Review

Killer toxin-like chitinases in filamentous fungi: Structure, regulation and potential roles in fungal biology

Georgios TZELEPIS\textsuperscript{a,}\*, Magnus KARLSSON\textsuperscript{b}

\textsuperscript{a}Department of Plant Biology, Uppsala BioCenter and Linnean Center for Plant Biology, Swedish University of Agricultural Sciences, Box 7080, S-75007 Uppsala, Sweden
\textsuperscript{b}Department of Forest Mycology and Plant Pathology, Uppsala BioCenter, Swedish University of Agricultural Sciences, Box 7026, S-75007 Uppsala, Sweden

A B S T R A C T

Fungal chitinases are hydrolytic enzymes responsible for degradation of chitin. Chitinases are involved in several aspects of fungal biology, including cell wall remodelling during hyphal growth, conidial germination, autolysis, mycoparasitism and nutrient acquisition. They are divided into three distinct phylogenetic groups; A, B and C. Chitinases from the C group show structural similarities with the killer toxin zymocin produced by the yeast Kluyveromyces lactis and it is speculated that they have a similar function in filamentous ascomycetes, by facilitating penetration of toxins into cells of competing individuals. Genome analyses show that certain fungal species with a mycoparasitic lifestyle contain high numbers of killer toxin-like chitinases, compared with specialized saprotrophs and plant pathogens. Recent developments within this research field have revealed considerable variation in the modular structure and regulation of killer toxin-like chitinases, suggesting more diverse roles than merely fungal-fungal interactions. In this review, we summarize the current knowledge about this intriguing class of chitinases, including their modular structure, evolution, gene regulation, and functional analyses in mycoparasitic as well as in saprotrophic species. We also propose important questions for future research.

© 2018 British Mycological Society. Published by Elsevier Ltd. All rights reserved.

1. Introduction

Fungal cells are surrounded by a dynamic matrix, the cell wall, which provides the appropriate strength for protection against environmental stress conditions, such as osmotic pressure, toxins, mechanical injuries and parasite attack, while at the same time allowing flexibility, and rigidity (Bowman and Free, 2006, Keegstra, 2010, Latge, 2007). The fungal cell wall mainly consists of chitin, mannans, β-1,3-glucan and proteins (Latge, 2007). Chitin is a biopolymer that consists of β-1,4-linked N-acetyl-D-glucosamine (GlcNAC) units. Chitin is not restricted to fungi, but is also found in the cuticle of insects and certain nematodes (Merzendorfer and Zimoch, 2003, Hill et al., 1991), in crustacean and mollusc...
Chitin is hydrolyzed by chitinases (EC 3.2.1.14), which cleave the β-1,4 glycan bond creating chitin oligomers or dimers (Goody, 1999). Chitinases are present in a wide range of organisms, eukaryotic and prokaryotic, playing a crucial and vital role in chitin recycling in both marine and terrestrial ecosystems (Keyhani and Roseman, 1999). Fungal genomes contain a plethora of genes putatively encoding chitinolytic enzymes (Karlsson and Stenlid, 2008, Seidl-Seiboth, 2012) that play multiple roles in fungal biology, including cell wall remodelling during growth, autolysis, hydrolysis of exogenous chitin for nutrient acquisition and attack of the cell wall of competing fungi for environmental niches (Kuranda and Robbins, 1991, Shin et al., 2009, Baker et al., 2009, Dunkler et al., 2005, Boldo et al., 2009). The most extreme form of competition can be found in mycoparasitic fungi that utilize chitinases to attack their fungal prey (Mamarabadi et al., 2008, Gruber and Seidl-Seiboth, 2012). According to their amino acid sequences, chitinases are classified in two distinct glycoside hydrolase families; 18 (GH18) and 19 (GH19) (Henrietta, 1991 Lombard et al., 2014). The latter is present in plant and bacteria, while fungal chitinases exclusively belong to GH18 (Karlsson and Stenlid, 2009). Furthermore, chitinases can be classified according to their preferred cleavage patterns into exochitinases, which cleave the chitin polymer from the exposed ends and endochitinases, which cleave the polymer at random positions (van Aalten et al., 2001, Horn et al., 2006). Phylogenetically, GH18 fungal chitinases are divided into three distinct groups (A, B and C) that can be further subdivided into subgroups (A-II, A-IV, A-V, B-I to B-VI, C-I, and C-II) (Seidl et al., 2005, Karlsson and Stenlid, 2008, Karlsson et al., 2016). Fungal GH18 enzymes from the B-V subgroup encode endo-β-N-acetylglucosaminidases (Stals et al., 2010, Tzelepis et al., 2014a, 2017).

Chitinases in group C are predicted to be large proteins with a molecular weight higher than 200 kDa and show sequence and domain similarity to the α/β subunits of the secreted zymocin killer toxin, which is produced by the dairy yeast Kluyveromyces lactis (Stark and Boyd, 1986, Magliani et al., 1997). The α-subunit in zymocin displays exochitinase activity, thus degrading the cell wall chitin in competing yeasts, and together with the β-subunit facilitates the penetration of a third subunit (γ) into the cytoplasm (Butler et al., 1991). The γ-subunit is a tRNase toxin responsible for inhibiting the proliferation of Saccharomyces cerevisiae by arresting cell division in the G3 phase (Stark et al., 1990). The α- and β-subunits are encoded and translated as a single polypeptide that is subsequently cleaved by a protease to produce two separate proteins, while the γ-subunit is encoded by a separate gene (Magliani et al., 1997). Because of this similarity with zymocin, group C chitinases are also referred to as killer toxin-like chitinases, and are hypothesized to function in fungal antagonism by permeabilization of fungal cell walls to allow penetration of antifungal molecules (Seidl et al., 2005). The aim of this review article is to summarize the current knowledge regarding the group C killer toxin-like chitinases in filamentous fungi and to identify important knowledge gaps that should be addressed in future research.
In addition, the TAL6 CBM50 module-containing protein from the mycoparasitic species *T. atroviride* is shown to inhibit germination of its own spores, thereby expanding the role of LysM proteins to regulation of fungal developmental processes (Seidl-Seiboth et al., 2013). The exact role of the CBM50 LysM modules present in group C chitinases has not been studied, but may function to enhance binding to the cell walls of fungal antagonists. In addition to CBM18 and CBM50 modules, certain subgroup C-II chitinases in *Aspergillus nidulans* and *N. crassa* are predicted to contain one or multiple transmembrane motifs in the C-terminal part (Tzelepis et al., 2012, 2014b), indicating that these proteins are likely localized in the plasma membrane such as gh18-8 in *N. crassa* and chIC2-3 in *A. nidulans* (Fig. 1A).

Interestingly, Stergiopoulos et al., 2012) reported that a small protein termed Hce2, which is homologous to the *C. fulvum* Ecp2 effector, is fused to the C-terminal of certain C-II group chitinases (Fig. 1A). These GH18-Hce2 proteins are found more frequently in saprotrophic species and in pathogens of humans, insects and mycoparasites, rather than in plant pathogens (Stergiopoulos et al., 2012). The Ecp2 effector protein was previously identified in phytopathogenic species such as *Mycosphaerella graminicola* and *M. fijiensis* (Stergiopoulos et al., 2010), and was shown to be a virulence...
factor during plant pathogenesis (Lauge et al., 1997). Although the exact function of the Ecp2 effector is not known, it is speculated that it triggers necrosis in host plants, similar to Avr2 in C. fulvum (Stergiopoulos et al., 2010). In addition, the ChiC18-10 group C chitinase from T. reesei also contains an epidermal growth factor-1-module (EGF-1), shown to be involved in protein-protein interactions (Wouters et al., 2005), at the C-terminal of the protein (Seidl et al., 2005). The functions of the Hce2 and EGF-1 modules are -unknown, but they may represent toxigenic peptides playing a role in antagonist interactions, in analogy with the zymocin γ-subunit.

3. Evolution and genomic organization of killer toxin-like chitinases

The highest numbers of group C chitinases are found in T. virens (15 genes), A. nidulans (11 genes), the truffle mycoparasite Tolycoladophium ophioglossoides (10 genes) and T. atroviride (9 genes) (Karlsson and Stenlid, 2008, Karlsson et al., 2018). Evolutionary analyses among Trichoderma species with predominantly saprophytic (T. reesei) or mycoparasitic (T. atroviride and T. virens) lifestyles, indicated selection for gene contractions and expansions, respectively. Specifically, T. reesei contains only four C-group chitinase genes, while T. atroviride and T. virens display an increased number of these type of chitinases, containing nine and fifteen genes, respectively as mentioned above (Ihrmark et al., 2010). High numbers of C-group chitinases in Trichoderma thus appears to be related with aggressiveness and broad host range mycoparasitism (Kubiczek et al., 2011). However, recent genome sequencing of more mycoparasitic species suggests that this correlation may not hold true, as the number of C-group killer toxin-like chitinases range from two to fifteen genes in different species (Karlsson et al., 2018), indicating reliance on different mechanisms during the mycoparasitic attack Karlsson et al., 2018. Selection for increased numbers of C-group chitinases is also implicated in other soil-borne ascomycetes including A. nidulans and Uncinocarpus resesi (Karlsson and Stenlid, 2008). Furthermore, low levels of sequence identity between C-group chitinases (Ihrmark et al., 2010), low number of orthologs (only five orthologs are identified between T. atroviride and T. virens) (Gruber et al., 2011b) and the localization in small non-syntenic clusters at scaffold ends (Gruber et al., 2011b), suggests that killer toxin-like chitinases evolve through a birth-and-death process followed by sequence diversification (Seidl-Seiboth et al., 2014).

Interestingly, C-group chitinase genes are frequently clustered with genes containing only one or multiple CBM50 LysM motifs but not any catalytic domains (Kubiczek et al., 2011, Gruber et al., 2011a). The function of these CBM50 LysM proteins remain unclear, but expression data show that they are perfectly co-regulated with their respective C-group chitinase genes in T. atroviride, indicating a role in fungal-fungal interactions, similar to LysM effectors in plant pathogens (de Jonge and Thomma, 2009). The LysM protein Tal6, whose gene is associated with the group C tac6 chitinase gene in T. atroviride, showed strong inhibition activity of T. atroviride conidial germination but not against conidia from other species, suggesting a role in regulation of fungal growth (Seidl-Seiboth et al., 2013).

4. Regulation of killer toxin-like chitinase genes

The transcriptional patterns of subgroup C-I and C-II chitinases have been studied thoroughly in saprotrophic model fungi such as N. crassa and A. nidulans but also in several mycoparasitic species. A summary of the available gene regulation data is presented in Table 1. In N. crassa, the C-II gh18-6 and gh18-8 genes were induced during interactions with the ascomycete Fusarium sporotrichioides as compared to growth on carbon rich media, while the gh18-8 gene was induced during carbon starvation conditions (Tzelepis et al., 2012). However, different expression patterns were observed upon interactions with other fungal species. For instance, the gh18-6 gene was induced during interactions with Botrytis cinerea, but not during confrontation with the basidiomycete R. solani, whereas gh18-8 was induced during N. crassa self-interaction (Tzelepis et al., 2012). The third group C gene in N. crassa, gh18-9 from subgroup C-I, was induced during carbon starvation conditions but not during fungal-fungal interactions (Tzelepis et al., 2012).

In A. nidulans, the four subgroup C-II genes chiC2-1, chiC2-2, chiC2-3 and chiC2-4 were all highly induced during interactions with B. cinerea and R. solani, while no induction was observed upon interaction with the oomycete Phytophthora niederhaurserii that lacks chitin in the cell wall (Tzelepis et al., 2014b). ChiC2-2 and chiC2-3 were also up-regulated during interactions with F. sporotrichioides. In contrast with the induced expression during interactions with living R. solani, all genes were down-regulated during growth on media where R. solani cell wall material was used as the sole carbon source. All genes were expressed, but not induced, during growth on chitin (Tzelepis et al., 2014b).

Expression patterns of group C chitinase genes in the aggressive mycoparasite T. atroviride have also been studied. Seidl et al., (2005) showed that the gh18-10 gene (later renamed to tac3) was induced during confrontation with R. solani mycelium and during growth on media containing R. solani cell wall material. However, in a later study, the eight group C chitinase genes in T. atroviride tac1-tac8 (including tac3) were all shown to be induced during confrontation with B. cinerea, but not with R. solani or during T. atroviride self-interactions (Gruber et al., 2011a). No expression was also observed on media containing R. solani cell wall material, except for the tac6 gene (Gruber et al., 2011a). Four genes, tac2, tac3, tac6 and tac7, were shown to be expressed on chitin-containing media (Gruber et al., 2011a). A possible involvement of the tac2 and tac6 gene products in hyphal growth and network formation comes from the induced expression in the central part of the mycelial colony but not in the peripheral zone (Gruber et al., 2011b). As mentioned previously, several tac CBM50 LysM genes that are clustered together with group C chitinases in the T. atroviride genome were also co-regulated with tac chitinase gene expression during interactions with B. cinerea (Gruber et al., 2011b).
<table>
<thead>
<tr>
<th>Gene name</th>
<th>Species</th>
<th>Subgroup</th>
<th>Conditions</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>gh18-6</td>
<td>N. crassa</td>
<td>C-II</td>
<td>Interactions with <em>F. sporotrichoides</em></td>
<td>Tzelepis et al., (2012)</td>
</tr>
<tr>
<td>gh18-8</td>
<td>N. crassa</td>
<td>C-II</td>
<td>Interactions with <em>F. sporotrichoides</em></td>
<td>Tzelepis et al., (2012)</td>
</tr>
<tr>
<td>gh18-9</td>
<td>N. crassa</td>
<td>C-I</td>
<td>Carbon starvation</td>
<td>Tzelepis et al., (2012)</td>
</tr>
<tr>
<td>chiC2-1</td>
<td>A. nidulans</td>
<td>C-II</td>
<td>Interactions with <em>B. cinerea</em></td>
<td>Tzelepis et al., (2014b)</td>
</tr>
<tr>
<td>chiC2-2</td>
<td>A. nidulans</td>
<td>C-II</td>
<td>Interactions with <em>R. solani</em></td>
<td>Tzelepis et al., (2014b)</td>
</tr>
<tr>
<td>chiC2-3</td>
<td>A. nidulans</td>
<td>C-II</td>
<td>Interactions with <em>F. sporotrichoides</em></td>
<td>Tzelepis et al., (2014b)</td>
</tr>
<tr>
<td>chiC2-4</td>
<td>A. nidulans</td>
<td>C-II</td>
<td>Interactions with <em>R. solani</em></td>
<td>Tzelepis et al., (2014b)</td>
</tr>
<tr>
<td>tac1</td>
<td>T. atroviride</td>
<td>C-I</td>
<td>Interactions with <em>B. cinerea</em></td>
<td>Gruber et al., (2011a)</td>
</tr>
<tr>
<td>tac2</td>
<td>T. atroviride</td>
<td>C-I</td>
<td>Interactions with <em>B. cinerea</em></td>
<td>Gruber et al., (2011a)</td>
</tr>
<tr>
<td>tac3</td>
<td>T. atroviride</td>
<td>C-II</td>
<td>Interactions with <em>B. cinerea</em></td>
<td>Gruber et al., (2011a)</td>
</tr>
<tr>
<td>tac4</td>
<td>T. atroviride</td>
<td>C-I</td>
<td>Interactions with <em>B. cinerea</em></td>
<td>Gruber et al., (2011a)</td>
</tr>
<tr>
<td>tac5</td>
<td>T. atroviride</td>
<td>C-I</td>
<td>Interactions with <em>B. cinerea</em></td>
<td>Gruber et al., (2011a)</td>
</tr>
<tr>
<td>tac6</td>
<td>T. atroviride</td>
<td>C-I</td>
<td>Interactions with <em>B. cinerea</em></td>
<td>Gruber et al., (2011a)</td>
</tr>
<tr>
<td>tac7</td>
<td>T. atroviride</td>
<td>C-II</td>
<td>Interactions with <em>B. cinerea</em></td>
<td>Gruber et al., (2011a)</td>
</tr>
<tr>
<td>tac8</td>
<td>T. atroviride</td>
<td>C-I</td>
<td>Interactions with <em>B. cinerea</em></td>
<td>Gruber et al., (2011a)</td>
</tr>
<tr>
<td>tvc2</td>
<td>T. virens</td>
<td>C-I</td>
<td>Interactions with <em>B. cinerea</em></td>
<td>Gruber et al., (2011b)</td>
</tr>
<tr>
<td>tvc3</td>
<td>T. virens</td>
<td>C-II</td>
<td>Interactions with <em>B. cinerea</em></td>
<td>Gruber et al., (2011b)</td>
</tr>
<tr>
<td>tvc4</td>
<td>T. virens</td>
<td>C-I</td>
<td>Interactions with <em>B. cinerea</em></td>
<td>Gruber et al., (2011b)</td>
</tr>
<tr>
<td>tvc5</td>
<td>T. virens</td>
<td>C-I</td>
<td>B. cinerea cell wall material</td>
<td>Gruber et al., (2011b)</td>
</tr>
<tr>
<td>tvc6</td>
<td>T. virens</td>
<td>C-I</td>
<td>B. cinerea cell wall material</td>
<td>Gruber et al., (2011b)</td>
</tr>
</tbody>
</table>

(continued on next page)
Investigating the expression profiles of group C chitinases in the mycoparasitic species *T. virens* showed that the regulation of these genes is complex and divergent from *T. atroviride* (Gruber et al., 2011b). Although *T. virens* possess fifteen group C chitinases, only four genes (*tvc2, tvc3, tvc4* and *tvc10*) were induced during confrontation with *B. cinerea* and *R. solani* compared with when *T. virens* was grown alone on solid medium, but notably, not compared with a *T. virens* self-interaction (Gruber et al., 2011b). In contrast, eleven out of the fifteen genes were up-regulated during growth on medium containing *B. cinerea* cell wall material, while four of them were induced on medium containing *R. solani* cell wall material and five during growth on *T. virens* cell walls (Gruber et al., 2011b). Even differences in chitin quality seem to influence group C chitinase gene expression in *T. virens*; seven genes were induced during growth on colloidal chitin versus three on crude chitin. Regulation by developmental stage appear to be important for group C chitinases in *T. virens* as all genes, except for the four putative pseudogenes *tvc1, tvc8, tvc13* and *tvc15*, were induced during hyphal network formation. *Tvc4* was also induced in conidia (Gruber et al., 2011b) (Table 1).

Gene expression of group C chitinases has also been studied in other mycoparasitic species. The subgroup C-I gene *chiC1* in *Clonostachys rosea* was induced during confrontation with *F. graminearum*, but not during interactions with *B. cinerea* (Nygren et al., 2018). However, neither *chiC1* nor the subgroup C-II gene *chiC2* were induced when co-cultivated with *F. graminearum* in liquid cultures (Tzelepis et al., 2015), again emphasizing that the developmental stage and physiological status may influence the regulation of group C chitinase genes. The *chiC1* gene was induced during growth on medium containing chitin as the sole carbon source (Tzelepis et al., 2015). A group C chitinase gene was up-regulated in the truffle mycoparasite *T. ophioglossoides* during growth on truffle tissue (Quandt et al., 2016) (Table 1). A putative group C chitinase gene was also induced in the thermophilic fungus *Myceliophthora thermophila* during growth on straw material from a range of plants (Kolbusz et al., 2014).

To summarize, a majority of group C chitinase genes are induced during interspecific interactions with other fungi (Table 1), supporting their possible roles as killer toxin-like facilitators of toxic compounds into the cells of competing individuals. However, induction of certain killer toxin-like chitinase genes upon growth on chitin or during self-interactions indicates that their chitinolytic activity has found additional uses in cell wall remodelling, autolysis and nutrient acquisition (Fig. 2).

5. Functional analyses of killer toxin-like chitinases

Structural modelling of the GH18 module of TAC2 from *T. atroviride* indicated that group C chitinases are processive exochitinases, due to the presence of a [SA]XGGW motif indicative of...
processivity and due to a deep and narrow catalytic cleft (Gruber et al., 2011a). Data derived from phenotypic analyses of group C chitinase gene deletion strains are limited. In N. crassa, deletion of the gh18-6 and gh18-8 genes did not have any impact on colony growth or morphology, conidiation, biomass production or responses to abiotic stress conditions (Tzelepis et al., 2012). Similarly, no differences in colony morphology or growth were observed in A. nidulans chiC2-1, chiC2-2, chiC2-3 or chiC2-4 deletion strains (Tzelepis et al., 2014b). Lack of phenotypes is possibly attributed to functional redundancy among the multiple chitinases present in these species. However, deletion of these genes led to increased A. nidulans biomass production, while deletion of chiC2-1, chiC2-2 and chiC2-4 led to more resistant phenotypes in abiotic stress conditions (Tzelepis et al., 2014b). Interestingly, deletion of the chiC2-2 gene, which carries the Hce2 peptide at the C-terminal, resulted in a mutant with reduced in vitro antagonistic ability (Tzelepis et al., 2014b), suggesting a specific role of Hce2 in fungal-fungal interactions.

Functional analyses of killer toxin-like chitinases have also been conducted in mycoparasitic species such as C. rosea and T. atroviride. Deletion of the chiC2 gene in C. rosea has an impact on its in vitro antagonistic ability (Tzelepis et al., 2015). Specifically, the chiC2 deletion strain had a reduced inhibitory activity against B. cinerea and R. solani, but not against F. graminearum (Tzelepis et al., 2015). However, the biocontrol ability of C. rosea against B. cinerea remains unaffected in the ΔchiC2 strain (Tzelepis et al., 2015). Interestingly, this deletion strain also shows a reduced conidiation rate as compared to wild type (Tzelepis et al., 2015). Similarly, deletion of the tac6 gene in T. atroviride is reported to result in altered conidiation patterns (Seidl-Seiboth et al., 2014). However, as mentioned previously, the tac6 gene carries mutations in its predicted catalytic motif that may result in loss of its chitinolytic ability (Gruber et al., 2011a).
6. Concluding remarks

Based on their sequence similarity with the yeast killer toxin zymocin, C-group chitinases have been hypothesized to function in fungal-fungal interactions by permeabilizing antagonist cell walls to facilitate diffusion of toxic compounds (Fig. 2A), either as secreted enzymes or as enzymes bound to the plasma membrane (Fig. 2B). Induction of many killer toxin-like chitinase genes during interspecific fungal interactions and functional studies in A. nidulans and C. rosea support this idea. However, more detailed studies of the molecular function of these proteins and their interaction with the fungal cell wall is needed before the killer toxin-like chitinase facilitated toxin entry hypothesis can be confirmed. On the other hand, regulation by developmental stage and nutritional stimuli and phenotypic effects on growth, conidiation and stress tolerance in A. nidulans, C. rosea and T. atroviride imply more diverse roles of killer toxin-like chitinases. It is important to investigate whether these examples constitute evidence of specific activities performed by killer toxin-like chitinases, or merely reflects the difficulties of the producers to handle and protect themselves against the action of these, presumably dangerous, enzymes. It is likely that co-localized, co-regulated CBM50 LysM proteins may bind to cell wall chitin of the producer in order to protect against the hydrolytic action of killer toxin-like chitinases. However, this hypothesis needs functional validation. Related to this question is whether killer toxin-like chitinases are proteolytically cleaved after secretion out of the fungal cell, in analogy with the α/β subunit of zymocin. If so, this could release presumably toxigenic peptides such as Hce2 and EGF-1 (Fig. 2), again in analogy with the zymocin γ subunit. In addition, this proteolytic cleavage may also release additional CBM50 LysM modules from subgroup C-II chitinases that may protect the producer from its own killer toxin-like chitinases. This post-translational release of protective proteins from the chitinase itself would represent the optimal solution for co-regulating CBM50 LysM proteins and killer toxin-chitinases and be an elegant way of minimizing self-inflicted damage. Finally, the rapid evolution of killer toxin-like chitinases, both in gene copy number changes and sequence divergence, may indicate co-evolution with their cell wall targets or with interfering proteins (proteases or inhibitors) produced by the antagonist in an effector arms race manner. Identification of these killer toxin-like chitinase interfering factors, if they exist, would open up new possibilities in agricultural and medical biotechnology. For example, compounds that specifically interfere with the defence against killer toxin-like chitinases in phytopathogenic fungi may be used to augment the efficacy of fungal biological control agents such as Trichoderma spp. and C. rosea.

Conflicts of interest

Both authors agree with the submission and they declare no conflict of interest.

Acknowledgements

We acknowledge financial support from the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (FORMAS, grant nos. 942-2015-368 and 942-2015-1128) and from the Centre for Biological Control (CBC) at the Swedish University of Agricultural Sciences.

References

Killer toxin-like chitinases in filamentous fungi


