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Metarhizium anisopliae chitinase CHIT30 is involved in heat-shock stress and contributes to virulence against *Dysdercus peruvianus*

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ABSTRACT

Entomopathogenic fungi are able to produce several chitinases, which serve a variety of biological functions, such as fungal cell wall organization and the degradation of exogenous chitin for nutrition or insect infection processes. In this study, we analyzed the contribution of the CHIT30 chitinase from *Metarhizium anisopliae* in morphogenetic development and virulence as a model of chitinase function. The analysis of *chi3* gene expression revealed transcript accumulation in response to heat-shock stress conditions as well as cultivation in medium supplemented with chitin. Null *chi3* mutants were constructed to determine the biological role of CHIT30. No substantial differences in the secreted chitinase activity could be detected between the wild type and the Δ*chi3* mutant. However, both endochitinase and exochitinase activities were diminished in the mutant strain following heat-shock treatment, suggesting that CHIT30 is involved in heat-shock adaptation. Mutants lacking CHIT30 chitinase showed reduced virulence against the cotton stainer bug *Dysdercus peruvianus*, indicating that the CHIT30 chitinase plays a role in the infection process of *M. anisopliae*.

Introduction

Chitinases catalyze the hydrolytic cleavage of chitin, a β-1,4 linked polymer of N-acetylglucosamine (GlcNAc). Chitin is one of the most abundant components of biomass in nature and is a common component of fungal cell walls, crustacean shells, and insect cuticles. Chitinases play nutritional roles in Bacteria and Archaea, defensive roles in plants and possibly humans, developmental roles in insects and morphogenetic, nutritional and parasitic functions in fungi (Adams 2004; Duo-Chuan 2006; Hartl *et al.* 2012). Thus, chitin metabolism is considered to be fundamental to the three domains of life. Fungal chitinases are classified as part of glycoside hydrolase family 18 according to the CAZy Database (Cantarel *et al.* 2009). Chitinases can be further classified as either endochitinases or exochitinases according to their mechanism of action and site of cleavage in the chitin polymer. Endochitinases randomly degrade chitin to generate GlcNAc oligomers, whereas the main products of exochitinases are GlcNAc dimers (Sahai & Manocha 1993). However, fungal chitinases with multiple

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activities, including endochitinase, exochitinase (da Silva *et al.* 2005; Pinto *et al.* 1997) and transglycosidase (Xia *et al.* 2001) activity, have been described.

Aided by extensive fungal genome sequencing, the complexity of the chitinolytic arsenal of filamentous fungi is becoming more evident (Hartl *et al.* 2012). For example, the genome of the mycopathogenic fungus *Trichoderma reesei* harbours 18 genes that encode for various chitinases. Based on their amino acid sequence and different conserved domains, these chitinases were classified into three phylogenetic groups (Seidl *et al.* 2005). Moreover, a genome-wide analysis of the human pathogen *Aspergillus fumigatus* revealed the presence of at least 18 chitinase genes (Alcazar-Fuoli *et al.* 2011). Up to now, the genome sequences of the entomopathogenic fungi *Metarhizium anisopliae*, *Metarhizium acridum* (Gao *et al.* 2011) and *Beauveria bassiana* (Xiao *et al.* 2012) have been determined. *In silico* analysis indicated that there are 30 putative chitinase genes in the broad-host-range *M. anisopliae* genome, 21 in the narrow-host specialist *M. acridum* (Gao *et al.* 2011), and 20 in the genome of *B. bassiana* (Xiao *et al.* 2012).

Despite the large collection of putative chitinase sequences from different genomes, there is little information on the role of single chitinases in filamentous fungi. The contribution of individual chitinases to different cellular processes has been described, such as autolysis in *A. fumigatus* and *Aspergillus nidulans* (Pocsi *et al.* 2009; Shin *et al.* 2009; Yamazaki *et al.* 2007) and a morphological role in *A. nidulans* (Takaya *et al.* 1998). However, as demonstrated by Alcazar-Fuoli *et al.* (2011), distinct members of a phylogenetic group of chitinases can provide compensatory effects in *A. fumigatus*, as mutant strains for each of the five chitinase genes from the same phylogenetic group, or even multiple gene deletion mutants, do not possess any defect in morphogenetic aspects compared to the parental strain (Alcazar-Fuoli *et al.* 2011).

Entomopathogenic fungal chitinases may be involved in pathogenesis, primarily during the penetration step when the cuticle is breached. The conidium adheres to the cuticle and applies turgor pressure, which is driven by appressorium formation and the secretion of a variety of enzymes that hydrolyze the main components of the cuticle, namely protein and chitin (Arruda *et al.* 2005; da Silva *et al.* 2005; Krieger de Moraes *et al.* 2003; Schrank & Vainstein 2010). The chitinolytic system of *M. anisopliae* is complex, and currently, only three genes have been cloned and characterized (Baratto *et al.* 2006; Bogo *et al.* 1998; da Silva *et al.* 2005; Schrank & Vainstein 2010). The gene *chit1* encodes a 42 kDa secreted endochitinase (Baratto *et al.* 2003), which can be detected during the stages of *Manduca sexta* cuticular penetration (St Leger *et al.* 1996). The overexpression of this gene, however, did not confer increased virulence to *M. anisopliae* but induced the early production of chitinases under inducing conditions relative to the wild type (WT) strain (Screen *et al.* 2001). The gene *chi2* produces two transcripts that undergo alternative splicing *via* intron retention to produce two proteins (Boldo *et al.* 2010). Moreover, assays using *chi2* gene deletion mutations and overexpression to evaluate the contribution of this gene product to the virulence of *M. anisopliae* against the insect *Dysdercus peruvianus* have indicated that there is a strong correlation between the *M. anisopliae* CHI2 chitinase levels and virulence (Boldo *et al.* 2009). The *chi3* gene product was the first characterized chitinase to display both endochitinase and exochitinase activities (Pinto *et al.* 1997) and is expressed during infection of the tick *Rhipicephalus* (*Boophilus*) *microplus* (da Silva *et al.* 2005). Here, to determine the relative contribution of the *chi3* gene product to the *M. anisopliae* infection process, null *chi3* mutant strains were generated, and their virulence was tested against a model host.

Materials and methods

Strains, chemicals, and culture media

Metarhizium anisopliae strain E6 was obtained from the ESALQ collection and maintained as previously described (Dutra *et al.* 2004). *Escherichia coli* TG2 was used in routine cloning, and *Agrobacterium tumefaciens* EHA105 was used to perform *Agrobacterium*-mediated transformation of *M. anisopliae* (Staats *et al.* 2007). Bacteria were obtained from the laboratory's own collection and maintained in Luria-Bertani (LB) medium with the appropriate antibiotics (Sambrook & Russell 2001). All chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA). The LB and Sabouraud media were purchased from Life Technologies (Grand Island, NY, USA) and Oxoid (Basingstoke, UK), respectively. The minimal medium (MM) used for *M. anisopliae* cultivation (6 g L^{-1} $NaNO_3$, 0.52 g L^{-1} KCl, 0.52 g L^{-1} $MgSO_4.7H_2O$, 1.52 g L^{-1} KH_2PO_4, 40 μg L^{-1} $Na_2B_4O_7.10H_2O$, 400 μg L^{-1} $CuSO_4.5H_2O$, 800 μg L^{-1} $FePO_4.2H_2O$, 800 μg L^{-1} $MnSO_4.2H_2O$, and 800 μg L^{-1} $Na_2MoO_4.2H_2O$) was prepared in distilled water and autoclaved. Colloidal chitin was prepared using chitin from Shrimp shells (Sigma–Aldrich – St. Louis, MO, USA) as previously described (Berger & Reynolds 1958).

Real-time PCR analysis

Metarhizium anisopliae was cultured in Sabouraud broth for 48 h at 28 °C on a rotary shaking platform (150 rpm) using an initial inoculum of 10^6 spores mL^{-1}. The mycelium were filtered through Miracloth and washed with a sterile 0.7 % NaCl solution. Standardized inoculum (1 g wet weight) was transferred to MM, MM + 1 % glucose (MMglc), MM + 1 M sorbitol (MMs), and MM + 1 % colloidal chitin (MMcc) and cultivated for 2 h at 28 °C while shaking on a rotary platform (150 rpm). For the heat-shock evaluations, a portion of the mycelium was also transferred to MM and incubated for 2 h at 42 °C while shaking on a rotary platform (150 rpm). Total RNA was extracted from the mycelium using Trizol (Life Technologies – Grand Island, NY, USA) according to the manufacturer's instructions and quantified using a Qubit Fluorometer (Life Technologies – Grand Island, NY, USA). RNA (2 μg) was treated with DNAse (Madison, WI, USA). An aliquot of the DNAse-treated RNA (500 ng) was used for oligo d(T)-primed cDNA synthesis using M-MLV reverse transcriptase (Life Technologies – Grand Island, NY, USA). The primers used for *chi3* transcript amplification and for the reference Tubulin *tub1* transcripts are listed in Table 1. Reactions were performed using Platinum® SYBR® Green qPCR SuperMix-UDG (Life Technologies – Grand Island, NY, USA) and analyzed with an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The amplification efficiency for