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Functional analysis of the C-II subgroup killer toxin-like chitinases in the filamentous ascomycete *Aspergillus nidulans*

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ABSTRACT

Chitinases are hydrolytic enzymes responsible for chitin polymer degradation. Fungal chitinases belong exclusively to glycoside hydrolases family 18 and they are categorized into three phylogenetic groups (A, B and C), which are further divided into subgroups (A-II to A-V, B-I to B-V and C-I to C-II). Subgroup C chitinases display similarity with the α/β-subunit of the zymocin yeast killer toxin produced by *Kluyveromyces lactis*, suggesting a role of these enzymes in fungal–fungal interactions. In this study, we investigated the regulation and function of 4 *Aspergillus nidulans* subgroup C-II killer toxin-like chitinases by quantitative PCR and by constructing gene deletion strains. Our results showed that all 4 genes were highly induced during interactions with *Botrytis cinerea* and *Rhizoctonia solani*, compared to self-interactions. In addition, *chiC2-2* and *chiC2-3* were also induced during contact with *Fusarium sporotrichoides*, while none of these genes were induced during interactions with *Phytophthora niederhauserii*. In contrast, no difference in expression levels were observed between growth on glucose-rich media compared with media containing colloidal chitin, while all genes were repressed during growth on *R. solani* cell wall material. Phenotypic analysis of chitinase gene deletion strains revealed that *B. cinerea* biomass was significantly higher in culture filtrate derived from the Δ*chiC2-2* strain compared to biomasses grown in media derived from *A. nidulans* wild type or the other chitinase gene deletion strains. The analysis also showed that all chitinase gene deletion strains displayed increased biomass production in liquid cultures, and altered response to abiotic stress. In summary, our gene expression data suggest the involvement of *A. nidulans* subgroup C-II chitinases in fungal–fungal interactions, which is further proven for ChiC2-2. In addition, lacking any of the 4 chitinases influenced the growth of *A. nidulans*.

1. Introduction

The fungal cell wall is a dynamic structure, which is composed mainly of glucans, glycoproteins and chitin (Bowman and Free, 2006). It provides the fungal hyphae with appropriate strength to withstand osmotic pressure and other environmental factors, while at the same time allowing cell growth and division (Latge, 2007). The cell wall also allows communication between the fungus and their extracellular environment (Bowman and Free, 2006). Chitin consists of *N*-acetylglucosamine (GlcNAc) monomers, linked by β-1.4-glucosidic bonds. The cell wall of filamentous fungi consists of approximately 10–20% chitin (de Nobel et al., 2000), while in yeasts only 1–2% of the cell wall is chitin (Klis et al., 2002). Although chitin is not the main constituent of the fungal cell wall, its contribution to cell wall rigidity and plasticity is vital. Disruption of chitin synthesis often results in osmotically unstable and malfunctioning cell walls (Specht et al., 1996).

Chitinases (EC.3.2.1.14) are hydrolytic enzymes that degrade the β-1.4 bond in chitin to release oligomeric or dimeric (chitobiose) products (Gooday, 1990). These enzymes are involved in several aspects of fungal biology, including degradation of exogenous chitin for nutritional purposes, in hyphal growth and development and in autolysis (Adams, 2004). Furthermore, chitinases play an important role in fungal–fungal interactions. Genome analyses have revealed that many soil-borne, highly competitive ascomycetes, such as *Trichoderma* spp., *Fusarium* spp. and *Aspergillus* spp., contain large arrays of chitinase genes (Gruber and Seidl-Seiboth, 2012; Karlsson and Stenlid, 2008).

Fungal chitinases belong exclusively to glycoside hydrolase family 18 (GH18) (Karlsson and Stenlid, 2009), according to the CAZy database classification (Cantarel et al., 2009). Chitinases can be categorized according to their cleavage patterns; endochitinases cleave the chitin polymer at random positions, while exochitinases cleave the chitin polymer from the exposed ends releasing chitobiose products (Horn et al., 2006). Fungal GH18 proteins are grouped into 3 phylogenetic groups, 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

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(Karlsson and Stenlid, 2008). Subgroup A-V contains members with exochitinase activity, while subgroups B-I, B-II and B-IV contain members with endochitinase activity. A recently characterized member of the A-II subgroup exhibits exo-β-*N*-acetylglucosaminidase (NAGase) activity, (van Munster et al., 2012), although it acts from the reducing end compared to NAGases in GH20 which act from the non-reducing end, while the B-V subgroup contains members with endo-β-*N*-acetylglucosamidase (ENGase) activity (Stals et al., 2010).

Group C chitinases display similarity with the α/β-subunits of the secreted zymocin killer toxin from the yeast *Kluyveromyces lactis* (Magliani et al., 1997; Stark and Boyd, 1986). The α-subunit has exochitinase activity and it is believed that its function is to degrade the chitin layer of antagonist yeast cell walls in order to facilitate the diffusion of the toxic γ-subunit into the antagonist cells (Butler et al., 1991). Therefore, it is hypothesized that certain C group chitinases may play a similar role in fungal–fungal interactions, by enhancing the permeability of antifungal compounds into antagonist cells (Seidl et al., 2005). Other group C genes are regulated by nutritional stimuli, developmental stage and during self-interactions, which suggest more diverse functional roles than merely in interspecific fungal–fungal interactions (Gruber et al., 2011a, 2011b; Tzelepis et al., 2012).

In general, group C chitinases are predicted to be targeted to the secretory pathway due to the presence of an N-terminal signal peptide. In addition to the GH18 catalytic module, subgroup C-II members are usually predicted to have 1 carbohydrate binding module (CBM) family 18 (chitin-binding) and 2 CBM50s (LysM, peptidoglycan-binding), while subgroup C-I members are predicted to lack CBM50s (Gruber et al., 2011b). Certain group C chitinases are predicted to contain transmembrane helices that indicates cell wall localization (Tzelepis et al., 2012), while others are predicted to contain the Hce2 fungal effector domain that interferes with host defence reactions (Stergiopoulos et al., 2012). Structural modelling of group C chitinases indicates processive exoenzyme activity (Gruber et al., 2011b).

Aspergillus nidulans is a saprophytic ascomycete that is used as a model species for the genus *Aspergillus*, which includes a plethora of species including human pathogens and industrially important species. The genome sequence of *A. nidulans* contains 20 GH18 genes, from which 4 belong to the C-II subgroup (Karlsson and Stenlid, 2008). In this study, we investigate the regulation and function of these 4 subgroup C-II killer toxin-like chitinases by quantitative PCR and by constructing gene deletion strains. Our results show that all 4 genes are induced during interspecific interactions with other fungi, but not with an oomycete or during self-interaction. Deletion of either gene results in growth-related effects. Deletion of *chiC2-2* results in reduced growth inhibitory activity of culture filtrates, which suggests a crucial role of ChiC2-2 in fungal–fungal interactions.

2. Materials and methods

2.1. Sequence analysis and primer design

A. nidulans GH18 subgroup C-II gene sequences were retrieved from the Broad Institute (http://www.broadinstitute.org/annotation/genome/aspergillus_group/MultiHome.html) database and screened for conserved domains using the SMART protein analysis tool (Letunic et al., 2009), InterProScan (Quevillon et al., 2005) and Conserved Domain Search (Marchler-Bauer et al., 2009). Signal P v.4.0 (Petersen et al., 2011) was used to predict signal peptides. Finally, TMHMM 2.0 (Krogh et al., 2001) and big-PI Fungal Predictor program (Eisenhaber et al., 2004) were used for transmembrane helices and glycosyl-phosphatidylinositol (GPI) plasma membrane anchor identification respectively. Primers were designed based on gene cDNA sequences using the PrimerSelect software implemented in the Lasergene 9 core suite package (DNAstar, Madison, WI). Primers used for quantitative PCR (qPCR) were designed to amplify 80–250 bp amplicons from predicted exons. The primers specificity against different fungal species and optimal annealing temperature were evaluated using gradient PCR techniques.

2.2. Strains and maintenance conditions

A. nidulans strains used in this study are summarized in Table 1. Wild type (WT) strain A4 and auxotrophic strains were obtained from the Fungal Genetic Stock Center (FGSC, Kansas City, MO) (McCluskey, 2003) and were maintained on *Aspergillus* Minimal Medium (AMM; pH 6.5; 6.0 g/l $NaNO_3$, 0.52 g/l KCl, 1.52 g/l KH_2PO_4, 22 mg/l $ZnSO_4 \times 7H_2O$, 11 mg/l H_3BO_3, 5.0 mg/l $MnCl_2 \times 4H_2O$, 5.0 mg/l $FeSO_4 \times 7H_2O$, 1.7 mg/l $CoCl_2 \times 7H_2O$, 1.6 mg/l $CuSO_4 \times 5H_2O$, 1.5 mg/l $Na_2MoO_4 \times 2H_2O$, 50.0 mg/l EDTA × Na_2, 0.52 g/l $MgSO_4 \times 7H_2O$) supplemented with 1% glucose and 1.5% agar and the appropriate supplements when needed (10 mM uridine, 0.5 μg/ml pyridoxine, 2.5 μg/ml riboflavin, 85 μM nicotinic acid, 2 μg/ml biotin) at 28 °C in darkness. *Botrytis cinerea* strain B05.10, *Rhizoctonia solani* strain SA1 and *Fusarium sporotrichoides* strain J26 were maintained on Potato Dextrose Agar (PDA, Sigma–Aldrich, St. Louis, MO), while *Phytophthora niederhauserii* strain P10617 was maintained on diluted granini juice agar (Hosseini et al., 2012) at 25 °C in darkness.

2.3. Gene expression analysis

For gene expression studies on different carbon sources, 100 ml Erlenmeyer flasks containing 50 ml AMM supplemented with 0.5% colloidal chitin, 0.5% *R. solani* cell wall material or 1% glucose (control sample), were inoculated with *A. nidulans* conidia to a final concentration of 1×10^6 conidia/ml and incubated on a rotary shaker for 24 h at 25 °C in darkness. Colloidal chitin was prepared by crab shell chitin (Sigma–Aldrich, St. Louis, MO) as described previously by Roberts and Selitrennikoff (1988) while *R. solani* cell wall material was prepared as described by Inglis and Kawchuk (2002). For gene expression studies during interspecific interactions 100 ml Erlenmeyer flasks containing 50 ml potato dextrose broth (PDB, Sigma–Aldrich, St. Louis, MO) were inoculated with *A. nidulans* conidia to a final concentration of 1×10^6 conidia/ml, and with 2 ml homogenized mycelia from *B. cinerea*, *F. sporotrichoides*, *R. solani* (derived from 7 days old cultures in PDB) or *P. niederhauserii* grown in lima bean broth (Hosseini et al., 2013) for 5 days at 25 °C in darkness. Control samples were inoculated with 2 ml PDB. Mycelia were harvested after 24 h, immediately frozen on liquid nitrogen and stored at −80 °C for a maximum of 2 weeks.

Total RNA was extracted using a phenol–chloroform-based method as previously described by Plumridge et al. (2010). Briefly, frozen mycelia were homogenized using a mortar and pestle in liquid nitrogen and suspended in 1 ml RNA extraction buffer (pH 4.0, 0.6 M NaCl, 0.2 M NaOAc, 100 mM EDTA, 4% SDS) and 1 ml acid phenol (pH 5.0, Sigma–Aldrich, St. Louis, MO), followed by a second phenol–chloroform extraction step. RNA was precipitated by isopropanol and treated with RNAse free DNase I (Fermentas, St. Leon-Rot, Germany). RNA concentration was determined spectrophotometrically using a NanoDrop (Thermo Scientific, Wilmington, DE), while RNA integrity was analyzed after DNase I treatment by electrophoresis on an Agilent Bioanalyzer, using the RNA 6000 Nano kit (Agilent Technologies, Santa Clara, CA). For cDNA synthesis, 1000 ng total RNA was reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) according to manufacturer's instructions using oligo $(dT)_{18}$ and random hexamer primers in a total volume of 20 μl, followed by 10-fold dilution of the