviride* is not a plant pathogen and to indicate that their potential functions, at least in *Trichoderma* spp., might be more general. The LysM proteins that were detected in the genome databases of

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Table II. LysM proteins in *T. atroviride*, *T. virens* and *T. reesei*

Protein[a]	Number of LysMs	Protein ID[b]
TAL1	4	43321
TAL2a	2	291370
TAL2b	3	45936
TAL4	3	267417
TAL5	3	85797
TAL6	6	297859
TAL50	1	29963
TAL51	1	127659
TVL1	4	160204
TVL2a	2	28703
TVL2b	3	222410
TVL3	4	156766
TVL4	4	201746
TVL6	4	124493
TVL7	1	79910
TVL8	1	215015
TVL9	3	66683
TRL18-1	2	54723
TRL50	1	123663
TRL51	1	121579
TRL52	4	105336

[a]The numbers of the proteins indicate the genomic position next to sgC chitinase genes; proteins with numbers >50 are not neighboring sgC chitinases.
[b]JGI database, v2.0 of the respective genomes.

T. atroviride, *T. virens* and *T. reesei* are listed in Table II. In *T. atroviride* and *T. virens*, 6 out of 8 and 6 out of 10 genes, respectively, encoding LysM proteins are neighboring or in close proximity to sgC chitinase genes. The genomic organization of the genes for sgC chitinases and LysM proteins is such that they share a common intergenic 5′ region. No LysM proteins were found next to subgroup A and B chitinases in the *Trichoderma* genomes.

We also screened other fungal genomes for the clustering of LysMs and GH18 proteins and could confirm it in *Aspergillus nidulans* (5 out of 7), *Neurospora crassa* (3 out of 5) and *Nectria haematococca* (*Fusarium solani*; 5 out of 11), less in *Aspergillus niger* (3 out of 9), *Cochliobolus heterostrophus* (4 out of 9), *Mycosphaerella graminicola* (1 out of 6) and not in *Magnaporthe grisea* (7 LysM proteins), *Fusarium oxysporum* (7 LysM proteins), *Phanerochaete chrysosporium* (6 LysM proteins).

LysMs show high sequence variability and evolve via a birth–death mechanism

A phylogenetic analysis of the aa sequences of LysMs from different fungal species revealed an extremely high inter- and intraspecies aa sequence variability and phylogenetically only weakly supported clades, even when only the genus *Trichoderma* was considered (Supplementary data, Figure S3). To characterize LysMs of the genus *Trichoderma* in more detail, HMM logos were created based on the SMART prediction algorithm and were compared with the PFAM consensus pattern (Figure 6). The SMART tool predicts longer LysMs than PFAM and the generated HMM logo shows interestingly three strongly conserved cysteine residues at positions 10, 38 and 52, which raises the question which of these cysteines participate in disulfide bridge formation. The only other two strongly conserved residues were an Asp at position 8 and an Asn at position 28. These two residues have already been reported to be conserved in prokaryotic LysMs.

The results of the phylogenetic analysis of the LysMs suggested that the LysMs undergo purifying selection without concerted evolution, probably by birth-and-death evolution (Nei and Kumar 2000). This mechanism implies that new genes are created by repeated gene duplication, in which some of the duplicate genes are maintained in the genome for a long time, whereas others are rapidly deleted or become non-functional. Consequently, nucleotide sequence differences between genes will primarily occur at synonymous sites. We therefore analysed the number of non-synonymous substitutions per non-synonymous site (K_a) and the number of synonymous, or silent, substitutions per synonymous, or silent, site (K_s) within the gene sequences of LysMs (which are intronless) of three selected, relatively stable clades (Supplementary data, Figure S4). In total, the number of differences at non-synonymous sites strongly exceeded those at synonymous sites, which shows that LysMs are indeed subject to a birth-and-death mechanism of evolution.

LysM genes are coregulated with sgC chitinase genes

Finally, we wanted to test whether the genes encoding LysM proteins, which we named TALs in *T. atroviride* (Table II), were coregulated with the respective sgC chitinase genes with which they clustered together at their genomic localization. LysM genes *tal1*, *tal2*, *tal4*, *tal5* and *tal6* were all found to be expressed during growth on *B. cinerea* cell walls. Their transcripts were absent on glucose, where only *tal2* and *tal6* were expressed (Figure 7A and B). This perfectly correlated with the transcript patterns of the respective chitinases *tac1*, *tac2*, *tac4*, *tac5* and *tac6*. However, differences in the expression patterns of *tals* and their corresponding *tacs* were detected on colloidal chitin, showing that this co-regulation is not strictly maintained under all growth conditions (Figure 7C).

Discussion

Fungal sgC chitinases are large proteins (up to 200 kDa) that contain a catalytic GH18 module in combination with CBM18 (chitin-binding) and LysMs (CBM50) and display strong sequence variations at their N- and especially C-terminal ends. The high sequence variability can be interpreted as the result of diversifying selection and could be due to an adaptation toward differences in cell wall composition in antagonistic species, as has been discussed by Karlsson and Stenlid (2008). In-depth analysis of the sequences and overall 3D structures of sgC chitinase GH18 modules revealed that all have deep substrate-binding clefts lined with numerous aromatic aa, which is characteristic for exo-processive enzymes (Figure 2 and Supplementary data, Figure S1). Chitinases with such properties are known to bind tightly to the substrate and release products predominantly consisting of dimeric chitooligosaccharides (chitobiose) (Sorbotten et al. 2005; Horn, Sikorski et al. 2006; Horn, Sorbotten et al. 2006; Sikorski et al. 2006). The processive architecture of the substrate-binding clefts and the

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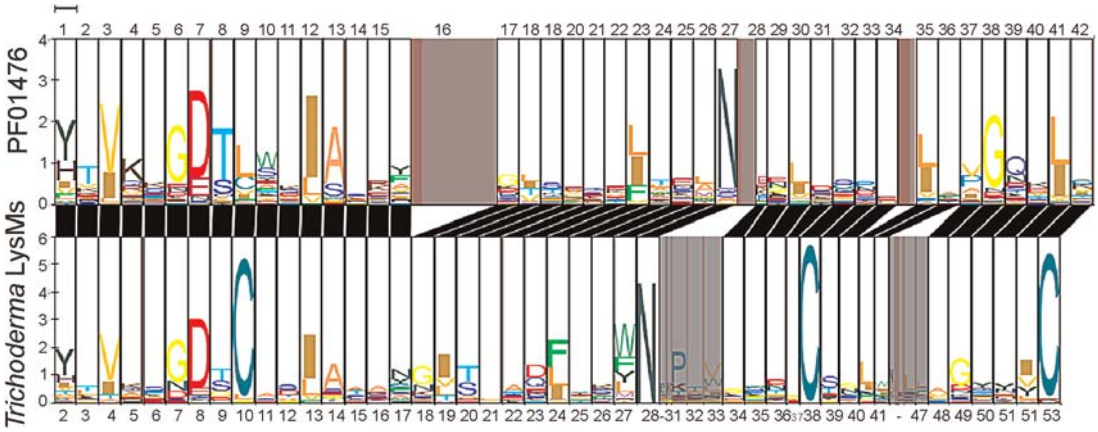

Fig. 6. Pairwise HMM logo comparison of the Pfam LysM logo (PF01476, upper panel) and a multiple alignment of LysMs (SM00257) from *T. atroviride*, *T. virens* and *T. reesei* (lower panel), created with LogoMat-P (Schuster-Böckler and Bateman 2005).

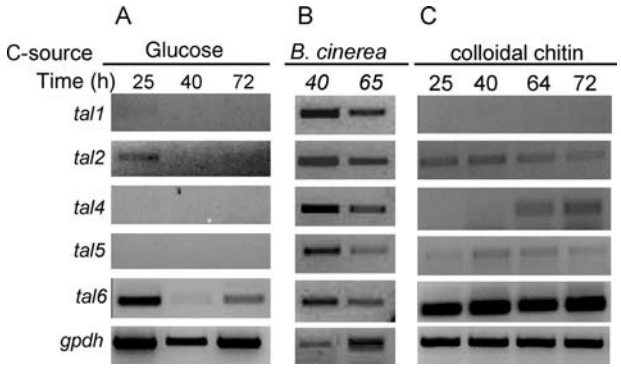

Fig. 7. Transcription of *tals*, encoding LysM-proteins, in *T. atroviride*. The numbers in the *tal*-gene names indicate the genomic position next to the corresponding sgC chitinase genes, e.g. *tal1* is next to *tac1* in the genome. *gpdh* was used as reference gene.

presence of multiple CBMs suggest that sgC chitinases might be particularly efficient in the hydrolysis of insoluble substrates and be able to degrade them locally, e.g. drill holes in fungal cell walls. The modular structure of the sgC chitinases is reminiscent of the α-subunit of the multimeric *Kluyveromyces lactis* killer toxin, which in total consists of three subunits (α, β, γ), of which α and β are encoded by the same ORF, and post-translationally processed, whereas the γ-subunit is the product of a separate ORF (Stark and Boyd 1986). The latter encodes the actual toxin, whereas the α-subunit encodes a chitinase responsible for the localized degradation of the host cell wall and the β-subunit, a protein that is involved in the translocation of the toxin into the host cell (Butler et al. 1991; Magliani et al. 1997). Although no counterpart for the γ-subunit of *K. lactis* killer toxins is known in *Trichoderma* spp., it is possible that the function of fungal sgC chitinases involves in addition to localized degradation of the substrate, e.g. the host cell wall, a transport mechanism. However, post-translational processing and the existence and cargo of possible transport mechanisms of sgC chitinases remain to be elucidated in filamentous fungi.

We could show in this study that despite their high numbers in mycoparasites, sgC chitinases are not solely involved in mycoparasitism, but have different roles in *T. atroviride*. The transcriptional profiles of the respective genes showed a fine-tuned expression, and genes encoding proteins with similar modular architectures were expressed under completely different growth conditions. Our results clearly demonstrated that information about the protein sequence, substrate-binding cleft and the modular structure of chitinases is not sufficient to determine the roles of the respective proteins, as no connection between the modular architectures or protein sequences of sgC chitinases and their transcript patterns was detected. However, it remains to be elucidated if the variable C-terminal parts of sgC chitinases are possibly the key to understanding the determinants for their respective detailed functions.

Transcriptional analysis revealed that all sgC chitinases were induced in mycoparasitism assays against *B. cinerea* and during growth on *B. cinerea* cell walls. No other growth condition was detected where all sgC chitinase genes were expressed. This suggests that sgC chitinases are involved in the degradation of complex, chitinous substrates such as fungal cell walls, which fits well to the aggressive, parasitic lifestyle of *T. atroviride*. It was conspicuous that during growth on chitin not all sgC chitinases were expressed, indicating that the expression of these genes during mycoparasitism—at least of the ones that are not inducible by chitin—might be triggered by a nonchitinous inducer. Generally, gene expression of sgC chitinases was not strong when compared with other chitinases such as *chi18-5* (=*ech42*), and their expression was also restricted to only a few culture conditions. In contrast, *ech42* is expressed under many different growth conditions including chitin degradation, mycoparasitism, starvation and autolysis (Mach et al. 1999; Brunner et al. 2003) and seems to be the most abundantly expressed, secreted chitinase in fungi. In our study, we found no gene expression of sgC chitinases during germination, starvation and autolysis and only a weak expression of *tac1*, *tac2*, *tac6* during sporulation (data not shown). However, for *tac2* and *tac6*, which were expressed on glucose during hyphal network formation,

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expression was even enhanced by chitin (Figures 3A, 4 and 5), which could indicate that these proteins are involved in remodeling processes of the fungus' own cell wall chitin and exogenous chitin sequestration if the correct inducer is present. The finding that *tac2* and *tac6* were abundantly expressed during hyphal network formation, but none of the other sgC chitinase genes were detected at all under such conditions is especially interesting in view of the facts that the modular structures of TAC2 and TAC6 do also not differentiate them from other sgC chitinases and that the gene cluster containing *tac2* was syntenically not conserved to *T. reesei*, what would be expected from a protein involved in fungal growth. It was also striking that the most strongly expressed sgC chitinase gene *tac6* does probably not even encode a catalytically active chitinase because TAC6 has potentially detrimental indels in the catalytic motif (Supplementary data, Figure S1). If TAC6 does not have enzymatic activity, the question arises what the role of this protein could be in hyphal network formation and what part the neighboring LysM protein could play in this context, which we found to be strongly expressed on colloidal chitin. It is possible that these proteins act as lectins, but their exact roles in fungal cell wall remodeling remain to be tested.

The finding that sgC chitinase genes were not induced by *R. solani* indicates that the cell walls of *B. cinerea* contain inducing compounds—or compounds that are converted into inducers—that are either not present or not accessible in *R. solani* cell walls. It is also possible that the degree of deacetylation in the fungal cell walls influences sgC chitinase gene expression. In a previous study (Seidl et al. 2005), we had cloned *T. atroviride chi18-10* (named *tac3* in this study) in strain P1 and found it to be induced by *R. solani*. However, for that study, cellophane overlays were used for plate confrontations assays, and under these conditions, we were also able to detect a weak band in this study with strain IMI206040 (data not shown), but no signal was found when the cellophane membrane was omitted. Our results emphasize that cellophane can influence the expression pattern of genes encoding hydrolytic enzymes, especially if rather low expression levels, as in the case of sgC chitinase genes, are investigated. With respect to different preys, we used a *Trichoderma gamsii* strain that was selected because it was able to antagonize the ascomycete *Fusarium graminearum* in another set of experiments, and we could also detect transcripts for sgC chitinase genes (Matarese and Seidl-Seiboth, unpublished results). *T. atroviride* is able to parasitize *R. solani* well, and the finding that sgC chitinases were not expressed under these conditions implies that sgC chitinases might be one component of the mycoparasitic attack, but are not essential for it.

Although *T. atroviride* and *T. virens* are both mycoparasites, they are phylogenetically members of different sections of the genus and not closely related within the genus *Trichoderma* (Kullnig-Gradinger et al. 2002). Growth on the preferentially used media for each of these fungi was not optimal for the other one, which made it difficult to directly compare them. Further, in mycoparasitism assays, *T. virens* does not overgrow itself in contrast to *T. atroviride*. Owing to the fine-tuned complex regulation of sgC chitinase genes that we found for *T. atroviride*, we therefore refrained in this work to compare the expression profiles of this group of genes between these two fungi. However, our preliminary analyses already indicated that the regulation of sgC chitinase genes is indeed different between *T. virens* and *T. atroviride* during mycoparasitism (Gruber and Seidl-Seiboth, unpublished results).

Genome analysis also revealed that in *Trichoderma* spp., including that which have a saprobic lifestyle, the genes upstream or downstream of chitinases or in close proximity to chitinases often contain LysMs (CBM 50). The expression patterns of the respective genes were similar to their neighboring sgC chitinase genes—only *tal2* and *tal6* were found on glucose, all tested *tals* were induced by *B. cinerea*—but gene expression on colloidal chitin did not perfectly correlate. However, it should be mentioned that only *tac3* and *tac7* were clearly induced by chitin, but no genomic adjacent LysM genes were found at these loci. For other gene pairs with bidirectional promoters that have already been studied in detail, such as the *niiA* and *niaD* genes in *A. nidulans* (Punt et al. 1995), it was found that some of the DNA-binding motifs in their shared promoter influence the expression of both genes, whereas others regulate act only on one of them. Similar effects might explain the partial coregulation of *tacs* and *tals* in *T. atroviride*.

The protein sequences of LysMs are highly variable, which possibly reflects diversity binding properties. In this context it is noteworthy, that even the LysM-sequences within one protein can be strongly different. TAL6 (protein id: 297859), for example, has six LysMs, of which four are highly similar to each other, but do not share this similarity with the other two modules (Supplementary data, Figure S3). The role of the LysM-protein Ecp6 in the plant pathogenic fungus *C. fulvum* as extinguisher of host immunity was recently shown (de Jonge et al. 2010). However, so far neither the targets nor the roles of LysM proteins in other fungi have been investigated. In mycoparasites, their functions could include the protection of the own fungal cell wall as well as the opposite role, an involvement in the mycoparasitic attack by aiding in the hydrolysis of the host cell wall, due to loosening the chitin structure, as has been described for other chitin-binding proteins (Vaaje-Kolstad et al. 2005). Our data would be consistent with both of these possibilities, but do not favor one of them. It will therefore be interesting to further study the functions of LysM proteins in the hydrolysis or protection of fungal cell walls and the roles of the different sgC chitinases in these processes.

Materials and methods

Bioinformatic analysis (protein models, modular analysis, alignments, phylogeny)

The genome sequences of *T. atroviride* IMI206040 (v2.0) and *T. virens* Gv29-8 (v2.0) are accessible at http://genome.jgi-psf.org/pages/fungi/home.jsf. The tools available on this website were used to analyze and refine the ORFs of genes encoding sgC chitinases and the resulting protein models. In view of the high numbers of chitinases, we had to compromise with respect to their naming. We are aware that orthologous

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proteins should ideally have the same name in different organisms and therefore the name of the organism should not be part of the protein abbreviation, but as described in the results section "Proteins with multiple LysMs and sgC chitinases show genomic clustering of their genes", some sgC chitinases have clear orthologs in other *Trichoderma* spp., whereas others seem to be unique for one species. If we would use the same abbreviation for sgC chitinases in general and then number them consecutively, some proteins that are clearly different based on their aa sequences would inevitably have had the same number. Modular analysis was carried out with InterProScan (http://www.ebi.ac.uk/Tools/InterProScan/). For structural modeling, a 3D model of the TAC2 sequence was generated with the Modeller v 9.4 software (Fiser and Sali 2003), using the human chitotriosidase structure as a template (PDB ID: 1GUV (Fusetti et al. 2002), 25% sequence identity to TAC2). Twenty individual TAC2 models were generated by Modeller and their structural integrity was analyzed by ProSa (Wiederstein and Sippl 2007) and the "Rampage" Ramachandran plot utility (Lovell et al. 2003). The best model, which was consequently used for further analysis, yielded a ProSa *Z*-score of −8.51 and showed three residues as outliers in the Ramachandran plot. The superposition of the final TAC2 model Cα atoms on the template structure gave a root mean square deviation of 0.21 Å. In summary, the evaluation parameters indicated a good quality model suitable as an aid for functional and sequence analysis. Multiple alignments were created with ClustalX 2.0 (Larkin et al. 2007), manually refined in GeneDoc (Nicholas et al. 1997) and phylogenetic analysis was carried out with MEGA 4 (Tamura et al. 2007) using the Neighbour Joining, a distance algorithmic method, and stability of clades was evaluated by 500–1000 bootstrap rearrangements. ORFs were experimentally verified using RACE–PCR as described in Seidl et al. (2005). To test the fit of the sequences to the model of neutral evolution, the *D*-test statistic proposed by Rozas et al. (2003) was computed with the DnaSP program. The extent of nucleotide divergence was estimated by using the uncorrected *p* distance (Tajima and Nei 1984). The proportions of synonymous (K_s) and non-synonymous (K_a) differences per site were calculated by the modified Nei-Gojobori method implemented in DNASp (Rozas et al. 2003).

Cultivation conditions

For gene expression analysis, strain *T. atroviride* IMI206040 (*Hypocrea atroviridis*) was used. To test mycoparasitic interactions, confrontation assays were performed. *T. atroviride* was grown on potato dextrose agar (BD Dicfo, Franklin Lakes, NJ) at 25°C and harvested when the mycelia were ca. 5 mm apart (before contact), at contact (contact) of the mycelia and after *T. atroviride* had overgrown (5 mm) the host fungus *R. solani* or *B. cinerea* (after contact). As a control, *T. atroviride* was confronted with itself and harvested at the same time points. To avoid the use of cellophane for these assays, peripheral hyphal zones were harvested from each confrontation stage by directly cutting out agar pieces, which were shock frozen in liquid nitrogen and stored at −80°C. For transcript analysis, hyphal zones from PDA plates were harvested as described for the confrontation assays.

For liquid standing cultures, minimal medium (Seidl et al. 2004) was used. Plastic Petri dishes with 9 cm diameter were filled with 15 mL of the medium and incubated with 6×10^5 spores/mL at 25°C in constant light. To ensure efficient germination, 0.05% (w/v) peptone were added to the medium and 0.5% (w/v) of the respective carbon sources—glucose, colloidal chitin, practical grade chitin and cell walls from *B. cinerea*, *R. solani* and *T. atroviride*—were used. Colloidal chitin and fungal cell walls were prepared as described in Seidl et al. (2005). Mycelia were harvested by filtration through Miracloth (Calbiochem, Merck, Darmstadt, Germany), briefly washed with cold tap water, squeezed between filter paper, immediately immersed in liquid nitrogen and stored at −80°C.

RNA isolation and RT–PCR

Mycelia from liquid cultures with glucose or cell walls as carbon sources were ground to a fine powder under liquid nitrogen and total RNA was isolated using the guanidinium thiocyanate method (Sambrook and Russell 2001). RNAs from cultures with chitin as carbon source were extracted following a protocol that was recently established for *T. reesei* cultures grown on insoluble cellulose (Tisch et al. 2010, manuscript submitted) to eliminate PCR-inhibiting compounds (that were otherwise present, even in the purified RNAs, and could not be detected using the devices mentioned

Table III. Primers used in this study for RT–PCR

Primer	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)
gpdh	CCAGAACATCATCCCCAGCAGC	GATGGAAGAGTTGTTGTTGCCGAG
tef1	GGTACTGGTGAGTTCGAGGCTG	GGGCTCAATGGCGTCAATG
chi18-5 (*ech42*)	CATGCCCATCTACGGACGAG	CTTCCCAGAACATGCTGCCTC
tac1	CAAGCGAGTTCTGTGGCA	CACCGAGGGCAATGAGTAG
tac2	GCCCTCGTGCTCCATCAG	GGTCTCGTAGTTGCCGGG
tac3	GATTCTCGTCGTCGCCG	CCCAGGTCTTCTTGTTCCAGTT
tac5	GCATTGGACTTGGAGGTGTGTTG	GCATAGCATCGGTGGGACAGAC
tac6	CGGGACTTATGGTTTGGGCGG	CGAACGGTCCAGATGCGGG
tac7	CAAGTTCGGAGATGGCTGTGC	GCTGCCCGACCTGGAGATTTGC
tac8	CGTCTCTCAATCCAATGTCAAC	CGCATTGTCCCGATTTTCCAC
tal1	GCTTACCAGGCAGACACTTCACT	GCCCTATGAGAAACCAGTCGG
tal2	CTGGCAAACTCTGTGTCGGCG	CCTCCCTCGCCGCCAATG
tal4	GCGAAAACTACAACAACCAAGG	TGAGAGATTCGCAGTTGTGATG
tal5	GGCTTGATACATACTACTGCGTCG	GGCAAAAGAACACAGGTAGATGG
tal6	GGCTCTTCAGGGTCAAATCTCTC	GGTGCCGTATTCGTAGGTAGAGC

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below). For this protocol, buffers and columns from the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) were used. Pieces of mycelia, frozen in liquid nitrogen, were added to a tube containing 800 µL of ice-cold buffer RLC contained in the kit supplemented with 10 µL/mL of 2-mercaptoethanol (Merck), 12 glass beads with 0.75–1.0 mm diameter and 2 glass beads with 2.85–3.3 mm diameter (Roth, Karlsruhe, Germany). The mycelium was ground twice for 30 s in a RETSCH MM301 Ball Mill (Retsch, Haan, Germany), and left on ice in between for 30 s. The tubes were incubated at 56°C for 3 min and then the content was transferred to a QIAshredder spin column (Qiagen). Thereafter, the procedure recommended by the manufacturer was followed including purification and concentration of the extracted RNAs.

For RNA extraction from agar, guanidinium thiocyanate cannot be used and we therefore adapted a protocol reported by Sokolovsky et al. (1990). Aliquots of mycelia, ground to a fine powder under liquid nitrogen, were added to 650 µL of ice-cold extraction buffer containing 0.6 M NaCl, 10 mM EDTA, 100 mM Tris–HCl, pH 8.0, 0.4% sodium dodecyl sulfate and 650 µL of phenol. The suspension was vortexed, incubated for 15 min on ice and centrifuged for 10 min at 4°C at 13,000×*g* for phase separation. The upper, aqueous layer was transferred to a fresh tube containing 750 µL of 8M LiCl and mixed briefly. The RNA was precipitated overnight at 4°C and pelleted by centrifugation at 13,000×*g*. Pellets were dissolved in 300 µL of RNase-free water containing 30 µL of 3 M sodium acetate (pH 5.2) and RNAs were precipitated with 750 µL of isopropanol for 2 h at −20°C and pelleted by centrifugation at 13,000×*g*, 4°C, washed with 70% ethanol, air-dried, dissolved in 100 µL of RNase-free water and stored at −80°C.

All isolated RNAs were treated with DNAse I (Fermentas, St Leon-Rot, Germany) and purified using the RNeasy MinElute Cleanup Kit (Qiagen). RNA quality and concentration were analysed with the Experion RNA StandardSense Kit (RQI classification: RNAs under 5.5 were excluded from our studies; Bio-Rad, Madison, WI) and the Nanodrop ND-1000 spectrophotometer (PEQLAB, Erlangen, Germany). For cDNA production, 5 µg of RNA/reaction were reverse-transcribed using the Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) and a mixture of random hexamer primer and oligo(dT)18 primer. RT–PCR (25–27 cycles) was performed using the gene-specific primers listed in Table III. Primers used for LysM-gene detection are numbered according to their adjacent chitinases genes and named *tal1–tal6*. The *tef1* gene (translation elongation factor 1-alpha, protein ID 300828) and the *gpdh* gene (glyceraldehyde-3-phosphate-dehydrogenase, protein ID 297741) were used as reference genes. Both genes could be detected with similar efficiency, but generally *gpdh* expression turned out to be more robust and thus *gpdh* was more suitable as control gene. In chitin cultivations, *ech42* (*chi18-5*, protein ID 131598) was used as positive control gene for the induction of the chitin degradation machinery.

Supplementary data

Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

Funding

This work was supported by the FWF Austrian Science Fund (T390 to V.S. and P20559). The work conducted by the US Department of Energy Joint Genome Institute is supported by the Office of Science of the US Department of Energy (Contract No. DE-AC02-05CH11231).

Conflict of interest statement

None declared.

Abbreviations

aa, amino acids; CBM, carbohydrate-binding module; GH, glycoside hydrolase; indels, insertions/deletions; LysMs, LysM modules; ORF, open-reading frame; RLK, receptor-like kinase; SgC, subgroup C.

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