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RESEARCH ARTICLE

Endochitinase CHI2 of the biocontrol fungus *Metarhizium anisopliae* affects its virulence toward the cotton stainer bug *Dysdercus peruvianus*

Juliano Tomazzoni Boldo · Angela Junges · Karina Bohrer do Amaral · Charley Christian Staats · Marilene Henning Vainstein · Augusto Schrank

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Abstract Chitinases have been implicated in fungal cell wall remodeling and play a role in exogenous chitin degradation for nutrition and competition. Due to the diversity of these enzymes, assigning particular functions to each chitinase is still ongoing. The entomopathogenic fungus *Metarhizium anisopliae* produces several chitinases, and here, we evaluate whether endochitinase CHI2 is involved in the pathogenicity of this fungus. We constructed strains either overexpressing or lacking the CHI2 chitinase. These constructs were validated by Southern, Northern and Western blot analysis, and chitinase production. To access the effects of CHI2 chitinase in virulence, the cotton stainer bug *Dysdercus peruvianus* was used as a host. CHI2 overexpression constructs showed higher efficiency in host killing suggesting that the production of this chitinase by a constitutive promoter reduces the time necessary to kill the insect. More significantly, the knock out constructs showed decreased virulence to the insects as compared to the wild type strain. The lack of this single CHI2 chitinase diminished fungal infection efficiency, but not any other detectable trait, showing that the *M. anisopliae* family 18, subgroup B endochitinase CHI2 plays a role in insect infection.

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J. T. Boldo · A. Junges · K. B. do Amaral · C. C. Staats · M. H. Vainstein · A. Schrank (✉)
Programa de Pós-graduação em Biologia Celular e Molecular (PPGBCM), Centro de Biotecnologia da Universidade Federal do Rio Grande do Sul (CBiot/UFRGS), Av. Bento Gonçalves, 9500, Bloco IV, Prédio 43-421, Bairro Agronomia, Porto Alegre, Rio Grande do Sul CEP 91501-970, Brazil
e-mail: aschrank@cbiot.ufrgs.br

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Introduction

Chitin is the major structural component of the invertebrate exoskeleton, and is an important component of the cell wall of filamentous fungi. This polysaccharide is an abundant linear insoluble polymer and is produced by a number of organisms. It is composed of several units of the acetylated amino sugar *N*-acetylglucosamine linked by β-1,4 bonds and is generated to provide stiffness to structures where it is deposited due to the rigidity of the polymer and by forming crosslinks in chitin fibers (Seidl 2008). In addition, it provides protective functions, acting as an initial barrier to hostile environments. In filamentous fungi, the rigid structure formed by chitin and other polysaccharides, which together represent almost 90% of the cell wall, is also useful for penetration of the hyphae into insoluble substrates for nutrition (Latge 2007). Fungi and other microorganisms can also use chitin as an exogenous carbon and nitrogen source and are, therefore, the main recyclers of this abundant polymer, second only to cellulose (Duo-Chuan 2006).

The enzymes that can fully degrade chitin into *N*-acetylglucosamine monomers are divided into *N*-acetylglucosaminidases [EC3.2.1.52, Glycoside hydrolase (GH) family 20] and chitinases (EC3.2.1.14, GH family 18 and 19). *N*-acetylglucosaminidases catalyze the release of GlcNAc monomers from GlcNAc dimers or from the non-reducing terminal end of GlcNAc multimers. Chitinases can be classified into two major classes: endochitinases that cleave the chitin polymer at any point inside the fiber and exochitinases that cleave from the non-reducing end of the polymer

and release *N*-acetylglucosamine (GlcNAc) dimers. All fungal chitinases analyzed so far (including all of the available fungal genomes) belong to GH family 18. Current classification divides GH family 18 chitinases into three subgroups. Subgroup A (former class V) chitinases contain catalytic domain, but no carbohydrate-binding module (CBM), and have molecular mass ranging from 40 to 50 kDa. Most of these chitinases possesses N-terminal signal peptide and are targeted to the secretory pathway, with some exceptions that have intracellular localization. Chitinases from subgroup B (former class III) also possesses N-terminal signal peptide and vary greatly in size, varying from 30 to 90 kDa. Smaller subgroup B chitinases contain a CBM, traditionally described as cellulose-binding domain (CBD), which has a broader polysaccharide binding spectrum and can also bind chitin. Larger subgroup B chitinases usually have serine/threonine rich domains and/or GPI-anchoring signal so that the mature proteins are bound to the plasma membrane. Subgroup C is composed by large chitinases that have a molecular mass of 140–170 kDa. They have N-terminal signal peptide and several features that distinguish them from other subgroups, like the peptidoglycan-binding regions. Filamentous fungi are prolific chitinase producers in terms of both quantity and variety. These enzymes have been implicated in many aspects of fungal biology such as conidial germination, hyphal growth and morphogenesis; in defense against niche competitors; and in nutrition by solubilizing exogenous chitin fibers (Seidl 2008).

Considering the participation of chitinases in nutrition, it is important to emphasize the lifestyle acquired by some filamentous fungi through evolution. Several filamentous fungi such as *Metarhizium anisopliae* have specialized in the acquisition of energy by penetrating and colonizing terrestrial arthropods, whose cuticle is composed of almost 30% chitin fibers (Duo-Chuan 2006). To acquire nutrients in nature, the entomopathogenic fungi have developed a complex infection process, involving mechanical pressure exerted by appressorium formation and the secretion of hydrolases, including chitinases (da Silva et al. 2005; Pedrini et al. 2007; Qazi and Khachatourians 2007; Wang and St Leger 2007). Until now, however, only one *M. anisopliae* chitinase has been experimentally shown to participate in the infection process (da Silva et al. 2005). *M. anisopliae* chitinases are induced in vitro when the fungus is grown in chitin-containing medium (Krieger de Moraes et al. 2003; Barreto et al. 2004; Baratto et al. 2006; da Silva et al. 2005). At least six chitinases have been detected, although only three genes have been isolated (Bogo et al. 1998; Freimoser et al. 2003; Baratto et al. 2006). The *chit1* gene encodes a 42-kDa endochitinase (Bogo et al. 1998; Baratto et al. 2003); the *chi2* gene also encodes a 42-kDa endochitinase (Baratto et al. 2006); and the *chi3* gene encodes a 30-kDa exo/endo-acting chitinase (Pinto et al. 1997; Freimoser et al. 2003; da Silva et al. 2005). Although chitinases are induced by chitin and host cuticle-containing media, the importance of these enzymes in the pathogenesis process is still unclear. The evaluation of constructs carrying deletions or overexpressing chitinase in bioassays could help the understanding of their function.

Few studies have been conducted to analyze whether chitinases participate in the infection process of entomopathogenic fungi. The overexpression of the *M. anisopliae chit1* gene did not alter fungal virulence toward *Manduca sexta* (Screen et al. 2001). In contrast, *M. anisopliae* CHIT30 chitinase (*chi3* gene) was found to be highly expressed during *Rhipicephalus* (*Boophilus*) *microplus* infection (da Silva et al. 2005), and the overexpression of the *Beauveria bassiana* endogenous *Bbchit1* gene enhanced fungal virulence toward aphids (Fan et al. 2007).

The *M. anisopliae chi2* gene was cloned from strain E6 (used as the wild-type strain in this work) and characterized (Baratto et al. 2006). The *chi2* gene comprises 1,524 bp, including two introns of 210 and 72 bp. The endochitinase encoded by this gene has a predicted size of 423 aa and a predicted mass of 42 kDa. CHI2 possesses a catalytic domain containing a substrate binding site and a fungal CBD at the C-terminus, as well a signal-peptide at the N-terminus, which is characteristic of GH family 18 subgroup B (Fig. 1a). Phylogenetically, the CHI2 chitinase clusters with the CHIT30 chitinase and other family 18 plant-like chitinases, subgroup B (Seidl et al. 2005). As suggested by Seidl et al. (2005), the *chi2* and *chi3* genes are related to mycoparasite chitinases since those chitinases clustered as a separated clade within subgroup B chitinases and potentially have a role in myco- and entomopathogenicity processes. Consistently, both *chi2* and *chi3* genes from *M. anisopliae*, as well as *chi18-13* from the mycopathogen *Trichoderma harzianum*, are upregulated in the presence of host cuticle (da Silva et al. 2005; Seidl et al. 2005; Baratto et al. 2006).

In order to assign a function to the *M. anisopliae* CHI2 chitinase, we constructed *chi2* overexpressing and knock out constructs and evaluated their virulence to insects.

Materials and methods

Plasmid constructs

For the construction of the CHI2 overexpression plasmid, a cDNA of the *chi2* gene from *M. anisopliae* (Baratto et al. 2006) was amplified using specific primers [chi2cdnaF: 5′ GGTACCAATGCATCATCTACGCGCTC 3′ (*Kpn*I site underlined); chi2cndaR: 5′ GGATCCCACCGGCATGA CGACCACCA 3′ (*Bam*HI site underlined)] and blunt

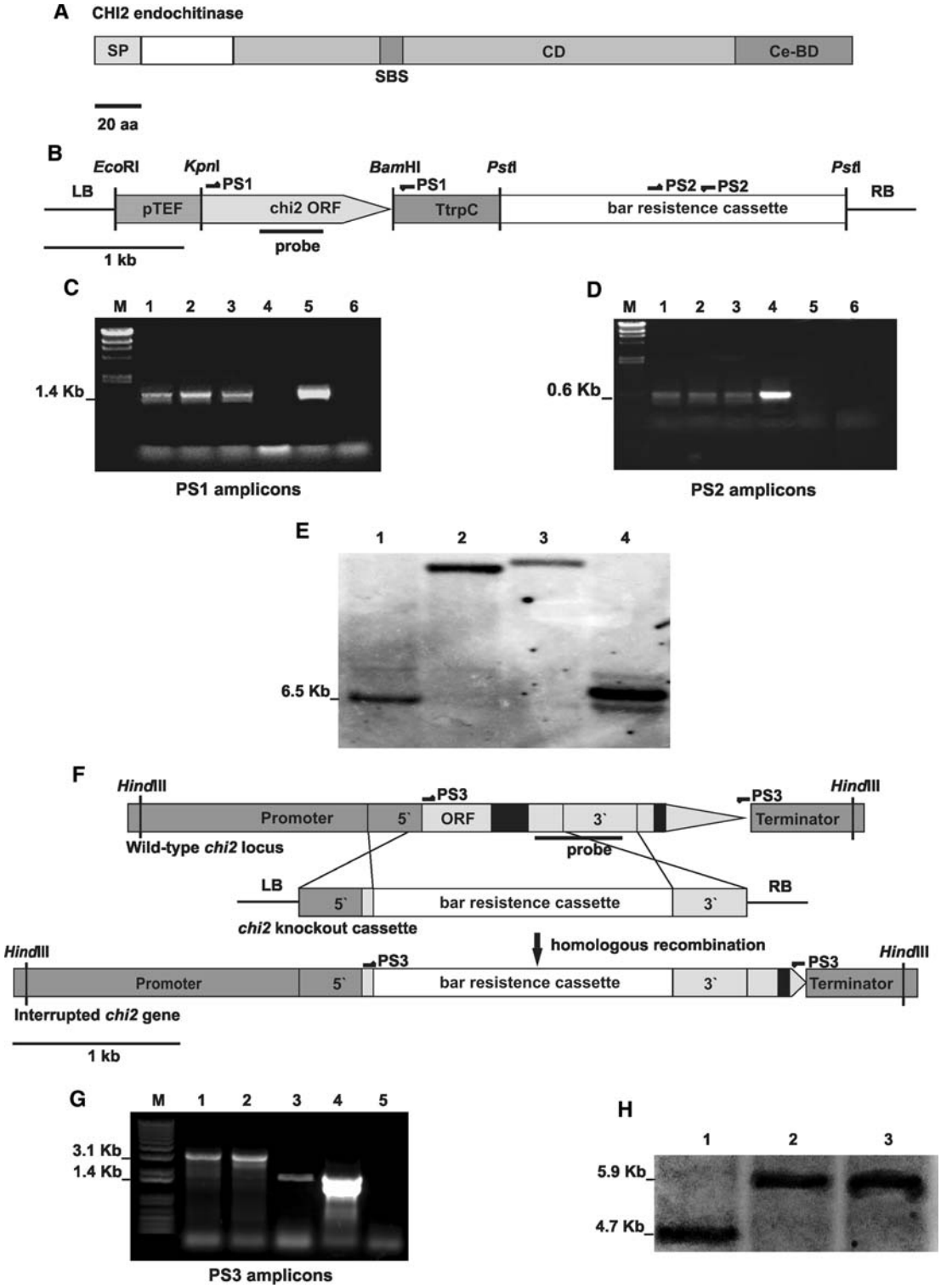

Fig. 1 Overexpression and knock out constructs. **a** CHI2 domain prediction. *SP* signal peptide; *CD* glycosyl hydrolases family 18 catalytic domain; *SBS* substrate binding site; *Ce-BD* fungal cellulose binding domain. **b** Overexpression cassette. **c** Overexpression transformants screened using PS1 primers: *1* T5; *2* T12; *3* T33; *4* E6; *5* positive control (pPZP::upchi2::bar plasmid); *6* no DNA template control. **d** Overexpression transformants screened using PS2 primers: *1* T5; *2* T12; *3* T33; *4* positive control (plasmid pPZP::upchi2::bar); *5* E6; *6* no DNA template control. **e** Southern blot analysis of the overexpression transformants: *1* E6; *2* T5; *3* T12; *4* T33. **f** Knock out cassette and schematic genome integration. **g** Knock out transformants screened using PS3 primers: *1* Δ22; *2* Δ25; *3* E6; *4* positive control (pPZP:: Δchi2::bar plasmid); *5* no DNA template control. **h** Southern blot analysis of the knock out cassettes: *1* E6; *2* Δ22; *3* Δ25. *PS* primer sets used for transformants screening PCR; *LB* binary vector left border; *RB* binary vector right border

cloned into the pUC18 *Sma*I site, generating the pUC::upchi2 plasmid. This plasmid was then digested with *Kpn*I and *Bam*HI restriction enzymes, and the *chi2* ORF was subcloned into the pTEF/TRPC plasmid digested with *Kpn*I and *Bam*HI (Nakazato et al. 2006), which contained the *M. anisopliae tef1-α* gene constitutive promoter and the *Aspergillus nidulans trpc* gene terminator. The pPZP2 01BK binary plasmid (Covert et al. 2004) was digested with *Eco*RI and *Hin*dIII, and the overexpression cassette of the *chi2* gene was inserted, generating the pPZP::upchi2 plasmid. The pPZP::upchi2 plasmid was digested with *Pst*I, and the resistance cassette carrying the *bar* gene, which confers resistance to the fungicide ammonium glufosinate (Nakazato et al. 2006), was inserted, generating the pPZP::upchi2::bar plasmid. The plasmid used to knock out the *M. anisopliae chi2* gene was constructed using fusion PCR methodology (Yu et al. 2004). The 5′ portion of the *chi2* gene, including 520 bp of the promoter region and 140 bp of the coding region, was amplified using primers 5CHI2F (5′ GCAAGACATCAGATCCTCGTGCCC 3′) and 5CHI2R (5′ AGTCACCGGTCACTGTACAGCAG TAGACGACATTTT 3′). The 3′ portion of the *chi2* gene, comprising 632 bp of the coding region, was amplified using primers 3CHI2F (5′ CATGCTCCTCTTCTTTAC TCTCGACATTGAAGTCAA 3′) and 3CHI2R (5′ AAGA TGGCATCGTGGTCGCG 3′). The 5CHI2R and 3CHI2F primers span the 5′ and the 3′ ends of a resistance cassette expressing the *bar* gene. Both PCR products were purified and used for PCR in a mix containing the resistance cassette and primers 5CHI2F and 3CHI2R. The expected product of 3.3 kb was purified and blunt cloned into the pPZP201BK vector, generating the pPZP::Δchi2::bar plasmid.

Agrobacterium tumefaciens-mediated transformation

Metarhizium anisopliae transformation mediated by *A. tumefaciens* was carried out as previously reported (Staats et al. 2007). Positive transformants were screened by PCR.

Southern blot analysis

According to PCR results, both overexpressing and knock out transformants were selected for Southern blot assays to further characterize cassette integration into the *M. anisopliae* genome. The selected transformants were grown on MCc media (Cove 1966) to obtain mycelia. The mycelia were grounded, and genomic DNA was extracted using standard protocols (Sambrook and Russel 2001). DNA samples were digested with the appropriate restriction endonucleases and subjected to electrophoresis in a 1.2% agarose gel for 16 h at 1 V cm^{-1}. After electrophoresis, the samples were electroblotted onto nylon membranes using a Trans-blot Electrophoretic Transfer Cell (Bio-Rad) and baked at 80°C for 2 h. A *chi2* gene probe (comprising nt 683–1185 (503 bp) of the *chi2* gene ORF—accession number DQ011663.2; Fig. 1b) was labeled with ^{32}P and hybridized for 16 h at 60°C. Gradual stringency washes were performed, and the membrane was exposed to the Kodak-K Screen. Digital images of the blots were obtained using the Pharos system (Bio-Rad).

Anti-serum production

To raise the anti-CHI2 polyclonal anti-serum, the coding region of the *chi2* gene was amplified using primers chi2_S_EC (5′ CCATGGATCATCTACGCGCTCTCG TCG 3′) and chi2_A_EC (5′ CTCGAGCCGGCATGAC GACCACCAAT 3′). The amplified product was blunt cloned into the pUC18 *Sma*I site. The pET-23d(+) vector was digested using *Nco*I and *Xho*I restriction enzymes, and the *chi2* coding region was subcloned. The recombinant plasmid was transformed into *Escherichia coli* BL21 (DE3)pLysS (Stratagene) cells for heterologous expression. Cells were cultivated on LB media containing 1% glucose for 2 h, and expression was induced by 0.1 M IPTG for 3 h. Cells were harvested by centrifugation, and expression was analyzed by SDS-PAGE. The recombinant protein was partially purified using a Hi-Trap chromatography column (GE Healthcare) following solubilization in 6 M urea. The eluted fraction with the recombinant chitinase was submitted to SDS-PAGE, and the corresponding band was excised from the gel and used to immunize rabbits. Antiserum against CHI2 was produced in New Zealand white rabbits, and reactivity to CHI2 chitinase was tested using standard procedures.

Northern blot analysis

The wt and constructs of *M. anisopliae* were cultured in media with 1% crystalline chitin or 1% glucose as the sole carbon sources for 96 h at 28°C and 150 rpm. The mycelia were filtered, and total RNA was extracted as previously described (Dutra et al. 2004). Total RNA samples were electrophoretically separated, and sample normalization was carried out using the Qubit system (Invitrogen). Northern blot analysis was carried out using standard procedures (Sambrook and Russel 2001) using radiolabeled *chi2* (same as used in the Southern analyses; Fig. 1b) and *tub* (comprising nt 347–864 (517 bp) of the *α-tubulin* ORF—accession number DQ393576.1) probes. After hybridization, the Kodak-K Screen (Kodak) was exposed to the membranes, and digital images of the hybridization signals were obtained using the Pharos system (Bio-Rad).

Western blot analysis

The wt and constructs from *M. anisopliae* were grown in media containing 1% crystalline chitin or 1% glucose as the sole carbon sources in a bench incubator for 96 h at 28°C and 150 rpm. The mycelia mat was filtered, and the supernatant was lyophilized. The pellets were suspended in one-tenth of the original volume, and approximately 300 μg of each sample was subjected to SDS-PAGE. The proteins were electroblotted onto PVDF membranes for 16 h at 100 V. The membranes were then blocked in blocking buffer for 2 h at room temperature, incubated with primary antibodies (1:1,000) in PBS/0.1% Tween-20 for 1 h at room temperature and washed three times for 10 min each with PBS/0.1% Tween-20. The membranes were subsequently incubated in the presence of secondary antibodies (anti-rabbit IgG conjugated to peroxidase—Sigma Chemicals; 1:2,000) for 1 h at room temperature and washed three times in PBS/0.1% Tween-20. The hybridization signal was generated using an ECL Plus kit (GE Healthcare), and a digital image was obtained using the VersaDoc system (Bio-Rad).

Total chitinase activity assay

A total of 0.25 mL of *M. anisopliae* culture supernatant of liquid media containing 1% crystalline chitin or 1% glucose as the sole carbon source was incubated in 0.5 mL of PBS (pH 7.0) buffer containing 0.5 g of colloidal chitin for 5 h at 37°C. The production of *N*-acetyl-D-glucosamine (GlcNAc) monomers was evaluated, and the total chitinase activity present in each assay was determined in triplicate. One nkat unit of chitinase was defined as the enzyme amount necessary to catalyze the release of 1 nmol s^{-1} of GlcNAc. A standard curve was generated using different concentrations of GlcNAc (1–500 μM). Statistical analyses of the results, which represent data from three different experiments, were done using Analyses of Variance (ANOVA) and Duncan's test ($P < 0.001$) performed by SPSS 13.0 software.

Endochitinase specific activity assay

The endochitinase specific activity assay was carried out using the 4-methylumbelliferyl-β-D-*N, N′, N″*-Tetraacetylchitotriose oligomer according to standard procedures (McCreath and Gooday 1992), with some modifications. Using a 96-well fluorimeter plate, 95 μL of McIlvain Buffer (0.1 M citric acid; 0.1 M Na_2HPO_4; pH 6.0) and 5 μL of 0.8 mM 4-methylumbelliferyl-β-D-*N, N′, N″*-Tetraacetylchitotriose were added and incubated for 5 min at 37°C. Then, 10 μL of *M. anisopliae* culture supernatant in liquid media containing 1% crystalline chitin or 1% glucose as the sole carbon sources was added and incubated for 30 min at 37°C. The reaction was stopped using 100 μL of Stop Buffer (1 M glycine/NaOH; pH 10.6) for 5 min at 37°C. Water was used in the control sample. The reading was performed using a fluorimeter set for 355 and 460 nm as the excitement and emission wavelengths, respectively. One nkat unit of chitinase was defined as the enzyme amount necessary to catalyze the release of 1 nmol s^{-1} of 4-MU. A standard curve was generated using different concentrations of 4-MU (0.1–10 μM). Statistical analyses of the results, which data from three independent experiments, were done using analyses of variance (ANOVA) and Duncan's test ($P < 0.001$) performed by SPSS 13.0 software.

Bioassays

The bioassays were conducted according to Lubeck et al. (2008) with modifications as follows. Ten male adults of the cotton stainer bug *Dysdercus peruvianus* (kindly provided by Dr. Célia Carlini, Centro de Biotecnologia, CBiot/UFRGS) were placed inside plastic containers with a water supply and cottonseeds for feeding (Staniscuaski et al. 2005). A fresh conidia suspension at 10^8 conidia/mL was produced, and each bug was immersed for 10 s in the suspension, except those used for the negative control, which were immersed in sterile distilled water. Each of the three biological replicates had three experimental replicates. Host mortality was recorded everyday for 10 days. The obtained data were analyzed by ANOVA, and groups were clustered by Duncan's test ($P < 0.001$) using SPSS 13.0 software.

Results

In order to elucidate the role of the *M. anisopliae* CHI2 endochitinase, we generated overexpression and knock out constructs for the *chi2* gene using the previously reported capability of *A. tumefaciens* to transfer T-DNA to *M. anisopliae* (Staats et al. 2007). The overexpression and knock out cassettes are shown in Fig. 1b, f, respectively. Fungal transformations using the pPZP::upchi2::bar plasmid yielded approximately 60 ammonium glufosinate resistant transformants per cellophane disk in five independent experiments. A total of 200 transformants were screened by PCR using primer set PS1 (chi2CDNAF—5′ GGTAC CAATGCATCATCTACGCGCTC 3′; PANDOWN—5′ TCCAGATTCGTCAAGCTGTTTGA 3′), which confirms the correct cassette assembly and insertion into *M. anisopliae* genome, and primer set PS2 (BARF—5′ CCATGG CCATGAGCCCAGAACGAC 3′; BARR—5′ GGATCCT CAGATCTCGGTGACGGG 3′), which amplifies a portion of the *bar* resistance gene. We detected 152 positive transformants with PS1 and PS2 PCRs, and 3 (T5, T12 and T33) clones were used for further analysis (Fig. 1c, d). Fungal

transformation using the pPZP::Δchi2::bar plasmid was less efficient and yielded an average of 40 transformants per cellophane disk in five independent experiments. Primer set PS3 (chi2CDNAF—5′ GGTACCAATGCATCATCTAC GCGCTC 3′; chi2CDNAR—5′ GGATCCTCACCGGCAT GACGACCACC 3′) was used to screen for positive transformants (Fig. 1g). Two positive transformants (Δ22 and Δ25) were identified out of 150 screened.

In order to analyze genomic integration of the overexpression cassette, DNA from *M. anisopliae* strain E6 and from the selected transformants, based on the PS1 and PS2 PCR experiments, was extracted and digested using *Eco*RV. The blotted DNA was hybridized with a probe comprising 0.5 kb of the coding region of the *chi2* gene. A single hybridization signal of 6.5 kb was detected in strain E6, indicating the presence of a single copy of the *chi2* gene (Fig. 1e). Transformants T5 and T12 showed distinct hybridization bands compared to the wt strain. The wt *chi2* locus was replaced with the overexpression cassette since no 6.5-kb signal was detected. In transformant T33 there were hybridization signals originating from both the wt *chi2* locus and the overexpression cassette, suggesting ectopic integration. Therefore, this transformant was chosen for further experiments. Transformant T33 also displayed the highest chitinase activity. Transformants from the knock out experiment selected by PS3 PCR experiments were grown, and DNA was extracted from mycelia. The DNA was digested with *Hin*dIII, separated by electrophoresis and blotted. The same probe mentioned above was used. We observed a 4.7-kb hybridization signal in the wt strain E6 (Fig. 1h). Transformants Δ22 and Δ25 did not show the wt *chi2* locus hybridization signal; instead, a 5.9-kb signal was detected, confirming that the wt locus was replaced by the knock out cassette.

Expression analyses were performed to confirm the overexpression and deletion of the *chi2* gene in the constructs generated. Chitinase activity, transcripts and CHI2 protein were assayed in the overexpression and knock out constructs and compared to wt levels. Total chitinase and endochitinase activities from constructs T5, T12, T33, Δ22 and Δ25 were assayed in culture filtrates from both glucose and crystalline chitin liquid media. All overexpression constructs showed chitinase activity that was significantly higher ($P < 0.001$) in both experiments compared to wt. The chitinase and specific endochitinase activities in the knock out constructs were slightly diminished in both media with different carbon sources when compared to the wt strain E6 (Fig. 2a, b).

Transcripts of the *chi2* gene were analyzed by Northern blot for the constructs as shown in Fig. 3a from RNA extracted from cultures in glucose and in the presence of chitin. The overexpressing transformant T33 produced *chi2* transcripts that were not suppressed by glucose. The knock out construct Δ22 did not produce *chi2* transcripts. These results were further confirmed by Western blot analysis using anti-CHI2 serum. Expression of CHI2 in construct T33 was detected in both culture media. No CHI2 recognition was observed in the Δ22 construct in medium containing 1% glucose as the sole carbon source (Fig. 3, panel 1% Glucose, lane 3). A faint recognition band, however, was observed for construct Δ22 in medium containing 1% chitin as the sole carbon source (Fig. 3, panel 1% Chitin, lane 3). This was probably caused by non-specific recognition of another isoform of chitinase once the chitinase system was activated in such medium, and no *chi2* transcript was observed in the Northern blot assays.

To analyze the participation of CHI2 chitinase in the infection process, both the *chi2* overexpression and knock out constructs were used in bioassays with the cotton stainer bug *D. peruvianus* as a host. A survival curve of the tested constructs is shown in Fig. 4. The LT_{50} and LT_{90} results demonstrated differences in the ability of the constructs to infect the host (Table 1). The wt strain had an LT_{50} of 156 h and an LT_{90} of 209 h. In contrast, the CHI2 overexpression construct T33 had an LT_{50} of 125 h and an LT_{90} of 154 h. This represents a drop of over 20% in the time required to kill the host, suggesting that the CHI2 chitinase enhances virulence. The knock out construct Δ22 had an LT_{50} of 186 h and an LT_{90} of 267 h, therefore requiring over 20% more time to kill the insect host. These results show that overexpression of CHI2 increased the efficiency of killing *D. peruvianus*, while the *chi2* knock out construct had reduced efficiency compared to wt. The overexpression of the CHI2 chitinase could represent a synergistic effect on the infection process. More significant, however, is the fact that the blocking of a single chitinase gene (CHI2 chitinase) had an important effect in the insect infection efficiency.

Discussion

Metarhizium anisopliae secretes several chitinases, including CHI2, an endochitinase of 42 kDa, which belongs to the subgroup B. Chitinases are thought to be key enzymes during the early stages of the infection process since chitin, associated with proteins, is one of the main compounds of the host cuticles and is highly insoluble. Another important factor is that chitinases are needed during the fungal morphogenesis process once chitin is present in the cell wall and must be remodeled in order to allow hyphae to grow and/or for cell differentiation into appressorium. *Metarhizium* has high chitinase diversity, and at least six chitinases have been observed in glycol-chitin zymogram assays after cultivation in chitin-containing medium (da Silva et al. 2005). In analyzing this complex enzyme family, the elucidation of each chitinase function will allow

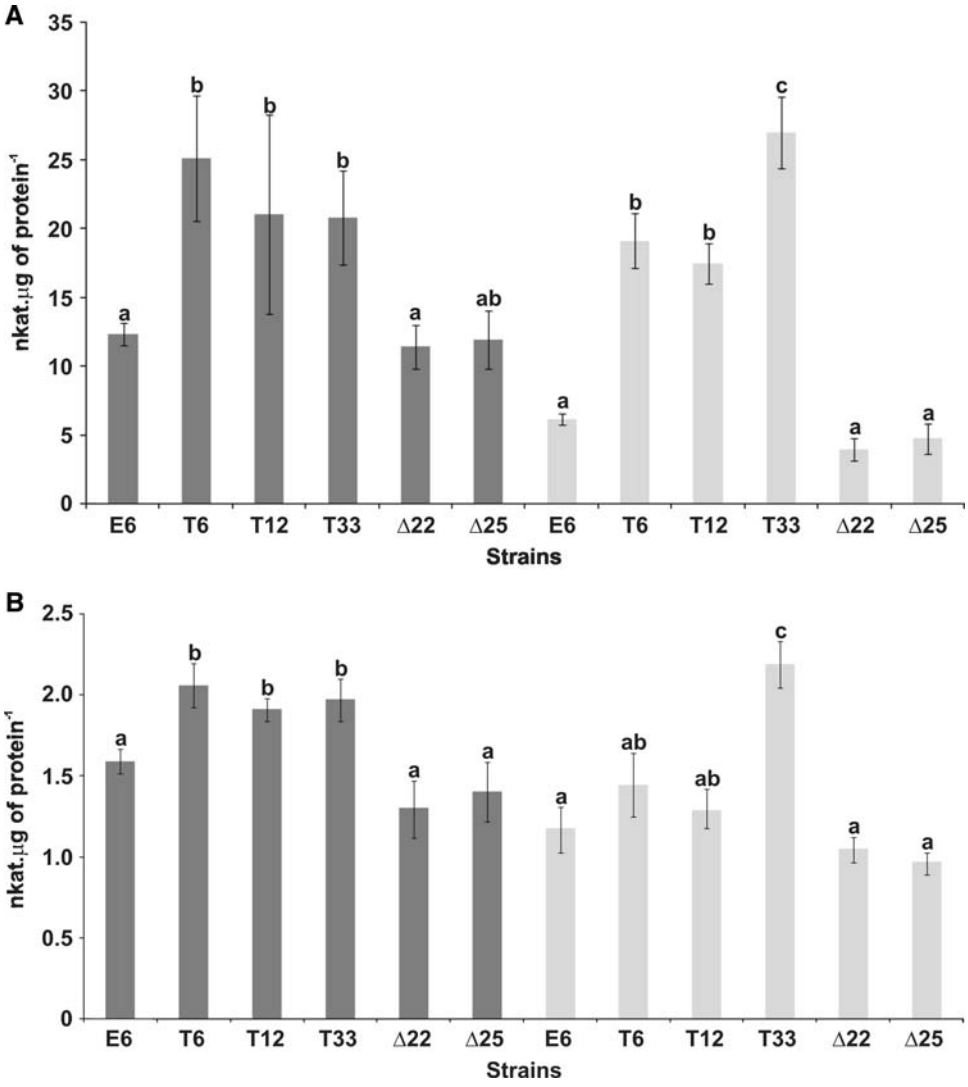

Fig. 2 Total chitinase and endochitinase activity of wild type and constructs. Total chitinase (**a**) and endochitinase activity (**b**) were detected in culture supernatants of *M. anisopliae* wild type strain (E6) and constructs overexpressing (T5; T12; T33) or knock out (Δ22; Δ25) for the *chi2* gene. Supernatants were obtained from fungi cultivated on medium containing 1% glucose (*dark gray bars*) or 1% chitin (*light gray bars*). *Bars* represent the average of three independent experiments and the *lines above bars* represent the standard deviation. *Bars* with the same letter do not differ statistically according to the Duncan's test ($P < 0.001$). One nkat unit of chitinase was defined as the enzyme amount necessary to catalyze the release of 1 nmol s^{-1} of GlcNAc for total chitinase activity experiments and 1 nmol s^{-1} of 4-MU for specific endochitinase activity experiments

a better understanding of the chitinolytic system. Although little is known about the participation of chitinases in the infection process of entomopathogenic fungi, the endochitinases seem to be responsible for initiating the chitin cuticle solubilization process due to their cleavage characteristics.

Since the *M. anisopliae* chitinase encoded by the *chi2* gene constitutes an endochitinase and is phylogenetically related to other endochitinases previously proven to be important in the infection process of other pathogenic fungi, specifically in mycopathogenic fungi (Seidl et al. 2005), it is probable that the CHI2 chitinase is also important for the *M. anisopliae* host infection process, as shown here. Considering the number of chitinases secreted by *Metarhizium*, it is expected that these enzymes act directly and synergistically along with other hydrolases throughout the infection process in order to solubilize the host cuticle, providing nutrition and allowing fungal penetration, to colonize the entire host. In this study, we demonstrated that overexpression and deletion of the chitinase encoded by the *chi2* gene alter fungal virulence toward the model host *D. peruvianus*.

Chitinases are a long-term subject of study in filamentous fungi. Genome-wide analyses have suggested that the chitin degradation enzymes are much more complex in these organisms (Seidl et al. 2005). Assigning function for putative chitinases is the actual challenge to understand if most of such diversity is simply redundant or if each chitinase has specific roles in fungal biology. This is particularly important for the entomopathogenic and mycopathogenic fungi that use chitinases during their life cycle and in the course of infection to digest the protective chitin barrier of their hosts.

Subgroups A and B chitinases have been implicated in exogenous chitin degradation, like the subgroup A chitinases *PjChi-1* from *Paecilomyces javanicus* (Chen et al. 2007), the *Crchi1* from the mycoparasitic fungus *Clonostachys rosea* (Gan et al. 2007); and subgroup B chitinases *chi2* and

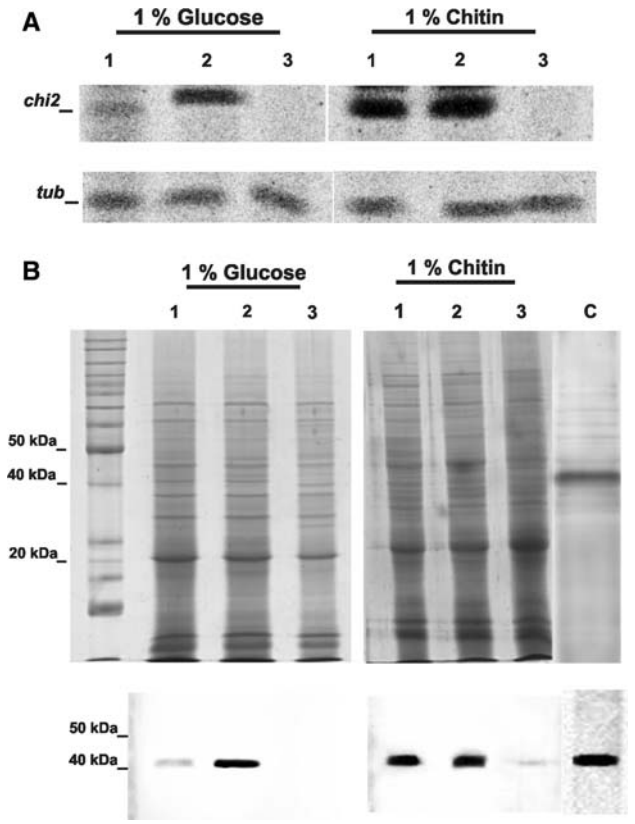

Fig. 3 Expression analysis of the *chi2* gene and CHI2 chitinase. **a** Northern blot analysis of *chi2* and *tub* gene expression in wild type and constructs: *1* E6; *2* T33; *3* Δ22. **b** Western blot analysis of CHI2 chitinase expression in wild type and constructs: *1* E6; *2* T33; *3* Δ22

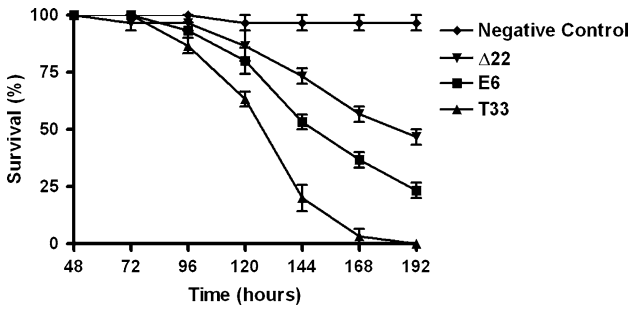

Fig. 4 Mortality of *Dysdercus peruvianus* in bioassays. Virulence of *M. anisopliae* wild type strain (E6) and constructs overexpressing (T33) or knock out (Δ 22) for the *chi2* gene was tested on *D. peruvianus* males. Groups of ten insects were treated with each *M. anisopliae* strain or construct (10^8 conidia mL^{-1}) and checked everyday until 100% mortality was achieved. *Bars* represent the standard error

chi3 from *M. anisopliae* (Baratto et al. 2006; da Silva et al. 2005). Both subgroups are also involved in cell wall remodeling. *A. nidulans chiA*, a subgroup B chitinase, is a large GPI-anchored chitinase and its deletion decreased germination rates and hyphal growth (Takaya et al. 1998; Yamazaki et al. 2008). Moreover, a subgroup A chitinase

Table 1 *M. anisopliae* LT_{50} and LT_{90} values obtained in bioassays using *D. peruvianus*

Strain/construct	LT_{50} (h)[#]	LT_{90} (h)[#]
E6	156 (150–162)[a]	209 (199–222)[a]
T33	125 (121–129)[b]	154 (149–161)[b]
Δ22	186 (172–208)[c]	267 (238–318)[c]

Values in parentheses represent the confidence interval (95%)

[#] Means followed by the same letter do not differ statistically according to a *t* test ($P < 0.001$)

from *A. nidulans*, *chiB*, is implicated in autolysis (Yamazaki et al. 2007). In this study, the deletion of the subgroup B chitinase *chi2* did not show morphological alterations, but diminished *M. anisopliae* virulence. It seems that chitinases from all subgroups are involved in hyphal-related processes and/or infection processes (Seidl 2008).

Some chitinases also have been shown to be important in the infection process of pathogenic fungi. The mycoparasitic fungus *Trichoderma* secretes chitinases (specially subgroup B) in order to break down chitin polymers of host fungi cell walls such as *Rhizoctonia solani* for nutrition, consequently interrupting their development (Seidl et al. 2005). Mutants of the fungus *C. rosea* lacking each of the three different endochitinases displayed altered mycoparasitic activity during interaction with *Fusarium culmorum* in liquid culture. The knock out of the endochitinase genes *cr-ech58* (possibly subgroup A), *cr-ech42* (subgroup A) and *cr-ech37* (subgroup B) each diminished virulence of *C. rosea*. Moreover, disruption of the *cr-ech42* gene showed the stronger effect in reducing the mycoparasitism, indicating that this gene is the most important of the three deleted endochitinases (Mamarabadi et al. 2008).

An acidic mammalian chitinase (subgroup not determined) seems to be important as a defense mechanism against the pathogenic fungi *Candida albicans* and *Aspergillus fumigatus* (Chen et al. 2009). In addition, plant endophytic bacterial and viral chitinases are probably involved in defense against pathogenic fungus and insect plagues (Corrado et al. 2008; Quecine et al. 2008; Silvar et al. 2008). The altered virulence of the constructs overexpressing and lacking the *chi2* gene demonstrated that the endochitinase CHI2 (subgroup B) participates in the infection process. This observation is in contrast to previous work in which the non-repressible expression of endochitinase CHIT1 (subgroup A) did not affect *M. anisopliae* virulence toward *M. sexta* (Screen et al. 2001). These authors hypothesized that the basal levels of endochitinase production were sufficient for the early period of infection once the chitin fibers were coated with proteins and somehow occluded, hampering the action of chitinases. As previously demonstrated (Limon et al. 1999; Bowman and Free 2006), however, the overexpression of a chitinase

(subgroup B) in *Trichoderma* spp. enhanced mycoparasitism despite of the protein coat in the host cell wall (Bowman and Free 2006; Latge 2007). It is more likely that a specific chitinase, or group of chitinases, may act differently depending on the host cuticle. Recently, a gene expression analysis comparing strains of *M. anisopliae* var. *anisopliae* and *M. anisopliae* var. *acridum*, which are specific or generalist when considering the ability to infect a narrow or a wide range of hosts, showed that CHI2 is present in the generalist strain Ma2575 and not in the specific strain Ma443 (Wang et al. 2009). This suggests that CHI2 participates in the infection processes and that its absence reduces or impairs the fungal capacity to infect certain hosts. As demonstrated by Fan et al. (2007), overexpression of the *Bbchit1* gene (subgroup B) induced high expression of the chitinase encoded by the aforementioned gene and enhanced *B. bassiana* virulence to aphids, diminishing both lethal conidial concentration and lethal time. In addition, genetically modified plants expressing different fungal/bacterial chitinases (subgroup A) have been found to be more resistant to phytopathogen fungi infection and insect feeding (Distefano et al. 2008; He et al. 2008; Kern et al. 2009; Shah et al. 2009).

Based on our findings, we propose that the endochitinase CHI2 (subgroup B) is important for pathogenicity by being secreted and directly acting in the penetration process of the host cuticle. Moreover, neither the overexpressing nor the knock out mutants presented detectable morphological alterations in spores, hyphae and appressorium formation (data not shown). These results strengthen the hypothesis of a specialization of the different chitinases in *Metarhizium*. However, other functions for the same chitinase cannot yet be ruled out at this point. The analysis of mutations in other chitinase genes as well as mutants lacking more than one chitinase should in the future allow a more comprehensive view of the chitin degradation system in *Metarhizium* and other fungi. Understanding fully the nature of chitinase functions could facilitate more effective utilization of *Metarhizium* in pest biocontrol strategies.

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