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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

Table 1 – Primers used in this study.

Primer	Sequence	Description
5CHI3F	5′TACAGGCTGCGCCAATGCTG 3′	Disruption construct for *chi3*, 5' flank
5CHI3R	5′TACAGCGGTCGTTAGCTCCCA 3′	Disruption construct for *chi3*, 5' flank
GFDBARF	5′CTGTACAGGACCGGTGACT 3′	Disruption construct for *chi3*, *bar* cassette
GFDBARF	5′GAGTAAAGAAGAGGAGCATG 3′	Disruption construct for *chi3*, *bar* cassette
3CHI3F	5′CATGCTCCTCTTCTTTACTCTATTCCTCGGCGGTAC 3′	Disruption construct for *chi3*, 3' flank
3CHI3R	5′TCTAGACAAGTTAGCCTTAATAATCTTCTGGTAATT 3′	Disruption construct for *chi3*, 3' flank
CHI3F	5′CCATGGAAATGTTTGTTAGAAACGCACTAGC 3′	RT-PCR
CHI3R	5′TTGGTCATGGAGGTAAC 3′	SiteFinding/RT-PCR
SiteFinding1	5′CACGACACGCTACTCAACACACCACCTCGCAC AGCGTCCTCAAGCGGCCGCNNNNNNGCCT 3′	SiteFinding
SPF1	5′CACGACACGCTACTCAACAC 3′	SiteFinding
TEF1F	5′CTTCAAGTACGCATGGGTTC 3′	RT-PCR
TEF1R	5′CACATAGGCTTGGAGGGAAC 3′	RT-PCR
qCHI3F	5′TAAACTCGGACGCTCAGGG 3′	qRT-PCR
qCHI3R	5′AAGGGGCTCGGATTGGTC 3′	qRT-PCR
qTUB1F	5′CATCTCTGGTGAACAGGGC 3′	qRT-PCR
qTUB1R	5′AGTTGTCGGGACGGAAAAG 3′	qRT-PCR

each primer-pair was determined based on a calibration dilution curve and slope calculation. Relative transcript level calculations were analyzed according to Pfaffl (2001). The results were statistically analyzed using analysis of variance (ANOVA) tests with the program Graphpad Prism software (La Jolla, CA, USA).

Plasmid construction

To construct the *chi3* gene inactivation cassette, the region upstream of the *chi3* coding sequence was cloned using the SiteFinding method (Tan *et al.* 2005). The primers used are listed in Table 1. PCR-fusion was used to construct the *chi3* gene inactivation cassette (Yu *et al.* 2004). Two fragments of approximately 500 bp that span the putative promoter region and the final portion of the coding sequence were amplified and purified. These fragments were mixed with a 2.2 kb DNA fragment containing the *bar* expression cassette and used in the PCR-fusion reaction. The final 3.2 kb DNA fragment was cloned with blunt ends into the *Eco*RV site of the vector pPZP201BK to generate pPZPΔchi3 (Fig 2A).

Mutant construction using Agrobacterium tumefaciens*-mediated transformation and characterization*

Metarhizium anisopliae transformation was mediated by *A. tumefaciens* harbouring the binary vector pPZPΔchi3 as previously reported (Staats *et al.* 2007). Transformants were selected on MMglc supplemented with 100 μg mL^{-1} ammonium glufosinate (Finale – Bayer CropScience, Monheim am Rhein, Germany) and screened by PCR using primers 5CHI3F and 3CHI3R (Table 1). Candidate deletion transformants were further analyzed by Southern blot analysis and RT-PCR.

Morphogenetic assays

Cell wall defects were evaluated by inoculating several different concentrations of *Metarhizium anisopliae* WT and Δ*chi3* spores onto solidified MMcc agar containing the cell wall stressors calcofluor white or Congo red. The plates were incubated at 28 °C for up to 5 d and analyzed for growth and sporulation. To analyze possible germination and polarization defects, conidia from WT and Δ*chi3* mutant strains were germinated at 28 °C for 4, 8, and 12 h on glass coverslips that were placed on the bottom of a plastic 100-mm Petri dishes containing MMglc. Coverslips with adherent hyphae were fixed for 30 min in phosphate-buffered saline (PBS) containing 3.7 % formaldehyde, washed three times with PBS, stained with 4′,6-diamidino-2-phenylindole (DAPI) (0.25 μg mL^{-1}) for 10 min and washed with PBS. Coverslips were mounted in *n*-propyl gallate and viewed using an Axioskop 40 fluorescent microscope (Carl-Zeiss, Jena, Germany). A total of 100 cells were evaluated for the presence of nuclei and germination.

Chitinase assays

Chitinase activity was measured in culture supernatants from *Metarhizium anisopliae* WT and Δ*chi3* strains that were inoculated (10^6 spores mL^{-1}) into MMcc (72, 96 or 120 h) and placed on a rotary shaking platform (150 rpm). Alternatively, to determine chitinase activity after the mycelium were exposed to heat-shock, the strains were first cultivated in Sabouraud broth for 48 h at 28 °C on a rotary shaking platform (150 rpm). The mycelium obtained from each strain was then filtered through Miracloth and washed with sterile 0.7 % NaCl solution, and 1 g (wet weight) was transferred to MM and subjected to a heat-shock treatment (42 °C for 1 h). The culture supernatants were collected, dialyzed against water and used for the chitinase assays. The determination of chitinase activity in the supernatants was essentially performed as previously described (Lubeck *et al.* 2008) using the substrates 4-methylumbelliferyl-β-d-N,N′-diacetylchitobioside [4-MU-$(GlcNAc)_2$] to detect exochitinases and 4-methylumbelliferyl-β-d-N,N′,N′-triacetylchitotrioside [4-MU-$(GlcNAc)_3$] to detect endochitinases. Experiments were performed in triplicate from three independent biological experiments. The chitinase activity was normalized to the protein content, quantified using Bradford reagent, and statistically analyzed using an ANOVA test with the program Graphpad Prism (La Jolla, CA, USA).

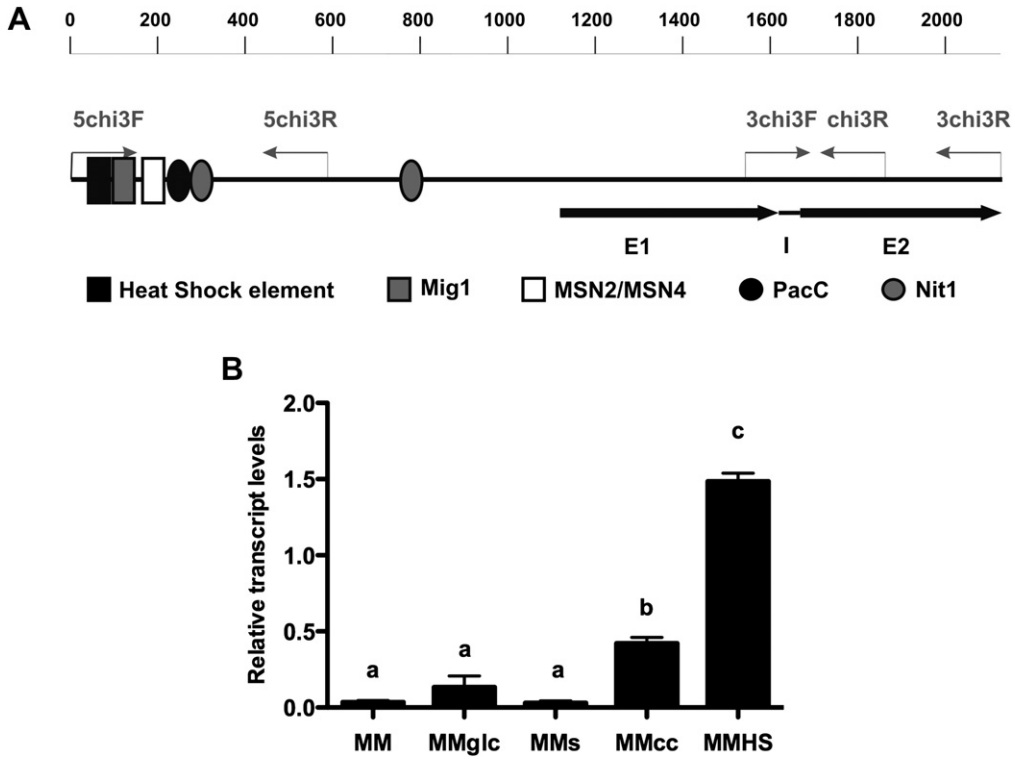

Fig 1 – Structure and transcriptional analysis of the *M. anisopliae chi3* gene. (A) Schematic representation of the *chi3* gene region showing the two exons (E1 and E2), the intron (I), the upstream regulatory sequence and the locations of the primer annealing sites. The consensus binding sites for known fungal transcription factors in the upstream noncoding region of the *M. anisopliae chi3* gene, as identified using MatInspector, are also represented. (B) Transcript profile of the *chi3* gene under several conditions as determined by Real-Time RT-PCR. WT mycelium was incubated in MM, MMglc, MMcc, MMs for 2 h at 28 °C. For the heat-shock experiments, the mycelium was incubated in MM for 2 h at 42 °C (MMHS). The bars represent the mean of three biological replicates, and the lines above the bars represent the standard deviation. The same letter above the bars indicates that there is no significant difference between samples according to the Tukey's multicomparison test ($P < 0.001$).

Virulence assays

The virulence of the WT and Δ*chi3* strains against the insect *Dysdercus peruvianus* was evaluated using bioassays as previously reported (Boldo *et al.* 2009). Ten male *D. peruvianus* adults (kindly provided by Dr Célia Carlini, Centro de Biotecnologia, CBiot/UFRGS) were reared inside plastic boxes with a water supply and cottonseeds for feeding. The insects were infected by immersion in a fresh conidia suspension (10^8 spores mL^{-1}) of each strain for 10 s. Three biological replicates were performed, and insect mortality was monitored daily. Median lethal time (LT_{50}) values were calculated using the Probit analysis of mortality with SPSS 13.0 software (SPSS Inc., Chicago, IL, USA).

Results

Cloning and expression analysis of the gene chi3

Based on the sequence of a clone isolated from an immunological screening of a cDNA expression library (GenBank accession AY545982; da Silva *et al.* 2005), primers were designed to isolate the 5′ flanking sequences of the *chi3* gene using SiteFinding PCR. A 1117 bp DNA fragment was cloned and sequenced (GenBank accession JN251037). *In silico* analysis using the MatInspector algorithm (Cartharius *et al.* 2005) indicated that there were several canonical binding sites for putative transcriptions factors including the catabolite repression element MIG1, the pH-responsive element PacC and the nitrogen-responsive element Nit1. Moreover, two stress-related elements were evident, i.e., a heat-shock binding factor and the stress response element MSN2/MSN4 (Fig 1A). These results prompted us to evaluate whether *chi3* transcript levels were regulated by stress conditions. We could detect *chi3* gene transcripts from mycelium grown in medium containing glucose (repressing condition) and chitin (inducing condition) as well as from mycelium grown under different stress conditions (low-nutrient conditions, osmotic shock and heat-shock). The heat-shock treatment showed a strong induction of *chi3* gene transcript levels with an approximately 20-fold increase compared to repressing conditions (Fig 1B).

Construction of chi3 *null mutants*

To elucidate the biological function of the *chi3* gene product, a mutant strain with an inactivation allele integrated at the *chi3* gene locus was constructed using *Agrobacterium*-mediated transformation (Fig 2A). One of the transformants generated

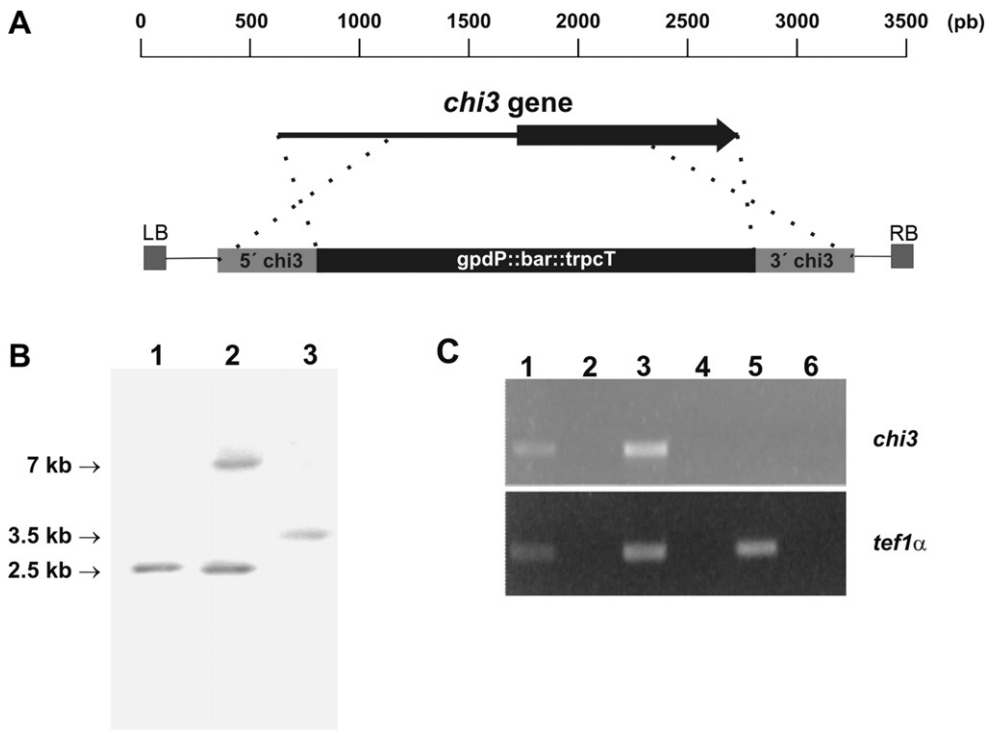

Fig 2 – Inactivation of the *M. anisopliae chi3* gene. (A) The genomic locus spanning the *chi3* gene (upper panel) and the inactivation cassette (lower panel). *gpdP* - *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase promoter. *bar* - ammonium glufosinate resistance gene. *trpCT* – *A. nidulans trpC* terminator. left border and right border - T-DNA left and right borders. (B) Genomic DNA digested with the restriction enzyme *Eco*RV was electrophoresed, transferred to nylon membranes (Hybond-N+, Amersham Biosciences) and probed with the *M. anisopliae* 5′ *chi3* gene-flanking fragment, which was used in constructing the gene inactivation cassette. Lane 1 - WT E6 strain, lane 2 - transformant with the ectopic integration of T-DNA and lane 3 - a candidate deletion mutant strain. (C) RT-PCR analysis of *chi3* transcript levels from the inactivated mutants. RNA samples that were extracted from WT, transformant with an ectopic integration of the inactivation cassette and two *chi3* mutants were reverse transcribed using M-MLV reverse transcriptase (Invitrogen). *chi3* cDNA was amplified with primers CHI3F and CHI3R (upper panel). The amplification of *tef-1α* cDNA was used as the RNA loading control (lower panel). Lanes 1, 3, and 5 are cDNA generated from WT, a transformant with ectopic integration of the T-DNA and the *chi3* mutant strain, respectively. Lanes 2, 4, and 6 are control reactions with no reverse transcriptase added. The numbers on the right represent DNA sizes based on a lambda HindIII molecular size marker.

in the *Agrobacterium*-mediated transformation experiments using the plasmid pPZPΔchi3, which contains the desired deletion construction, was selected, and its T-DNA integration pattern was evaluated using Southern blot analysis to confirm the deletion of the *chi3* allele (Fig 2B). In addition, the expression of *chi3* in the selected mutant was analyzed using RT-PCR. Amplicons from the *chi3* gene could only be detected for cDNA synthesized from RNA extracted from WT cultivated in MMcc. As expected, *chi3* amplicons could not be detected in the Δ*chi3* mutant strain cultivated under similar conditions (Fig 2C).

chi3 *mutant morphogenetic assays*

Because morphogenetic functions have been attributed to fungal chitinases (Seidl 2008), the possible role of CHIT30 in the cell wall architecture and growth was evaluated. The growth rate of the Δ*chi3* mutant was indistinguishable from that of the WT strain in various media (Sabouraud, MMglc, MMcc) based on colony radial growth-rate measurements (data not shown). In addition, assays employing the cell wall stressors Congo red (200 μg mL^{-1}) and calcofluor white (200 μg mL^{-1}), which are used to analyze cell wall defects, were performed to evaluate changes in growth and sporulation. These assays did not indicate any differences in the sensitivity of the WT and Δ*chi3* mutant strains to these stressors (data not shown). Polarization and germination were also evaluated, and there were no significant differences between the WT and Δ*chi3* mutant strains at any of the analyzed time points (4, 8, and 12 h), when considered the number of conidia germinated and nuclei number (not shown). These data confirm that the absence of CHIT30 does not result in major alterations to the cell architecture or morphogenetics processes of *Metarhizium anisopliae* under the analysis conditions.

Chitinase assays

To evaluate the contribution of CHIT30 chitinase to the total secreted chitinase activity in the supernatants of chitin-

amended cultures, a time course assay was performed comparing supernatants from WT and the Δ*chi3* mutant cultures supplemented with colloidal chitin. There were no significant differences detected at any of the analyzed time periods (Fig 3). Given that *chi3* transcript levels increased after exposing the *Metarhizium anisopliae* mycelium to heat-shock treatments, the chitinase activity of temperature-treated culture supernatants was also evaluated. The Δ*chi3* mutant displayed a reduction in both endochitinase and exochitinase activity following thermal stress compared to WT (Fig 4). These results indicate that CHIT30 activity constitutes a considerable fraction of the total chitinase production during heat-shock stress.

Virulence assays

Because that the CHIT30 chitinase was previously detected during *Metarhizium anisopliae* infection (da Silva *et al.* 2005), virulence assays using a cotton stainer bug *Dysdercus peruvianus* infection model were performed with both the WT and Δ*chi3* mutant strains. Although the two strains resulted in 100 % mortality of the host, the insects infected with the WT strain had an LT_{50} of 6.17 d (confidence interval 5.82–6.52), whereas the group treated with the Δ*chi3* mutant strain had an LT_{50} of 8.49 d (confidence interval 8.12–8.92), indicating that the mutant strain takes approximately 40 % longer to kill the same number of hosts (Fig 5).

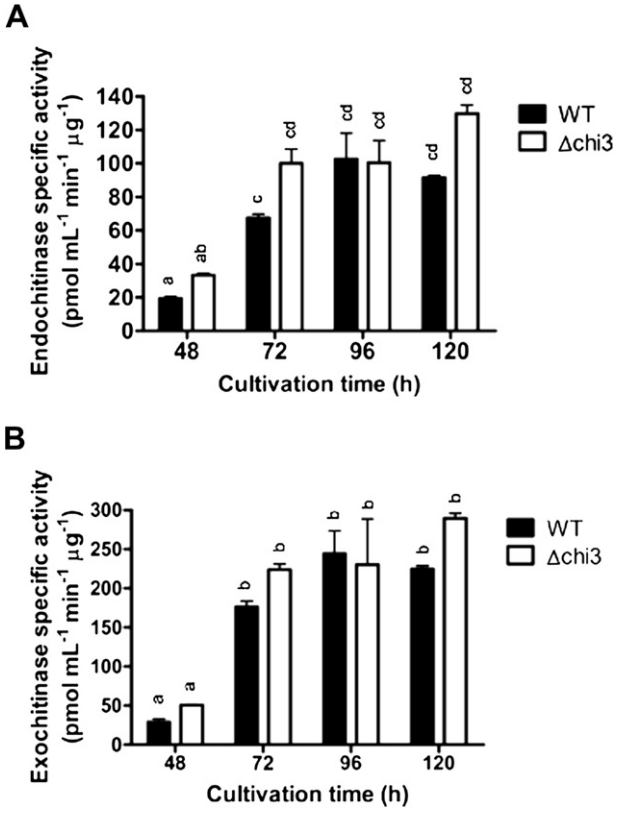

Fig 3 – Analysis of secreted chitinase activity *M. anisopliae* strains grown under chitinase-inducing conditions. The endochitinase (A) and exochitinase (B) activities of culture supernatants of WT and Δchi3 mutant strains were assayed. Supernatants were collected from cultures grown in medium supplemented with 1 % colloidal chitin. Data represent the mean (bars) and standard deviation (lines above bars) of three independent experiments. An ANOVA followed by a Tukey's multiple comparison test was used to compare the means between the WT and Δchi3 mutant strains. Bars with the same letter are not statistically significant.

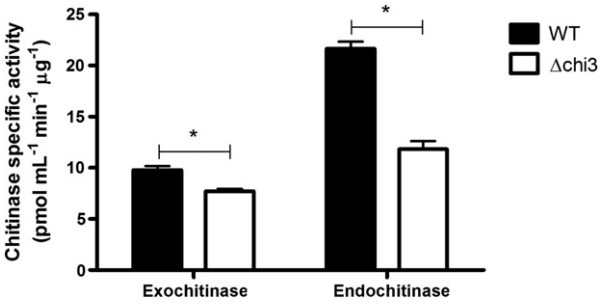

Fig 4 – Analysis of secreted chitinase activity *M. anisopliae* strains following heat-shock exposure. Supernatants were collected from mycelium incubated at 42 °C for 1 h in MM. Data represent the mean (bars) and standard deviation (lines above bars) of three independent experiments. A student's *t*-test was used to compare the means between the WT and *Δchi3* mutant strains. An * denotes a statistically significant difference, $P < 0.05$.

Discussion

The genomes of filamentous fungi are characterized by a large collection of chitinases genes (Gao *et al.* 2011; Seidl *et al.* 2005; Taib *et al.* 2005). Therefore, a reasonable method of functionally characterizing the role of each chitinase in different biological processes is to generate deletion mutant strains. The search for chitinases involved in virulence can be based on an analysis of their sequence similarity because phylogenetically related fungal chitinases have been associated with similar functions, and group B chitinases have been implicated in the degradation of chitin from extracellular sources (Seidl *et al.* 2005). Considering that both the *chi2* and *chi3* genes cluster with the subgroup B chitinases and that the lack of CHI2 chitinase reduces the virulence of *Metarhizium anisopliae*

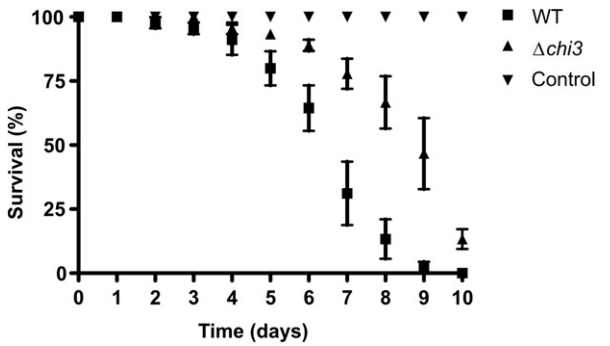

Fig 5 – Virulence assays. The virulence of *M. anisopliae* WT and *Δchi3* mutant strains was evaluated on *D. peruvianus* males. Bars represent the standard deviation.

(Boldo *et al.* 2010), it is therefore reasonable to consider that the CHIT30 chitinase might also contribute to the *M. anisopliae* infectious process. In addition, the immunolocalization of this protein to host cuticle regions that surround the invading hyphae strongly suggests that CHIT30 production is associated with penetration (da Silva *et al.* 2005). Our results are consistent with this assumption because *M. anisopliae* mutants that lack the CHIT30 chitinase displayed reduced virulence against *Dysdercus peruvianus*.

One notable feature of the *chi3* gene is its transcript accumulation after exposure to heat-shock treatment. The thermal regulation of chitinase production has also been observed for the mycoparasite *Trichoderma harzianum chit33* (de las Mercedes Dana *et al.* 2001) and *ech42* genes (Mach *et al.* 1999). Moreover, the products of these *T. harzianum* genes were characterized as heat-resistant chitinases (Haran *et al.* 1995). The effect of heat-shock treatments on filamentous fungal chitinases is poorly understood, although the reduction in chitinase activity of the *chi3* null mutant strain following heat-shock treatment reinforces the assumption that the CHIT30 chitinase could play a role in *M. anisopliae* adaptation to heat-shock. CHIT30 is a specialized chitinase that possesses both endochitinase and exochitinase activity (da Silva *et al.* 2005; Pinto *et al.* 1997). Because both group A and group B chitinases have been implicated in both cell wall remodelling and exogenous chitin degradation (Seidl 2008), group B chitinases may act in remodelling the cell wall to adapt it to specific stringent conditions. This may be the case for CHIT30 chitinase, whose activity in the culture supernatants is substantially increased after *M. anisopliae* has been exposed to heat-shock treatment. Additional studies will be necessary to clarify this hypothesis.

Based on the findings presented here, the activity of the endo/exochitinase CHIT30 is important for virulence. In addition, there were no morphological alterations to the spores, hyphae or appressorium formation that could be detected in the Δ*chi3* mutant strain. However, a consistent reduction in the total chitinase activity could be detected in *chi3* null mutant strains following heat-shock treatment. These results support the hypothesis of chitinase specialization in *Metarhizium* species, in which the variety of chitinases has not been associated with redundant functions. The functional analysis of other chitinase genes will provide a more detailed understanding of the chitinolytic system in *M. anisopliae* and other entomopathogenic fungi. Further analysis of CHIT30, as subcellular localization assays and the determination kinetics of expression during the *M. anisopliae* infectious process, should yield more information about the role of CHIT30 and related chitinases during the host–pathogen interaction.

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