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Functional differentiation of chitinases in the white-rot fungus *Phanerochaete chrysosporium*

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ABSTRACT

Enzymes that act on chitin, including glycoside hydrolases (GH) family 18 (chitinases) and family 20 (NAGases), may have multiple roles in fungal biology. Here, we describe the number, structure and expression of the eleven GH18 and four GH20 genes identified in the wood-rotting basidiomycete *Phanerochaete chrysosporium*, and measure endochitinase and NAGase activities *in situ*. A phylogenetic analysis placed a predicted Chi18D protein in a separate, novel subgroup that was named B-VI. Chitinolytic activity was induced at the hyphal front of *P. chrysosporium* both during combative interactions with *Heterobasidion irregulare* and during secondary colonization of dead *H. irregulare* mycelium. Gene expression of *chi18I* and *chi18K* was induced during combative interactions with *H. irregulare*, but not during secondary colonization of dead mycelium. In conclusion, our data suggests that *P. chrysosporium* uses a consortium of different chitinolytic enzymes for nutrient acquisition and for defence of territorial boundaries.

1. Introduction

The fungal cell wall obtains its strength from fibrils of carbohydrates including chitin, which is a polymer of β-1,4-linked *N*—acetylglucosamine (GlcNAc) units. Chitin, which is also produced by arthropods and some planktonic algae, is one of the most common biopolymers on Earth (Gooday, 1990), and constitutes an important resource for many microorganisms, both in terrestrial and aquatic environments. Wood decay basidiomycetes (Agaricomycotina) are more likely to depend on cellulose than on chitin to cover their metabolic carbon demand. Nevertheless, the nitrogen content of wood is often lower than optimal for fungal growth (Boyle, 1998), and wood decay basidiomycetes may exploit chitin as a source of nitrogen (Lindahl and Finlay, 2006).

On the scale of individual hyphae, all fungi need to decompose their own cell walls, to expand and organise their mycelia. Hyphal extension requires that the newly formed cell wall at the tip is soft and yielding, potentially because chitin and β-1,3-glucan fibrils have yet not been linked (Fontaine et al., 2000; Bartnicki-Garcia, 2006). To form lateral branches from established hyphae, to enable hyphal fusion, or allow the formation of clamp connections, it is speculated that the cell wall has to be softened by enzymatic activity. On a larger scale of morphogenesis, the old mycelium senesces and breaks down, often leaving rhizomorphs to maintain the physical integrity of the mycelium (Dowson et al., 1986). When nitrogen is in short supply, an important benefit of this tightly regulated morphological differentiation is that it enables recycling of nitrogen, preventing accumulation of cell wall chitin in the redundant mycelium. Thus, wood decay basidiomycetes may benefit from enzymatic recycling of their own chitin (Lindahl and Finlay, 2006).

Basidiomycetes prosper in environments with limited disturbance, such as logs and forest podzols, where they often form relatively large mycelia. In the terminology of Grime (1977), further developed by Cooke and Rayner (1984), many basidiomycetes may be described as C-strategists with high competitive ability and efficient resource utilisation (Sterkenburg et al., 2015). Concordantly, basidiomycetes often display antagonism against other fungi and have a high capacity for interference competition for territory (Boddy, 2000; Lindahl et al., 2001). Interspecific fungal

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interactions often result in the superior competitor gaining access to territory previously held by another fungus (Holmer and Stenlid, 1993; Boddy, 2000). Chitinases play a central role for the successor both in direct antagonism towards other fungi (Seidl, 2008), but also for assimilation of resources incorporated in the cell walls of previous colonisers (Lindahl and Finlay, 2006).

Chitinases (EC.3.2.1.14) are hydrolytic enzymes that cleave the β-1,4-bond, releasing oligomeric, dimeric (chitobiose) and monomeric (GlcNAc) products. Chitobiose can be further cleaved by β-*N*-acetylhexosaminidases (NAGase, EC.3.2.1.52) to release GlcNAc monomers. In the Carbohydrate Active Enzymes database (Lombard et al., 2014), fungal chitinases are classified as family 18 glycoside hydrolases (GH18), while fungal NAGases belong to glycoside hydrolase family 20 (GH20) (Seidl, 2008). Based on sequence variation of the GH18 catalytic module, the fungal GH18 gene family is phylogenetically divided into clusters A, B and C (Seidl et al., 2005), which have been further subdivided into groups A-II to A-V, B-I to B-V and C-I to C-II (Karlsson and Stenlid, 2008). Basidiomycete chitinases belong to groups A-III, A-V, B-III and B-V (Karlsson and Stenlid, 2008). Group B-V genes encode enzymes with mannosyl glycoprotein endo-*N*-acetyl-β-D-glucosaminidase (ENGase)-type activity (EC.3.2.1.96), associated with deglycosylation of high-mannose-type glycoproteins (Hamaguchi et al., 2010). The catalytic module of GH18s and GH20s may be connected to one or several substrate binding modules (CBMs) (Seidl, 2008; Karlsson and Stenlid, 2009), which enhance binding of enzymes to insoluble substrates. The amino acid sequence of these enzymes may also contain a N-terminal secretion peptide, a C-terminal glycosylphosphatidylinositol (GPI) anchor signal for attachment to the plasma-membrane, or N- or O-linked glycosylation sites for oligosaccharide modifications (Bowman and Free, 2006).

In the current study, we aim to (a) identify GH18 and GH20 chitinase and NAGase genes in the genome of *Phanerochaete chrysosporium* and describe their modular structure and phylogenetic relationships, and (b) to test if *P. chrysosporium* expression of specific chitinase and NAGase genes as well as over-all chitinase and NAGase activity is associated with mycelial morphogenesis, recycling of senescent mycelium, antagonism, and mobilisation of resources during secondary colonization.

2. Material and methods

2.1. Sequence analysis, primer design and phylogenetic analysis

The *P. chrysosporium* strain RP78 genome (Martinez et al., 2004; Ohm et al., 2014) version 2.2 was screened for the presence of GH18 and GH20 genes, following an iterative BLAST (Altschul et al., 1997) approach (Karlsson and Stenlid, 2008). Conserved domains were predicted using the SMART protein analysis tool (Letunic et al., 2015) and Conserved Domain Search (Marchler-Bauer et al., 2015). Signal P 4.1 (Petersen et al., 2011) was used to predict signal peptides, and the big-PI Fungal Predictor program (Eisenhaber et al., 2004) was used to predict GPI-anchor sequences. PCR primers were designed using PrimerSelect, and protein molecular masses were predicted using EditSeq, both implemented in the Lasergene 10 core suite (DNAstar, Madison, WI).

Predicted GH18 and GH20 proteins were also retrieved from the genomes of *Phanerochaete carnosa* (Suzuki et al., 2012), *Ganoderma lucidum* (Binder et al., 2013), *Fomitopsis pinicola* (Floudas et al., 2012), *Heterobasidion irregulare* (Olson et al., 2012), *Coprinopsis cinerea* (Stajich et al., 2010) and *Ustilago maydis* (Kämper et al., 2006). Amino acid sequences (≥200 amino acid residues) were aligned with MUSCLE (Edgar, 2004), and subsequent phylogenetic analyses were 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 pairwise deletion of gaps. Bootstrap analysis was performed using 500 replicates.

2.2. Strains and media

P. chrysosporium strain RP78 and *H. irregulare* strain TC32-1 were maintained on solid Hagem medium (Stenlid, 1985). Sterile bioassay trays (240 × 240 × 25 mm) were filled with 1% water agar (1 cm thick). A central area of agar was removed, leaving a 4 cm wide rim along the edges of the tray. Strips of spruce veneer (200 × 20 × 1 mm) were wetted with deionized water, autoclaved and placed in bioassay trays so that the ends were supported by the agar rim. After sub-culture of fungal strains on water agar, the veneer was inoculated by placing rectangular (20 × 5 mm) pieces of culture agar close to the end of the veneer strips.

Experiment 1 (Exp1) was designed to provide samples for investigation of enzyme production and gene expression during hyphal morphogenesis and recycling of senescent mycelia. *P. chrysosporium* was inoculated at one end of the veneer strip, and samples were harvested after 14 d when the mycelia had extended about 15 cm. The first cm of the strip that contained the inoculation plug, as well as the non-colonized distal part, was discarded. The remaining part with fungal mycelia was divided in half (for practical reasons) and subjected to enzyme activity measurements. Parallel veneer strips for RNA extraction were divided in two parts (Fig. 1; SGO = solitary growth old mycelia, SGF = solitary growth hyphal front), which were immediately frozen in liquid nitrogen and stored at −80 °C.

Experiment 2 (Exp2) was designed to provide samples for investigation of enzyme and gene expression associated with mobilisation of resources during secondary colonization of fungal mycelia. *H. irregulare* was inoculated at one end of the veneer strip. After 14 d, when *H. irregulare* had extended about 10 cm, the veneer was dried at 60 °C for 72 h. The veneer strip, now containing dead *H. irregulare* mycelium, was rewetted on water agar for 24 h and placed in new bioassay trays. The strip was inoculated with *P. chrysosporium* at the end opposing *H. irregulare* and harvested after another 14 d, when *P. chrysosporium* had extended across the dead *H. irregulare* mycelia. The first cm at each end of the strip that contained the two inoculation plugs was discarded. Then, a 4 cm section was sampled at each end, one section containing only *P. chrysosporium* (NC = non-interacting control), the other containing *P. chrysosporium* overgrowing dead *H. irregulare* (SC = secondary colonization) (Fig. 1). Samples were subjected to enzyme activity measurements, or frozen in liquid nitrogen and stored at −80 °C for RNA extraction.

Experiment 3 (Exp3) was designed to provide samples for investigation of enzyme production and gene expression associated with interspecific antagonism. *H. irregulare* was inoculated at one end of the veneer strip. After 5 d, *P. chrysosporium* was inoculated at the opposing end. After an additional 14 d, the fungi had made physical contact. According to visual examination under a dissection microscope, the interaction resulted in deadlock, and a 10 cm piece with the interaction in the middle was sampled for enzyme activity measurements (Fig. 1). In parallel, a 4 cm piece around the interaction that covered 3 cm of *P. chrysosporium* and 1 cm of *H. irregulare* was sampled for RNA extraction (Fig. 1; CA = combative antagonism). All treatments were performed in 5–7 biological replicates.

2.3. Enzyme activity assays

Spatial distribution of endochitinase activity and NAGase activity were assayed according to Lindahl and Finlay (Lindahl and