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Review

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

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ABSTRACT

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.

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 injures 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

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shells (Kurita, 2006, Peters, 1972), and in protozoa and algae (Mulisch, 1993, Kapaun and Reisser 1995), thereby making it the second most distributed polymer in nature after cellulose (Gortari and Hours, 2013). However, it is absent from vertebrates and plants, which makes chitin a suitable target for drugs and pesticides (Spindler *et al.*, 1990, Chaudhary *et al.*, 2013). Plants can also recognize chitin from invading fungi by specific receptors and thereby activate basal defence responses leading to pattern-triggered immunity (PTI) (Boller and Felix, 2009, Newman *et al.*, 2013). Chitin only constitutes 10–20 % of the total dry biomass in filamentous fungi, while it is even lower in yeasts (1–2 %) (Bartnicki-Garcia, 1968, de Nobel *et al.*, 2000, Klis *et al.*, 2002). Despite the relatively low proportion of chitin in fungal cell walls, it plays a crucial role for cell wall functioning by balancing plasticity and rigidity (Specht *et al.*, 1996).

Chitin is hydrolysed by chitinases (EC.3.2.1.14), which cleave the β-1,4 glycan bond creating chitin oligomers or dimers (Gooday, 1990). 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, Gruber and 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) (Henrissat, 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.

2. Phylogeny and modular structure of killer toxin-like chitinases

Group C chitinases have been identified only in filamentous ascomycetes (Karlsson and Stenlid, 2009). Based on phylogenetic analyses of the GH18 module, group C chitinases are divided into two distinct subgroups, i.e. C-I and C-II (Seidl *et al.* 2005, Karlsson and Stenlid 2008). This sequence diversification of the GH18 module is also reflected in differences in the modular structure of the members of the two subgroups. Members from both subgroups typically contain a GH18 module with an intact catalytic DXXDXDXE motif indicating that they are active chitinolytic enzymes (van Aalten *et al.*, 2001, Gruber *et al.*, 2011a,b, Tzelepis *et al.*, 2012, 2014b). They also typically have a N-terminal endoplasmic reticulum-targeting signal peptide, indicating that they are secreted out of the cell (Gruber *et al.*, 2011a,b, Tzelepis *et al.*, 2012, 2014b). A few group C chitinases, such as Chi18-1 from *Trichoderma reesei* andTAC6/TVC6 from the mycoparasites *T. atroviride* and *T. virens*, respectively, carry amino acid substitutions in the catalytic motif that may render them inactive (Karlsson and Stenlid, 2009, Gruber *et al.*, 2011a). Subgroup C-I chitinases are further characterized by the presence of chitin-binding family 18 carbohydrate-binding modules (CBM18) together with the GH18 catalytic module located at the N-terminal of the protein (Fig. 1A). In contrast, subgroup C-II chitinases contain both CBM18 and peptidoglycan/chitin-binding CBM50 LysM modules, located at the N-terminal side of the centrally located GH18 module (Fig. 1A). Analysis of predicted 3D structures of zymocin and the C-II subgroup gh18-8 from *Neurospora crassa* showed high structural similarities between zymocin and C-II subgroup chitinases (Fig. 1B).

CBM18 modules are shown to bind to chitin (Boraston *et al.*, 2004) and may function to increase the adherence of -group C chitinases to insoluble chitin substrates (Seidl-Seiboth *et al.*, 2014). CBM50 modules were first studied in prokaryotic organisms and display binding affinity towards both chitin and peptidoglycans (Buist *et al.*, 2008). They were subsequently described in many eukaryotic organisms including plants and fungi (Zhang *et al.*, 2009, Seidl *et al.*, 2005). In phytopathogenic fungi, LysM effector proteins may facilitate pathogen infections by binding and thus masking small chitin fragments from the host immune system, as is the case with the Ecp6 effector from the tomato pathogen *Cladosporium fulvum* (de

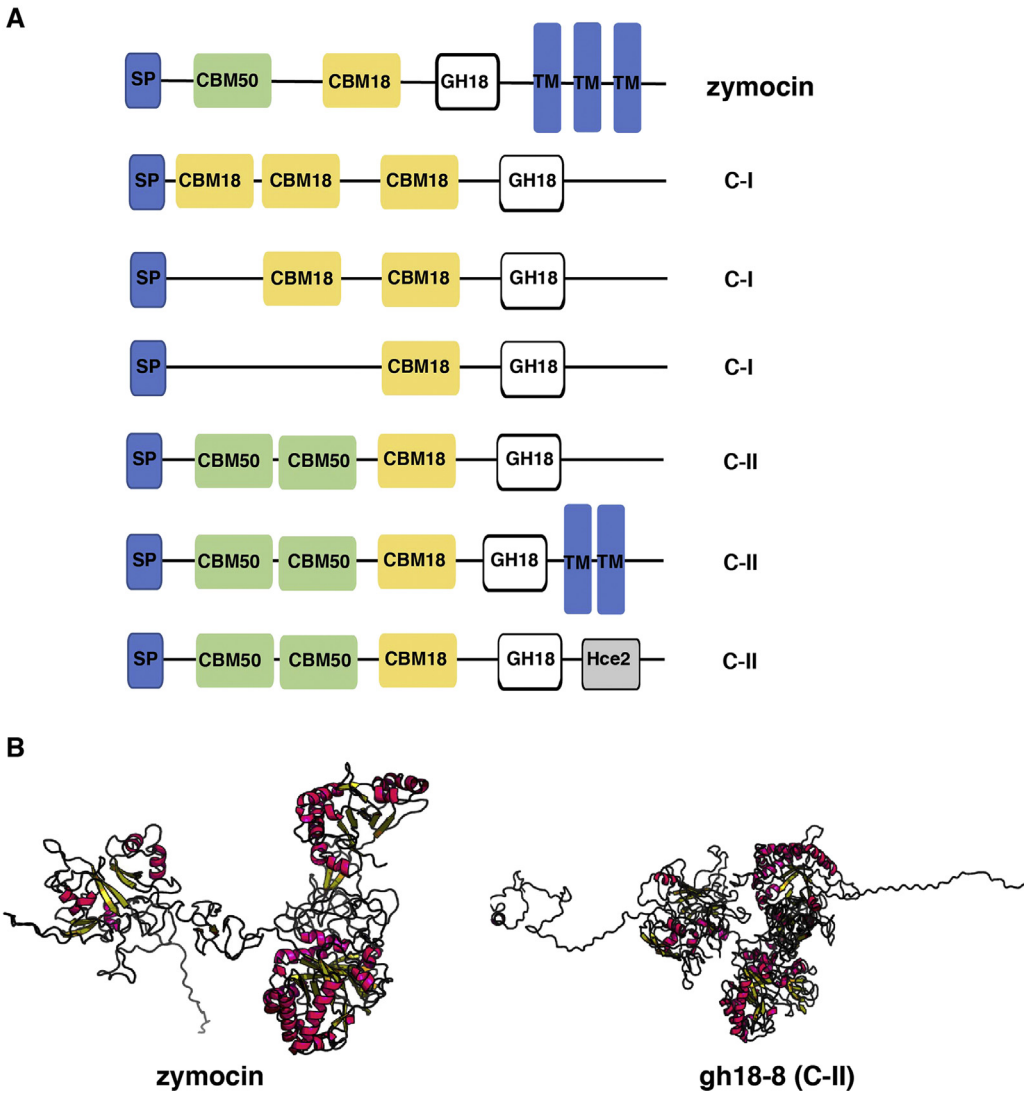

Fig. 1 – Modular architecture of fungal group C killer toxin-like chitinases. A) Domain structures of zymocin and fungal C group chitinases as predicted by SMART software. B) 3D protein model of zymocin and *Neurospora crassa* gh18-8 (C-II) chitinase as predicted by raptorX software. Their modular structures contain the chitinase catalytic domain (GH18), a single secretion peptide (SP) and one or two chitin-binding family 18 carbohydrate-binding modules (CBM18). Chitinases at the C-II subgroup contain two additional LysM peptidoglycan-binding family 50 carbohydrate-binding modules (CBM50). In some cases, additional modules are identified including transmembrane domains (TM) and the Hce2 homologue of the Ecp2 effector. Domain structure is drawn schematically and not to scale.

Jonge *et al.*, 2010). 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 Chi18-10 group C chitinase from *T. reesei* also contains an epidermal growth factor-1-like 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 *Tolypocladium 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 this 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 (Kubicek *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 reesii* (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 (Kubicek *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 niederhauserii* 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 *tal* 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 1 – List of induced genes encoding for putative C-group chitinases in filamentous fungal species.

Gene name	Species	Subgroup	Conditions	Reference
gh18-6	*N. crassa*	C-II	Interactions with *F.sporotrichoides* Interactions with *B. cinerea*	Tzelepis *et al.*, (2012)
gh18-8	*N. crassa*	C-II	Interactions with *F.sporotrichoides* *N. crassa* self-Interactions Carbon starvation	Tzelepis *et al.*, (2012)
gh18-9	*N. crassa*	C-I	Carbon starvation	Tzelepis *et al.*, (2012)
chiC2-1	*A. nidulans*	C-II	Interactions with *B. cinerea* Interactions with *R. solani* *R. solani* cell wall material	Tzelepis *et al.*, (2014b)
chiC2-2	*A. nidulans*	C-II	Interactions with *B. cinerea* Interactions with *R. solani* Interactions with *F.sporotrichoides*	Tzelepis *et al.*, (2014b)
chiC2-3	*A. nidulans*	C-II	Interactions with *B. cinerea* Interactions with *R. solani* Interactions with *F.sporotrichoides*	Tzelepis *et al.*, (2014b)
chiC2-4	*A. nidulans*	C-II	Interactions with *B. cinerea* Interactions with *R. solani*	Tzelepis *et al.*, (2014b)
tac1	*T. atroviride*	C-I	Interactions with *B. cinerea*	Gruber *et al.*, (2011a)
tac2	*T. atroviride*	C-I	Interactions with *B. cinerea* Chitin-containing media	Gruber *et al.*, (2011a)
tac3	*T. atroviride*	C-II	Interactions with *B. cinerea* Chitin-containing media	Gruber *et al.*, (2011a)
tac4	*T. atroviride*	C-I	Interactions with *B. cinerea*	Gruber *et al.*, (2011a)
tac5	*T. atroviride*	C-I	Interactions with *B. cinerea*	Gruber *et al.*, (2011a)
tac6	*T. atroviride*	C-I	Interactions with *B. cinerea* *R. solani* cell wall material Chitin-containing media	Gruber *et al.*, (2011a)
tac7	*T. atroviride*	C-II	Interactions with *B. cinerea* Chitin-containing media	Gruber *et al.*, (2011a)
tac8	*T. atroviride*	C-I	Interactions with *B. cinerea*	Gruber *et al.*, (2011a)
tvc2	*T. virens*	C-I	Interactions with *B. cinerea* Interactions with *R. solani* *B. cinerea* cell wall material *T. virens* cell wall material Colloidal chitin Hyphal network formation	Gruber *et al.*, (2011b)
tvc3	*T. virens*	C-II	Interactions with *B. cinerea* Interactions with *R. solani* *B. cinerea* cell wall material *T. virens* cell wall material Colloidal chitin Carbon starvation Hyphal network formation	Gruber *et al.*, (2011b)
tvc4	*T. virens*	C-I	Interactions with *B. cinerea* Interactions with *R. solani* *B. cinerea* cell wall material *R. solani* cell wall material *T. virens* cell wall material Colloidal chitin Crude chitin Carbon starvation Conidia formation Hyphal network formation	Gruber *et al.*, (2011b)
tvc5	*T. virens*	C-I	*B. cinerea* cell wall material Colloidal chitin Carbon starvation Hyphal network formation	Gruber *et al.*, (2011b)
tvc6	*T. virens*	C-I	*B. cinerea* cell wall material Colloidal chitin Carbon starvation Hyphal network formation	Gruber *et al.*, (2011b)

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Table 1 (*continued*)

Gene name	Species	Subgroup	Conditions	Reference
tvc7	*T. virens*	C-II	*B. cinerea* cell wall material *R. solani* cell wall material *T. virens* cell wall material Colloidal chitin Crude chitin Carbon starvation Hyphal network formation	Gruber *et al.*, (2011b)
tvc9	*T. virens*	C-II	*B. cinerea* cell wall material *R. solani* cell wall material *T. virens* cell wall material Hyphal network formation Carbon starvation	Gruber *et al.*, (2011b)
tvc10	*T. virens*	C-II	Interactions with *B. cinerea* Interactions with *R. solani* *B. cinerea* cell wall material Colloidal chitin Crude chitin Carbon starvation Hyphal network formation	Gruber *et al.*, (2011b)
tvc11	*T. virens*	?	*B. cinerea* cell wall material Hyphal network formation	Gruber *et al.*, (2011b)
tvc12	*T. virens*	C-I	*B. cinerea* cell wall material Carbon starvation Hyphal network formation	Gruber *et al.*, (2011b)
tvc14	*T. virens*	C-II	*B. cinerea* cell wall material Hyphal network formation	Gruber *et al.*, (2011b)
chiC1	*C. rosea*	C-I	Interactions with *B. cinerea* Interactions with *F. graminearum* colloidal chitin	Nygren *et al.*, (2018) Tzelepis *et al.*, (2015)
TOPH09828	*T. ophioglossoides*	?	Truffle mycoparasitism	Quandt *et al.*, (2016)
94536	*M. thermophila*	C-II	Plant straw material	Kolbusz et al. (2014)

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

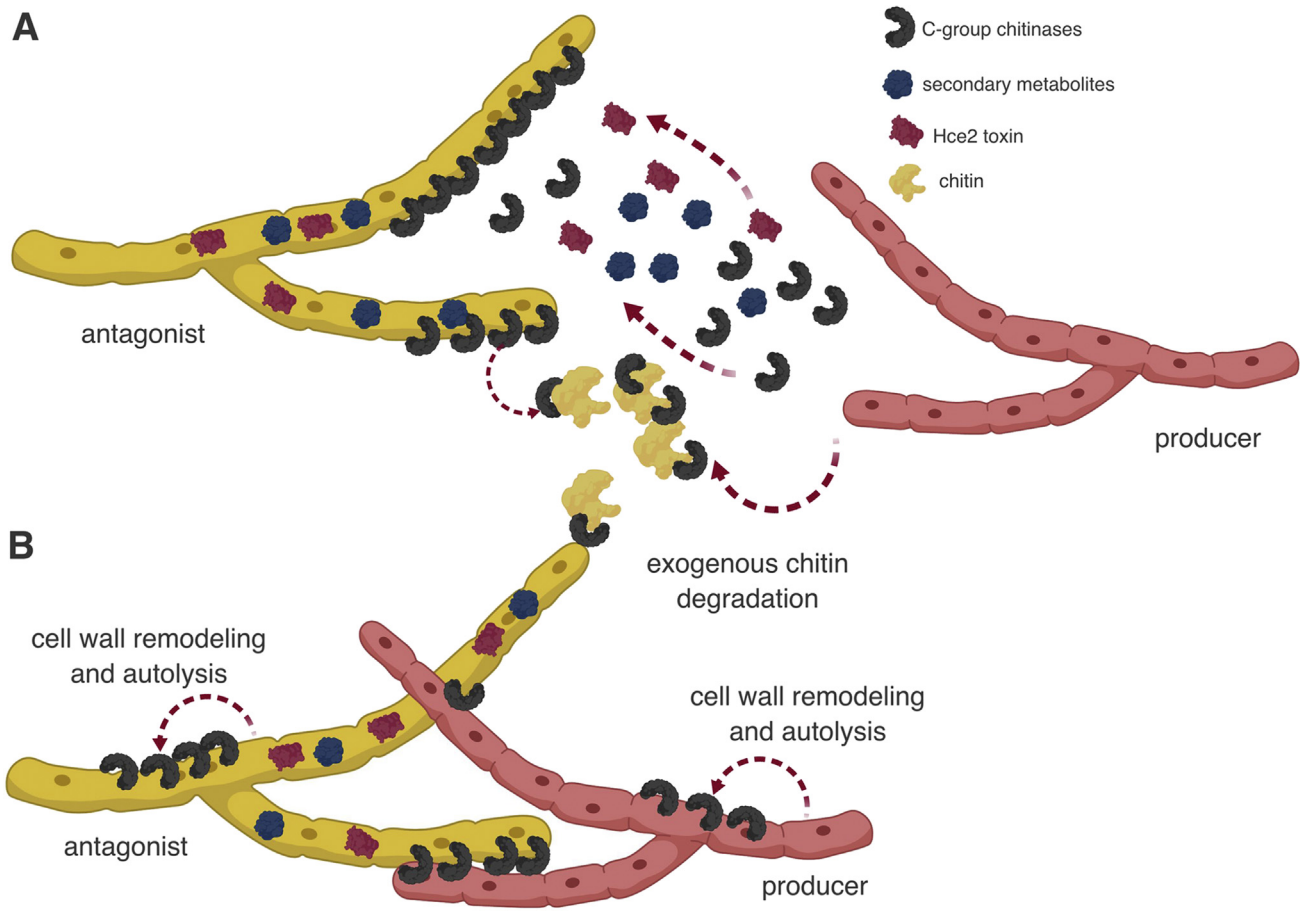

Fig. 2 – Proposed function of group C killer toxin-like chitinases during fungal-fungal interactions. A) Killer toxin-like chitinases are secreted from the producer, bind to cell wall chitin of the antagonist through their CBM18 or CBM50 modules and facilitate the penetration of secondary metabolites or the Hce2 protein into the cytoplasm of the antagonist. The Hce2 protein is presumably released from certain group C chitinases by hitherto unknown proteases. Secreted group C chitinases can also be involved in degradation of exogenous chitin for nutrition purposes. B) Killer toxin-like chitinases with transmembrane domains are presumably located in the cell wall of the producer and would require physical contact with the antagonist cell wall for their activity. Additional function in cell wall remodelling, hyphal branching or exogenous chitin degradation can be speculated. Dashed arrows show the dynamic flow of the molecules. Figure was created with BioRender.

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 Δ*chiC2* 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*.

Conflict of interest

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

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