Identifying glycoside hydrolase family 18 genes in the mycoparasitic fungal species *Clonostachys rosea*

Georgios Tzelepis, Mukesh Dubey, Dan Funck Jensen and Magnus Karlsson

*Clonostachys rosea* is a mycoparasitic fungal species that is an efficient biocontrol agent against many plant diseases. During mycoparasitic interactions, one of the most crucial steps is the hydrolysis of the prey’s fungal cell wall, which mainly consists of glucans, glycoproteins and chitin. Chitinases are hydrolytic enzymes responsible for chitin degradation and it is suggested that they play an important role in fungal–fungal interactions. Fungal chitinases belong exclusively to the glycoside hydrolase (GH) family 18. These GH18 proteins are categorized into three distinct phylogenetic groups (A, B and C), subdivided into several subgroups. In this study, we identified 14 GH18 genes in the *C. rosea* genome, which is remarkably low compared with the high numbers found in mycoparasitic *Trichoderma* species. Phylogenetic analysis revealed that *C. rosea* contains eight genes in group A, two genes in group B, two genes in group C, one gene encoding a putative ENGase (endo-β-N-acetylglucosaminidase) and the ech37 gene, which is of bacterial origin. Gene expression analysis showed that only two genes had higher transcription levels during fungal–fungal interactions, while eight out of 14 GH18 genes were triggered by chitin. Furthermore, deletion of the C group chiC2 gene decreased the growth inhibitory activity of *C. rosea* culture filtrates against *Botrytis cinerea* and *Rhizoctonia solani*, although the biocontrol ability of *C. rosea* against *B. cinerea* was not affected. In addition, a potential role of the CHIC2 chitinase in the sporulation process was revealed. These results provide new information about the role of GH18 proteins in mycoparasitic interactions.

**INTRODUCTION**

Mycoparasitism is the interaction between fungi where one living fungus acts as a nutrient source for another (Jeffries, 1995). Degradation of the fungal prey’s cell wall by the mycoparasite is an important step for establishment of mycoparasitic interactions. The fungal cell wall provides cells with the appropriate rigidity to cope with different environmental stress factors, and the opportunity to communicate with the extracellular environment. It is mainly composed of glucans, chitin and glycoproteins (Bowman & Free, 2006). The cell wall of filamentous fungi contains 10–20 % chitin (Bartnicki-Garcia, 1968; de Nobel et al., 2000), which is a biopolymer composed of 1,4-β-linked units of N-acetylglucosamine. Interference with chitin synthesis by disruption of chitin synthases resulted in osmotically susceptible conidia in *Aspergillus nidulans* (Specht et al., 1996).

Chitinases (EC 3.2.1.14) are hydrolytic enzymes responsible for cleavage of the 1,4-β-glycosidic linkage in chitin polymers (Gooday, 1990). According to the carbohydrate-active enzymes (CAZy) classification, fungal chitinases belong exclusively to glycoside hydrolases (GH) family 18 (Cantarel et al., 2009; Karlsson & Stenlid, 2009). They are categorized, depending on the cleavage patterns, as endochitinases, which can cleave the chitin chain at random positions, and as exochitinases, which are able to degrade the chitin polymer from the exposed termini releasing chitobiose products (Horn et al., 2006). Fungal GH18s are phylogenetically arranged in three distinct groups referred to as A, B and C (Seidl et al., 2005), which are further divided into subgroups A-II to A-V, B-I to B-V and C-I to C-II (Karlsson & Stenlid, 2008). Group A is predicted to include mainly exochitinases (Gruber & Seidl-Seiboth, 2012), although the A-II subgroup member CfcI from *Aspergillus*
**niger** exhibits exo-β-N-acetylglucosaminidase activity (van Munster et al., 2012). Characterized group B chitinases are mostly endo-acting enzymes, while the B-V group contains members with endo-β-N-acetylglucosaminidase (ENGase) activity (Stals et al., 2010).

Chitinases from the C group are predicted to be exo-acting (Gruber et al., 2011b), although no member is biochemically characterized so far. These chitinases display similarities with the α/β subunit of the secreted zymocin killer toxin from the yeast *Klyuyveromyces lactis* (Magliani et al., 1997; Stark & Boyd, 1986), which enhances the permeability of the target yeast cell wall to allow penetration of the toxic γ subunit into the cell (Magliani et al., 1997). It is hypothesized that certain C group chitinases in filamentous ascomycetes perform a similar function, enhancing penetration of toxic metabolites into the antagonist cells during fungal–fungal interactions (Seidl et al., 2005). The mycoparasitic lifestyle of *Trichoderma virens* is correlated with selection for increased numbers of group C chitinases (Ihrmark et al., 2010; Seidl-Seiboth et al., 2014). Several C group chitinase genes in *Trichoderma* spp., in *Neurospora crassa* and in *As. nidulans* are induced during fungal–fungal interactions (Gruber et al., 2011b; Tzelepis et al., 2012, 2014), and deletion of *chiC2-2* in *As. nidulans* results in reduced growth inhibitory activity of culture filtrates (Tzelepis et al., 2014). Yet, other group C chitinase genes are developmentally regulated and may be involved in cell wall modification during growth (Gruber et al., 2011b; Tzelepis et al., 2014).

*Clonostachys rosea* (teleomorph *Bionectria ochroleuca*) is a soil-inhabiting, mycoparasitic ascomycete fungus that is reported to be an effective biological-control agent against several plant-pathogenic fungi (Luongo et al., 2005; Sutton et al., 1997). This has also been demonstrated with our *C. rosea* strain IK726 (Jensen et al., 2000, 2004; Moller et al., 2003). IK726 was isolated from *Fusarium culmorum*-infected barley roots (Knudsen et al., 1997). *C. rosea* produces extracellular chitinases during growth on *Fusarium equisetii* and *Pythium ultimum* cell wall material (Inglis & Kawchuk, 2002), and three chitinase genes (*ech37*, *chi1* (also referred to as *ech42*) and *ech58*) have been identified in *C. rosea* (Gan et al., 2007; Mammarabadi et al., 2008a).

The *ech37* gene is of bacterial origin and is present in *C. rosea* through horizontal gene transfer (Ubbayasekera & Karlsson, 2012). Gene deletions of *ech37*, *ech42* and *ech58* resulted in lower *in vitro* antagonistic activity towards *F. culmorum*, but showed no effect on the biocontrol ability of *C. rosea* to control *F. culmorum* on barley or *Alternaria radicina* on carrots (Mammarabadi et al., 2008a).

The aim of this study was to screen the *C. rosea* IK726 genome for GH18 genes, to cluster them in phylogenetic groups and to study their regulation. Based on this information, the killer toxin-like C group gene *chiC2* was chosen for further functional characterization by construction and analysis of gene deletion strains. We identified 14 GH18 genes, eight in group A, two in group B, two in group C, one subgroup B-V ENGase and *ech37*. The *ech37* and *chiB1* genes were induced during fungal–fungal interactions, while higher transcript levels of eight genes were observed in chitin media compared to carbon-rich conditions. Moreover, the *ΔchiC2* strain displayed reduced growth inhibitory activity of culture filtrates, implying a role in fungal–fungal interactions, while an additional role of this chitinase in sporulation was revealed.

**METHODS**

**C. rosea** genome analysis and primer design. The *C. rosea* genome sequence (GenBank/EMBL/DBJ accession no. PRJEB4240; Karlsson et al., 2015) was screened for the presence of GH18 genes using tblastn (Altschul et al., 1997) with *Trichoderma atroviride* chitinase amino acid sequences (Table S1, available in the online Supplementary Material) as queries. Putative *C. rosea* GH18 genes were analysed for similarity to characterized proteins in the non-redundant database at the National Center for Biotechnology Information using the blastp algorithm (Altschul et al., 1997). Identification of conserved domains was performed using the SMART protein analysis tool (Letunic et al., 2003). Several C group chitinase genes in *Trichoderma* spp., in *Neurospora crassa* and in *As. nidulans* were evaluated using gradient PCR techniques. Primer specificity and optimal annealing temperature were evaluated using gradient PCR techniques.

**Phylogenetic analysis of *C. rosea* chitinases.** *C. rosea* GH18 amino acid sequences and GH18 sequences derived from *T. atroviride*, *T. virens*, *Trichoderma roseae*, *N. crassa* and *Fusarium graminearum* (Table S1) were aligned with clustalw (Thompson et al., 1994). Phylogenetic analysis based on GH18 catalytic domains was carried out using the neighbour-joining method implemented in the mega v.6.06 software (Tamura et al., 2013). The JTT substitution model was used (Jones et al., 1992), with uniform rates among sites and complete deletion of gaps. Bootstrap analysis was performed using 1000 replicates.

**PCR conditions and sequencing.** Initial phylogenetic analyses indicated that protein ID BN869_T00005750 (*chiB2*) contained a sequence gap. PCR for gap closing was performed with the primers listed in Table S2 and the following conditions: 95 °C for 1 min; 30 cycles of 95 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min; followed by final extension at 72 °C for 10 min. Sequencing was performed by Macrogen, and the resulting sequence was deposited in GenBank/EMBL/DBJ under accession number KM510224.

**Fungal strains and maintenance conditions.** *C. rosea* strain IK726, *F. graminearum* strain 1104-14, *Rhizoctonia solani* strain SA1 and *Botrytis cinerea* strain B05.10 were maintained on potato dextrose agar (PDA; Sigma-Aldrich) at 25 °C in darkness. PDA plates were inoculated from stock cultures preserved in 10 % (w/v) glycerol at –80 °C.

**Gene expression analysis.** For transcription analysis of chitinase genes during interspecific interactions, Erlenmeyer flasks containing 50 ml potato dextrose broth (PDB; Sigma-Aldrich) were inoculated...
with an agar plug of actively growing C. rosea mycelia, and incubated at 25 °C in darkness for 3 days. Subsequently, 2 ml homogenized mycelia from B. cinerea, F. graminearum or R. solani (derived from 7-day-old cultures in PDB) were added to the Erlenmeyer flasks and further incubated at 25 °C for 24 h. Control flasks were inoculated with 2 ml fresh PDB. For gene expression analysis in different nutrient conditions, C. rosea mycelia were grown in 100 ml Erlenmeyer flasks containing 50 ml SMS (synthetic minimal salts) medium (Dubey et al., 2012) supplemented with 1 % (w/v) glucose for 5 days. Then, mycelia were washed three times with autoclaved distilled water and reincu-
lated on SMS medium supplemented with 1 % (w/v) glucose (carbon rich), 0.1 % (w/v) glucose (carbon starvation) or 1 % (w/v) colloidal chitin (Sigma-Aldrich) as a sole carbon source at 25 °C for 24 h. Mycelia from all treatments were immediately frozen in liquid nitrogen after harvest and stored at −80 °C for a maximum period of 2 weeks.

Frozen mycelia were homogenized using pestle and mortar with liquid nitrogen, and total RNA was extracted using the RNeasy Plant Mini kit (Qiagen) according to the manufacturer’s instructions. RNA concentration was determined spectrophotometrically using a NanoDrop machine (Thermo Scientific), while RNA integrity was analysed after DNase I treatment by electrophoresis on an Agilent Bioanalyser, using the RNA 6000 Nano kit (Agilent Technologies). For cDNA synthesis, 1000 ng total RNA, after treatment with DNase I (Fermentas), was reversed transcribed using an iScript cDNA synthesis kit (Bio-Rad) using oligo(dT)18 and random hexamer primers in a total volume of 20 μl, followed by 10-fold dilution of the cDNA and storage at −20 °C. Synthetic kanamycin positive control RNA (Promega) was included in the cDNA synthesis to monitor the efficacy of the reverse transcription reaction. The following protocol was used for reverse transcription: 5 min at 25 °C, 30 min at 42 °C, followed by enzyme inactivation at 85 °C for 5 min. In order to in-
vestigate possible contaminations by genomic DNA a control sample without the reverse transcriptase polymerase (−RT control) was prepared, while a non-template control (NTC) was used in order to identify the presence of unintended amplification products.

Transcript levels were quantified by RT-qPCR in an iQ5 qPCR system (Bio-Rad) using the primers listed in Table S2, as described previously (Tzela
pis et al., 2014), while primer amplification efficacy was deducted from amplification of standard curves usingserial dilutions of C. rosea genomic DNA. Amplification of the kanamy
cin positive control was performed as described previously (Karlsson et al., 2005). Expression of GH18 genes was normalized by actin (act) and β-tubulin (tub) gene expression as these were reported as suitable RT-qPCR reference genes in C. rosea strain IK726 (Mamarabadi et al., 2008b; Zapparata, 2014). Relative expression values were calculated from the threshold cycle (Ct) values and the primer amplification efficiencies by using the formula described by Pfaffl (2001). Finally, this analysis was conducted for at least four biological replicates each based on two technical replicates.

**Construction of the gene deletion cassette.** Genomic DNA from C. rosea WT was extracted using a hexadecyl-trimethyl-ammonium bromide-based protocol (Nguyen et al., 2008). Approximately 1200 bp of 5’ and 3’ flanking regions of the chiC2 gene were amplified from genomic DNA by PCR using the primer sequences listed in Table S2, while the hygromycin-resistance gene (hygB) was amplified from the pCCT7 vector (Lorang et al., 2001) as described previously (Dubey et al., 2013). The deletion cassette was constructed using the gateway cloning technique according to the manufacturer’s instructions (Invitrogen), while the pPm43GW vector (Karimi et al., 2005) was used as the destination vector in order to construct the pPm43GW-03743-ko deletion vector.

**Deletion of the chiC2 chitinase gene and mutant validation.** The disruption vector was used to transform Agrobacterium tumefa-
ciens strain AGL-1, following a freeze–thaw procedure and positive clones were selected on yeast extract peptone agar (YEP) plates containing rifampicin (35 μg ml⁻¹) and spectinomycin (100 μg ml⁻¹). Deletion of the chiC2 chitinase gene in C. rosea was performed through an Ag. tumefaciens mediated protocol as described elsewhere (Utermark & Karlovsky, 2008). Hygromycin-resistant colonies were subcultured on PDA three times, followed by inoculation on hygromycin-containing PDA in order to test for mitotic stability.

Mitotically stable, hygromycin-resistant colonies were screened for correct homologous integration of the deletion cassette by PCR, using primers located approximately 100 bp upstream and downstream of the cassette, in combination with primers specific to the hygB gene (Table S2). Furthermore, WT and deletion strains were tested for expression of the chiC2 gene by reverse transcription PCR (RT-qPCR) using the same primers as for RT-qPCR (Table S2). All phenotypic analyses were conducted using three independent positive transformants in order to confirm that any phenotypic impact was attributed to the chiC2 gene deletion and not to ectopic insertions.

**Antagonism and biocontrol assays.** The antagonistic ability of C. rosea WT and the chiC2 deletion strains against F. graminearum, B. cinerea and R. solani were investigated using a plate confrontation assay and in liquid cultures. For plate confrontations, fungi were inoculated on Czapek–Dox agar (Sigma-Aldrich) at opposite sides of 9 cm diameter Petri plates and incubated at 25 °C in darkness. Due to its different mycelial growth rate, C. rosea was inoculated 5 days prior to the other species and growth was recorded daily.

For the antagonistic assay in liquid cultures, 100 ml Erlenmeyer flasks containing 50 ml PDB were inoculated with a 5 mm diameter C. rosea agar plug and incubated for 3 days. Then, culture filtrates were made by filter-sterilization using a 0.45 μm cellulose acetate membrane (VWR) and 20 ml medium was reinoculated with a 5 mm diameter agar plug from 5-day-old R. solani, F. graminearum or B. cinerea cultures, and incubated at 25 °C in darkness on a rotary shaker (150 r.p.m.). Dry weight biomass production was recorded 7 days after inoculation. Both the plate confrontation assay and the liquid culture filtrate assay were performed in triplicates.

The biocontrol ability of C. rosea WT and ΔchiC2 strains against B. cinerea was evaluated in an Arabidopsis thaliana detached leaf bioassay as described previously (Dubey et al., 2014a). Briefly, C. rosea conidia (1 × 10⁸ spores) derived from WT and chiC2 deletion strains were inoculated in A. thaliana (ecotype Colombia-0) leaves, followed by inoculation with B. cinerea (5 × 10⁵ conidia). Leaves were placed on water agar plates and the necrotic lesion surface was measured 65 h post-inoculation using a DeltaPix camera and software (DeltaPix).

**Growth and sporulation rates.** For growth rate measurements under different carbon conditions, C. rosea WT and the deletion strains were inoculated on SMS medium with 1 % (w/v) glucose, 1 % (w/v) colloidal chitin (Sigma-Aldrich) or 1 % (w/v) R. solani cell wall material (Inglis & Kawchuk, 2002) as the sole carbon source, and containing 1.5 % agar. The agar plates were incubated at 25 °C in darkness and mycelial growth rates were recorded daily. For abiotic stress analysis, plates containing Czapek–Dox agar supplemented with 0.5 M NaCl, 0.02 % (w/v) SDS, 1.5 M sorbitol or 0.1 % (w/v) caffeine were inoculated with C. rosea WT and the deletion strains and incubated 25 °C in darkness. Growth rates were recorded 5 days after inoculation. Conidiation rates of C. rosea WT and chiC2 deletion strains were determined by harvesting conidia from 10–day-old PDA cultures, grown in 25 °C in darkness, and counting using a Bright-Line haemocytometer (Sigma-Aldrich). All growth and conidiation experiments were performed in three biological replicates.

**Statistical analysis.** ANOVA (one way) was conducted on gene expression and phenotypic data using a General Linear model im-
plemented in SPSS 20 (IBM). Pairwise comparisons were performed using the Tukey’s or Fisher’s method at the 95 % significance level.
RESULTS

Genome analysis and phylogeny of C. rosea chitinases

Genome analysis of C. rosea strain IK726 revealed that it contained 14 genes with a predicted GH18 module. BLASTP analysis of these genes showed high similarity (between 42 and 83 % identity, Table S3) with chitinases from fungal species that belong to the order Hypocreales, while ENG1 showed 61 % identity to the putative EAA75614 ENGase from F. graminearum. In order to categorize the predicted C. rosea chitinases into phylogenetic groups, a phylogenetic analysis was performed with chitinases from other Sordariales fungal species. Our data revealed that C. rosea contained eight chitinases in phylogenetic group A (Fig. 1): CHIA5 and CHIA6 in subgroup A-II, CHIA1 and CHIA2 in subgroup A-IV, and CHIA3, CHIA4, ECH42 and ECH58 in subgroup A-V. Two chitinases were categorized in the B group: CHIB1 and CHIB2 (Fig. 2). Finally, C. rosea contained two chitinases in the C group: CHIC1 grouped in the C-I subgroup and CHIC2 grouped in the C-II subgroup (Fig. 3). The presence of ECH37 and one putative ENGase, ENG1, was also confirmed.

Modular structure of C. rosea chitinases

The GH18 module of all C. rosea chitinases contained the conserved DXXDXDXXE motif essential for catalytic activity, with the exception for CHIA2 that contained the motif DXXDXAXE. The molecular masses of chitinases from group A were predicted to range between 32 and 67 kDa. In addition to the single GH18 module, CHIA4, CHIA5, CHIA6, ECH42 and ECH58 were predicted to contain an N-terminal secretion signal peptide (Fig. 4). Furthermore, CHIA2 was predicted to be localized in the mitochondria according to Target P and PSORT II prediction. After gap closing by PCR and sequencing, the predicted CHIB2 protein contained a single GH18 module and a signal peptide at the N-terminal (Fig. 4). The second member of the B group, CHIB1, was predicted to contain only the GH18 catalytic domain. However, the phylogenetic analysis showed that CHIB1 was orthologous to plasma membrane anchored chitinases with a complex modular structure that is conserved in filamentous ascomycetes (Tzelepis et al., 2012; Yamazaki et al., 2008), indicating that the C. rosea chiB1 gene model was partial. The predicted C group chitinase CHIC1 protein contained only the GH18 domain. Again, alignments with other C-I group chitinases from closely related species (data not shown) revealed a conserved modular structure including carbohydrate binding module (CBM) 18s (accession no. IPR001002; chitin binding), indicating that the CHIC1 gene model was partial also. The CHIC2 protein was predicted to have the typical C-II group killer toxin-like chitinase modular structure containing a signal peptide, two CBM50 modules (accession no. IPR018392; LysM peptidoglycan binding), one CBM18 module and the GH18 module (Fig. 4). However, alignment with full-length C-II chitinases from N. crassa and As. nidulans indicated that the chiC2 gene model was missing the C-terminus. Finally, ECH37 was predicted to have a signal peptide followed by the GH18 module (Fig. 4), while ENG1 contained only a single GH18 module (Fig. 4).

Gene expression analysis of C. rosea chitinase genes

We investigated the transcriptional regulation of GH18 genes under different conditions relevant to chitinase
functions, using RT-qPCR. The analyses of standard curves revealed that the primer efficiencies ranged between 77 and 92%. The melt curve analyses showed single product amplification in each sample. No amplification was observed in NTC or −RT control samples. None of the group A genes was induced during interspecific interactions.
Fig. 3. Phylogeny of group C chitinases. Analysis was conducted using neighbour-joining with JTT substitution model and complete deletion of missing data, based on CLUSTAL W alignment of GH18 catalytic domain amino acid sequences. Numbers at nodes indicate the bootstrap value. Bar, indicates the number of amino acid substitutions. Protein identifiers include protein name (if available) or protein ID accession numbers from the respective genome projects. Subgroup names are indicated. C. rosea chitinases are indicated in bold type.
in comparison to self-interactions (data not shown). However, ech42 was induced during growth on chitin media compared to carbon starvation and growth on carbon-rich media (P<0.001, Fig. 5b). The chiA2 gene showed higher transcript levels during carbon-starvation conditions and in media where chitin was the sole carbon source, compared with carbon-rich media (P<0.023, Fig. 5b). Finally, higher transcript levels were observed in chiA5 and chiA6, when C. rosea grew on chitin media compared to carbon-rich conditions (P≤0.014, Fig. 5b).

The B group chiB1 gene showed higher transcript levels during interspecific interactions with R. solani compared to the control (P=0.020, Fig. 5a), and it was upregulated during growth on chitin media comparing with carbon-rich conditions (P=0.001, Fig. 5b). The second member of this group, chiB2, was highly induced on chitin media compared to carbon-rich and carbon-starvation conditions (P<0.001, Fig. 5b). The C-I subgroup chitinase gene, chiC1, showed higher transcript levels during growth on chitin media compared to carbon-rich conditions (P=0.022, Fig. 5b), while the C-II subgroup member chiC2 was constitutively expressed in all conditions tested (data not shown). The ech37 gene was upregulated during contact with B. cinerea mycelia compared with the control (P=0.001, Fig. 5a). High induction of this gene was also observed during growth on chitin media compared to carbon-rich conditions (P<0.001, Fig. 5b).

Fig. 4. Domain structure of C. rosea GH18 proteins. Amino acid sequences were analysed for the presence of conserved domains using SMART and InterProScan. Question marks indicate putatively partial sequences. Bar, indicates a length of 100 amino acids and refers to total protein length but not to conserved domains. CBM-18, chitin binding domain (IPR001002); CBM-50, peptidoglycan binding LysM domain (IPR018392); GH18, glycoside hydrolase family 18 catalytic domain; SP, signal peptide.
Generation and validation of C. rosea chiC2 deletion strains

The C. rosea killer toxin-like C-II subgroup member chiC2 gene was replaced with the hygB gene conferring resistance to hygromycin by homologous recombination (Fig. S1) using an Ag. tumefaciens-mediated protocol. More than 100 positive colonies were observed on plates. Twenty transformants were isolated and ten of them were tested in order to confirm that the deletion cassette was inserted at the correct locus. For mutant validation, PCR was conducted using primers located upstream or downstream in combination with primers located

---

Fig. 5. Expression profiles of C. rosea GH18 genes. (a) Expression profiles during interactions with different fungal species: C. rosea mycelia grown in 50 ml PDB medium were triggered with 2 ml homogenized mycelia from B. cinerea (Cr/Bc), R. solani (Cr/Rs) or F. graminearum (Cr/Fg), while 2 ml PDB was added in control samples (control). (b) Expression profiles in different carbon sources: Crich, SMS + 1 % glucose; Cstarv, SMS + 0.1 % glucose; and chitin, SMS + 1 % colloidal chitin. Cultures were grown at 25 °C in darkness and mycelia were harvested 24 h post-inoculation. Relative expression levels in relation to actin gene (act) expression are calculated from Ct values and according to Pfaffl's method. Error bars represent SD based on at least four biological replicates. Different capital letters (A, B) indicate statistically significant differences according to Tukey's test (P≤0.05).
in the selection cassette (Table S3). Amplification of fragments with the expected size was observed in all transformants. Fragments were absent in WT (Fig. S2a, b), while amplification of the actin gene (act) was observed in all strains in order to validate the genomic DNA quality (Fig. S2c). Furthermore, RT-PCR analysis revealed the complete loss of chiC2 gene expression in the mutants (Fig. S2d), while expression of the actin gene was detected in all strains in order to validate the cDNA quality (Fig. S2e). Previous studies have showed that expression of hygB in C. rosea does not result in any detectable growth- or biocontrol-related phenotypes (Dubey et al., 2014b; Lübeck et al., 2002).

**Antagonism and biocontrol assays of C. rosea chiC2 deletion strains**

The antagonistic behaviour of C. rosea WT and ΔchiC2 strains during interactions with R. solani, F. graminearum, B. cinerea, and A. thaliana leaves. Leaves were inoculated with C. rosea conidia, derived from WT and ΔchiC2 strains, 30 min before application of B. cinerea conidia, and were incubated for 72 h in darkness at 25 °C. Leaves inoculated only with B. cinerea conidia were used as a negative control (control). (e) Conidiation rate of C. rosea WT and chiC2 chitinase deletion strains 10 days after inoculation on PDA plates at 25 °C in darkness. Different capital letters (A, B) indicate statistical significant differences according to Fisher’s test (P≤0.05). Error bars represent the SD of three biological replicates.

**Fig. 6.** Phenotypic analysis of C. rosea WT and chiC2 chitinase deletion strains. Biomass is shown as mg of (a) B. cinerea, (b) R. solani and (c) F. graminearum grown in C. rosea culture filtrates 7 days post-inoculation. C. rosea mycelia were grown in 50 ml flasks containing PDB for 3 days at 25 °C in darkness and culture filtrates were filter-sterilized using a 0.45 μm cellulose acetamide membrane and reinoculated with 5 mm agar plug from the above fungal species. (d) Measurement of necrotic lesions caused by B. cinerea in Ar. thaliana leaves. Leaves were inoculated with C. rosea conidia, derived from WT and ΔchiC2 strains, 30 min before application of B. cinerea conidia, and were incubated for 72 h in darkness at 25 °C. Leaves inoculated only with B. cinerea conidia were used as a negative control (control). (e) Conidiation rate of C. rosea WT and chiC2 chitinase deletion strains 10 days after inoculation on PDA plates at 25 °C in darkness. Different capital letters (A, B) indicate statistical significant differences according to Fisher’s test (P≤0.05). Error bars represent the SD of three biological replicates.

http://mic.sgmjournals.org
or B. cinerea was assessed in dual cultures. Seven days after the co-cultures started, an inhibition zone was observed in all cases. No macroscopic differences in antagonistic ability were observed between C. rosea WT and the ΔchiC2 deletion strains (Fig. S3). Since chiC2 is predicted to be secreted, the biomass production of R. solani, F. graminearum and B. cinerea were assessed in culture filtrates derived from C. rosea WT and ΔchiC2 deletion strains. Our data showed that B. cinerea and R. solani produced higher biomass in culture filtrates from the ΔchiC2 strains compared to WT filtrates (P ≤ 0.010, Fig. 6a, b), while no significant differences were observed for F. graminearum (Fig. 6c). All C. rosea strains produced the same amount of biomass in liquid culture (Fig. S4). Interestingly, no differences in biocontrol ability between WT and ΔchiC2 strains against B. cinerea on detached Ar. thallana leaves were observed (Fig. 6d).

Colony morphology, sporulation and growth rates of C. rosea chiC2 deletion strains

The colony morphology of C. rosea on PDA plates was indistinguishable between WT and chiC2 deletion strains (Fig. S5). No differences in growth rate between C. rosea WT and ΔchiC2 strains were identified in any media or abiotic stress condition (Fig. S6). However, the conidiation rate was significantly lower in the ΔchiC2 strains compared to WT (P < 0.001, Fig. 6e).

DISCUSSION

C. rosea is a mycoparasitic species from the same order (Hypocreales), but from a different family (Bionectriaceae) than Trichoderma spp. (Hypocreales). It is therefore highly relevant to compare the evolutionary trajectory of the chitinase gene family in Trichoderma spp. with the situation in C. rosea, as to gain a better understanding of the interaction mechanisms employed by different mycoparasites. The mycoparasitic lifestyle of Trichoderma spp. is correlated with selection for high numbers of group B endochitinases and group C killer toxin-like chitinases (Seidl-Seiboth et al., 2014). The switch from a mycoparasitic to saprotrophic lifestyle in T. reesei is accompanied by a rapid loss of group C killer toxin-like chitinases. In contrast, no selection for either gene gains or losses of group A exochitinases is detected among Sordariomycete fungi (Seidl-Seiboth et al., 2014). Therefore, the remarkably low number of group B and group C chitinases in C. rosea is an important observation that suggests fundamental differences in how different mycoparasites attack the cell wall of the fungal prey. One interpretation is that cell wall degradation may not be a prominent feature of the mycoparasitic attack in C. rosea. However, a low number of genes in a particular isozyme gene family do not necessarily imply a low capacity to degrade a particular substrate. For example, the genome of the well-known cellulose and hemicellulose producer T. reesei contains fewer cellulose- and hemicellulose-encoding genes than most ascomycete species (Martinez et al., 2008).

The higher transcription levels of the two subgroup A-II genes chiA5 and chiA6 in C. rosea when chitin was the sole carbon source may suggest that these chitinases play a role in nutrient acquisition. The ech1 gene, member of the A-II group in As. niger, is expressed under carbon-limitation conditions (van Munster et al., 2012). In contrast, previous studies in N. crassa and in T. atroviride show that A-II chitinase genes are constitutively expressed (Seidl et al., 2005; Tzelepis et al., 2012).

The C. rosea A-IV subgroup chitinase CHIA2 is predicted to be localized in mitochondria and to carry amino acid substitutions in its catalytic module that is predicted to result in an inactive enzyme. Orthologues to CHIA2 in N. crassa (gh18-3) and T. reesei (chi18-3) are also predicted to be localized in mitochondria and carry similar mutations (Karlsson & Stenlid, 2009; Seidl et al., 2005). High transcript levels of the chiA2 gene were observed on chitin and during carbon-starvation conditions. Orthologous genes are differentially regulated in different species. In N. crassa, expression of the gh18-3 gene was induced during interactions with Fusarium sporotrichoides (Tzelepis et al., 2012), while in T. atroviride the chi18-3 gene was constitutively expressed (Seidl et al., 2005).

The C. rosea A-V subgroup member ECH42 represents a highly conserved and highly expressed endochitinase present in ascomycetes (Seidl, 2008). However, our study showed that this gene is not induced during interspecific interactions, which is in contrast with the data reported by Mamarabadi et al. (2008b), where induction of ech42 was observed during contact with B. cinerea on strawberry leaves. The ech42 gene is highly induced during growth on chitin media. Similar expression patterns, induction by chitin, have been reported for ech42 orthologues in several Trichoderma spp. (Carsolio et al., 1994; Mach et al., 1999; Zeilinger et al., 1999), indicating that this chitinase plays a role in exogenous chitin degradation. Deletion of ech42 in C. rosea results in lower in vitro antagonistic activity towards F. culmorum, but has no effect on its biocontrol ability towards F. culmorum on barley (Mamarabadi et al., 2008a), while the role of this chitinase in Trichoderma species biocontrol efficacy is controversial (Carsolio et al., 1999; Woo et al., 1999). In the current work, the C. rosea ech58 gene is not induced during interspecific interactions (including F. graminearum), which is in agreement with data from growth on F. culmorum cell wall material (Mamarabadi et al., 2008a). Deletion of the ech58 does not impair the biocontrol ability of C. rosea towards F. culmorum on barley (Mamarabadi et al., 2008a).

As regards the chitinases in group B, the chiB1 gene is triggered during fungal–fungal interactions, while high induction of this gene was also observed in colloidal chitin. In N. crassa the chit-1 gene, homologous to chiB1, was also triggered by colloidal chitin (Tzelepis et al., 2012). CHIT-1 is predicted to be a GPI-anchored cell wall protein,
and deletion of the *chit-1* gene resulted in a slower growth rate in carbon-rich media (Tzelepis et al., 2012). Similarly, the B group *chiA* chitinase in *As. nidulans* is also a GPI-anchored protein and increased expression levels of this gene was observed during early germination stages (Yamazaki et al., 2008). Regarding the second member of group B, *chib2*, it was highly induced by colloidal chitin. Induction by chitin of the B-II subgroup chitinase gene *chit33* in *Trichoderma harzianum* was also observed (De las Mercedes Dana et al., 2001), implying a function of these chitinases in exogenous chitin degradation for nutrient purposes.

The similarity of group C killer toxin-like chitinases with the *K. lactis* zymocin, the selection for increased gene copy numbers in *T. viruns* (Ihrmark et al., 2010; Seidl-Seiboth et al., 2014), and the transcriptional induction of many studied members during interspecific interactions in *T. atroviride*, *T. viruns*, *N. crassa* and *As. nidulans* (Gruber et al., 2011a; Tzelepis et al., 2012, 2014), suggests a cell wall permeabilizing role of these enzymes to facilitate entry of toxic secondary metabolites into antagonist cells (Karlsson & Stenlid, 2008; Seidl et al., 2005). The situation in *C. rosea* is markedly different, as only two C group members are present and neither gene is induced during interspecific interactions. However, in *T. atroviride*, *T. viruns* and *N. crassa* there are several group C chitinases that are developmentally regulated, or induced by chitin (Gruber et al., 2011a, b; Tzelepis et al., 2012). Deletion of the *As. nidulans* C-II subgroup member gene *chiC2-2* results in reduced growth inhibitory activity of culture filtrates against *B. cinerea*, which confirms the aggressive role of certain killer toxin-like chitinases in fungal–fungal interactions (Tzelepis et al., 2014). These results can now be extended to a mycoparasitic species, as deletion of the C-II subgroup gene *chiC2* in *C. rosea* results in reduced growth inhibitory activity of culture filtrates against *B. cinerea* and *R. solani*.

We also demonstrated a role of CHIC2 during conidiation. Similarly, deletion of the C-I subgroup *tac6* gene in *T. atroviride* is reported to alter the conidiation patterns (Seidl-Seiboth et al., 2014). However, in contrast with CHIC2, the predicted TAC6 protein carries amino acid substitutions in the catalytic site that are predicted to result in an inactive chitinase (Gruber et al., 2011b). The role of chitinases in conidiation is reported in budding yeasts since deletion of the CTS2 chitinase gene in *Saccharomyces cerevisiae* results in abnormal spore synthesis (Giaever et al., 2002). In filamentous fungi, chitinase inhibitors affect the fragmentation of *Acremonium chrysogenum* hyphae in atrochonin (Sánder et al., 1998). The *C. rosea* C-I subgroup member gene *chiC1* is induced by chitin, similar to *tac7* in *T. atroviride* (Gruber et al., 2011b) and to *gh18-9* in *N. crassa* (Tzelepis et al., 2012), suggesting an involvement in nutrient acquisition.

In conclusion, we showed that the mycoparasite *C. rosea* contains only 14 GH18 genes, which is markedly different from the high numbers found in the mycoparasitic *T. atroviride* and *T. viruns* (29 and 36 genes, respectively). By construction of gene deletion strains, we showed that the *C. rosea* C-II subgroup chitinase CHIC2 has an aggressive role during interactions with *B. cinerea* and *R. solani*. The *chiC2* deletion also reduced the conidiation rate in *C. rosea*, implying an involvement of this chitinase in sporulation.

**ACKNOWLEDGEMENTS**

This work was financially supported by the Department of Forest Mycology and Plant Pathology, the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (FORMAS, grant numbers 229-2009-1530 and 229-2012-1288), and the Carl Trygger Foundation (grant numbers 08:189, 11:230 and 12:233). Sequencing of the *C. rosea* genome was performed by the SNP&SEQ Technology Platform in Uppsala, Sweden. The facility is part of the National Genomics Infrastructure (NGI) Sweden and Science for Life Laboratory. The SNP&SEQ Platform is also supported by the Swedish Research Council and the Knut and Alice Wallenberg Foundation.

**REFERENCES**


Edited by: A. Herrera-Estrella