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Isolation, Characterization, and Transcriptional Analysis of the Chitinase *chi2* Gene (DQ011663) from the Biocontrol Fungus *Metarhizium anisopliae* var. *anisopliae*

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Abstract. *Metarhizium anisopliae* infects arthropods via a combination of specialized structures and cuticle degradation. Hydrolytic enzymes are accepted as key factors for the host penetration step and include chitinases. The characterization of the *chi2* chitinase gene from *M. anisopliae* var. *anisopliae* is reported. The *chi2* gene is interrupted by two short introns and is 1,542-bp long, coding a predicted protein of 419 amino acids with a stretch of 19 amino acid residues displaying characteristics of signal peptide. The predicted chitinase molecular mass is 44 kDa with a mature protein of 42 kDa and a theoretical pI of 4.8. The comparison of the CHI2 predicted protein to fungal orthologues revealed similarity to the glycohydrolase family 18 and a phylogenetic analysis was conducted. The *chi2* gene is up-regulated by chitin as a carbon source and in conditions of fungus autolysis, and is down-regulated by glucose. This regulation is consistent with the presence of putative CreA/CreI/Crr1 carbon catabolic repressor binding domains on the regulatory sequence.

Metarhizium anisopliae is a well-known, broad-range arthropod pathogen, which is applicable in the biological control of several insect pests, including vectors for human diseases, and ticks [13, 14, 16]. During fungal penetration through the host cuticle, hydrolytic enzymes such as proteases, chitinases, and lipases are produced and secreted and are proposed to be important for the initiation of the infection process, leading to cuticle transposition [28]. This range of extracellular enzymes that degrade the components of the host cuticle is produced when *M. anisopliae* is grown in arthropod cuticle or chitin as the sole carbon and nitrogen source [3, 18]. Chitinases are among these extracellular enzymes and some have been purified and characterized [15, 23, 29].

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In fungi, chitinases have a physiological role in hyphal growth and morphogenesis [30] and have also been shown to be produced during host infection by entomopathogenic fungi [8]. Analysis of secreted chitinases in *M. anisopliae* revealed at least six isoforms (30, 33, 43.5, 45, 60, and 110 kDa) and only one has both the protein and the gene isolated and characterized (*chi3* gene and CHIT30 chitinase) [8]. Three genes coding for chitinases were described in *Metarhizium*: *chit1* gene and the ortholog *chi1*, code a 42-kDa endochitinase [2, 5, 26]; *chi2* (partial sequence, AJ293217); and, *chi3*, which codes for an endo/exo-acting 30-kDa chitinase (CAC07217.1) [8]. However, the role of chitinases in arthropod pathogenesis is still not completely understood.

One approach to understand their function is the isolation of chitinase genes and the evaluation of their overexpression in bioassays. Thus, the overexpression of

the *M. anisopliae chit1* gene did not show altered pathogenicity to *Manduca sexta* [26]. In contrast, the *M. anisopliae* CHIT30 chitinase (*chi3* gene) was shown to be produced during tick infection [8] and the overexpression of a *Beauveria bassiana* chitinase, gene *Bbchit1*, enhanced the virulence for aphids [12]. These three chitinases, CHIT1, CHIT30, and Bbchit1, share very low levels of similarity and da Silva et al. [8], analyzing the sequences from chitinases whose function in cell morphogenesis/growth or in pathogenesis was assigned, showed that chitinases with similar cellular roles may diverge in sequence. In *Metarhizium*, only one of the chitinase genes, the *chit1* gene, was fully characterized [5]. For genes *chi2* (AJ293217) and *chi3* (AJ293218), only ESTs sequences are deposited.

Aiming to contribute to the investigation of the role of *Metarhizium* chitinase genes in the host infection process, we isolated and characterized the genomic and cDNA copies of the *chi2* ortholog from *M. anisopliae* var. *anisopliae.* We also studied its transcription regulation under different culture conditions, including the use of host cuticle as a carbon/nitrogen source.

Materials and Methods

Organisms and growth conditions. *M. anisopliae* var. *anisopliae* strain E6 from the Microbial Genetics Group (Escola Superior de Agronomia Luiz de Queiroz, USP, Brazil) was maintained in complete Cove's medium (MCc) media as previously described [9]. For RNA extraction, the fungus was grown in liquid Cove's medium [9] with $NaNO_3$ 0.6%, supplemented with glucose (1%), *N*-acetylglucosamine, GlcNAc, (0.1%), *Boophilus microplus* cuticle (1%) [9], or chitin (0.8%). *E. coli* XL1-Blue (Stratagene, La Jolla, CA, USA) was used for genomic library construction and propagation of pUC18 plasmid and clones. Bacterial cultivation was in LB agar or LB broth [24].

Southern hybridization and library construction. Genomic DNA from *M. anisopliae* was extracted from mycelium [4] and (10 μg) digested with BamHI, EcoRI, HindIII, KpnI, PstI, or XbaI restriction endonucleases and fractionated on 0.8% agarose gel electrophoresis. The DNA was transferred to nylon blotting membrane Hybond^{TM}-N^{+}, probed with a 615-bp amplicon from *chi2* gene and hybridized using the ECL kit. The probe was generated using primers (Chi2F-GTGTTGGCCTTGTTGGCCTG and Chi2R-TACTGGCCAATTTG CTCGGC) (Invitrogen, São Paulo, Brazil) based on the reported ortholog *chi2* gene partial sequence from *M. anisopliae* var. *acridum* [AJ293217].

Nucleotide sequencing and computational analysis. Inserts and amplicons were sequenced at the ACTGene Laboratory (Centro de Biotecnologia, UFRGS, Porto Alegre, RS, Brazil in an ABI-PRISM3100 Genetic Analyzer and analyzed by Blast using the NCBI server at http:www.ncbi.nlm.nih.gov/BLAST/ [1]. Signalscan Program (at http://www.dna.affrc.go.jp/PLACE/signalscan.html) was used to find a putative signal peptidase cleavage site. Chitinase amino acid sequences from fungi (CAC07216.1; AAB81998; AAN41259.1; CAG86633.1; EAL03025.1; EAL00460; CAG62749.1; BAA36223.1; AAS55554; NP_013388; AAA92642.1) were aligned using ClustalX [31] and a phylogenetic tree was constructed using the Molecular Evolutionary Genetics Analysis (MEGA) software [19] by the neighbor-joining method. Phylogenetic tree architecture confidence was evaluated by 10,000 bootstrap replications.

RT-PCR analysis and characterization of transcription start site. Total RNA extraction was performed as described [9]. First-strand cDNA synthesis was performed with M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol with oligo dT as primer, using 1 μg of RNA. Amplification of the *chi2* gene transcripts was performed as described above and RNA quantity was normalized by the amplification of *tef1*-α gene [21]. Amplicons were resolved by electrophoresis in 1.0% agarose gel. For isolation and characterization of 5′ ends from the *chi2* gene, a 5′ RACE System was used (Version 2.0, Invitrogen), with 1 μg of RNA extracted from mycelium grown for 48 h with chitin as carbon source. Primers were Chi2R (see above) and an antisense primer (Chi2IR-GAATTGGGTTGGCAGTAC). The amplified product was purified and sequenced.

Results and Discussion

In order to clone the *M. anisopliae* var. *anisopliae* strain E6 complete *chi2* gene sequence, PCR fragments were amplified (615 bp in size) using primers derived from the previously reported ortholog *M. anisopliae* var. *acridum chi2* gene [AJ293217]. A recombinant clone with a 5.3-kb insert encompassing the *chi2* gene was selected by colony hybridization from about 1,000 colonies from a plasmid library carrying *M. anisopliae* var. *anisopliae* strain E6 genomic DNA. The complete nucleotide sequence of the *chi2* gene was determined (DQ011663) and the 419 amino acid residue ORF (CHI2) shows high similarity (97%) to the putative chitinase ortholog from *M. anisopliae* var. *acridum* [AJ293217]. The transcription initiation site was determined by sequencing a 311-bp cDNA amplicon generated by 5′ RACE reaction. The transcription initiation (G + 1) was identified and the ATG start codon was positioned at 95 bp from the transcription initiation site. The transcription initiation environment is ACATCAAG, which is similar to the consensus TCATCANC [10]. The *chi2* gene is 1,542 bp long and is interrupted by two introns (210 and 72 bp long). In silico analysis of the 5′ flanking region revealed canonical CAAT and TATAA putative controlling elements at the appropriate distances. In addition, a consensus motif was found for the CreA/Crel/Crr1 carbon catabolic repressor, a negative regulator mediating carbon catabolism repression in *A. nidulans* and *M. anisopliae* (Fig. 1) [7, 25, 31].

Chitinase CHI2 has a predicted molecular mass of 44 kDa and a putative signal peptidase cleavage site at V^{19}, rendering a mature protein of 42 kDa with theoretical pI of 4.8. A chitinase with a similar molecular mass is coded by the *chit1* gene from *M. anisopliae* var. *anisopliae* (CHIT42 endochitinase, AF027498) [2].

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ggcaagacatcagatcctcgtgcccgacaaggccaatgaaaccactcaagccaacgcaggaacattcaac   70
tctcaggcacgcatcagctagtccctgcaaaccccccacgaatggccgatagcttacacggcaaaagc   140
gactacgggttctcggcacgccgtgtgcctcatgttgtcactcgacgctcctcgagctcgtcacggggag  210
gcaattgaagatggatgacacagcttcattcctgtccatgcaacgtctcctcctaaacgtggtgctgtct  280
cgcgtgcctctcagctcgcaatgccgttccacgtgtcgacttgggagcttggtatgtagccgggctcctc  350
gtgcgctggatggatgagtgttgtgaaaatgatatataacaggcccgaattaactgcggatggacggcca  420
tgtattgcacatcaagttggtagtttgagcttgtgtatattctcttcacttctctgtacatgttacatct  490
ttattttgtgtatatcatcgatATG
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Fig. 1. *M. anisopliae* var. *anisopliae* strain E6 *chi2* gene 5′ flanking sequence. Capitalized letters mark the first ATG. Putative CAAT and TATAA sequences and a putative CREA element (CCCCAC) are underlined. The transcription start site is shown in bold and the conserved transcription initiation sequence is shaded.

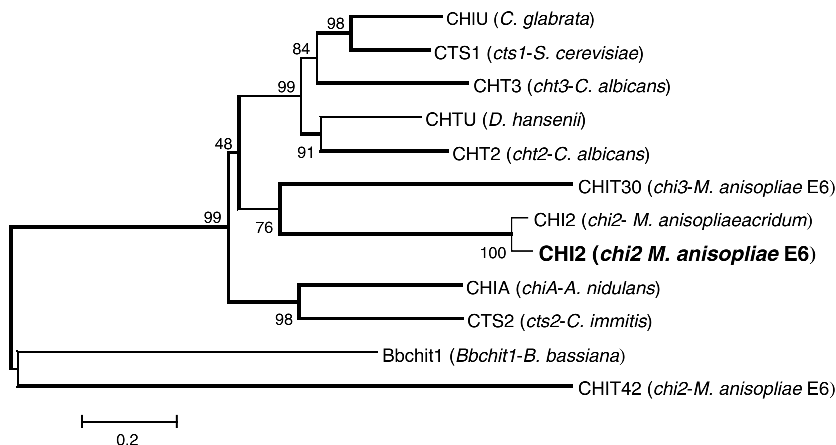

Fig. 2. Phylogenetic neighbor-joining tree of *M. anisopliae* var. *anisopliae* strain E6 *chi2* deduced amino acid sequences. Tree confidence was confirmed by 10,000 bootstraps and the numbers on the branches represent values for the bootstrap. The scale bar indicates the number of amino acid substitutions. For the GenBank accession number for the chitinase sequences analyzed, see Materials and Methods.

However, there is very little amino acid identity between the two chitinases (CHIT42 and CHI2) and their theoretical pIs differ (Fig. 2) [5, 26].

The comparison of the predicted CHIT2 chitinase to fungal orthologs revealed a similarity to the glycohydrolase family 18 [Pfam database; 11]. The consensus motif SXGG corresponding to a substrate-binding site was identified; however, the catalytic domain consensus motif (D1XXD2XD3XE), highly conserved among fungal chitinases [22, 25], has one amino acid substitution (D1 $\rightarrow$ N) in the CHI2 sequence. A characteristic fungal-type cellulose-binding domain (CBD) present in the *chi2* C-terminal sequence is similar to that of the 33-kDa chitinase gene in *Trichoderma virens*, predicted to encode a protein C-terminus with homology to the conserved family I cellulose-binding domain [17]. Apparently, the CDB in endochitinases increases hydrolytic activity towards insoluble substrates such as chitin-rich fungal cell walls in *Trichoderma harzianum* strains [20].

In order to evaluate the evolutionary relationships and to classify the predicted chitinase CHIT2 into bacterial-like or plant-like classes, a neighbor-joining phylogenetic tree was constructed. As shown in Figure 2, the tree collapsed in two clusters, one encompassing *Metarhizium* CHIT2 orthologues and CHIT30, as a plant-like class, and the other cluster with CHIT42 (coded by *chit1* gene), a bacterial-like chitinase [8].

Previously, we reported the effect of different carbon sources on both total chitinase synthesis and secretion in *M. anisopliae* and the dual regulation depending on the GlcNAc concentration in the culture medium [3, 5, 18, 23]. To investigate the regulation of the *chi2* gene, RT-PCR was conducted using RNA extracted from cultures amended with different carbon sources: 1% glucose, 0.1% GlcNAc, 1% tick cuticle, or 0.8% chitin. In glucose-added cultures, the sugar was supplemented every 24 hours to ensure its availability throughout fungal growth. The primers were targeted to a region spanning the first intron of the *chi2* gene, generating an amplicon of 402 bp when cDNA was used as template for amplification, and a 615-bp amplicon for genomic DNA. To normalize RNA quantities, a 1,031-bp amplicon generated by primers directed to the *tef 1-*α gene (AY445082) was used. As shown in Figure 3, after 48 h *M. anisopliae* culture, *chi2* gene transcripts were only detected when chitin was the carbon source. After 72 h, *chi2* gene transcripts were also detected in cultures in the presence of GlcNAc or tick cuticle whilst transcripts were still not detected in the presence of glucose. Similar results were reported for the *chit36* gene from *T. harzianum* [32] and for the *Bchit1*gene from *B. bassiana* [12]. In early cultures (18 or 30 h), *chi2* gene transcripts were not detected (data not shown). In cultures with 0.1% GlcNAc, the *chi2* gene transcripts were only detected after 72 hours of

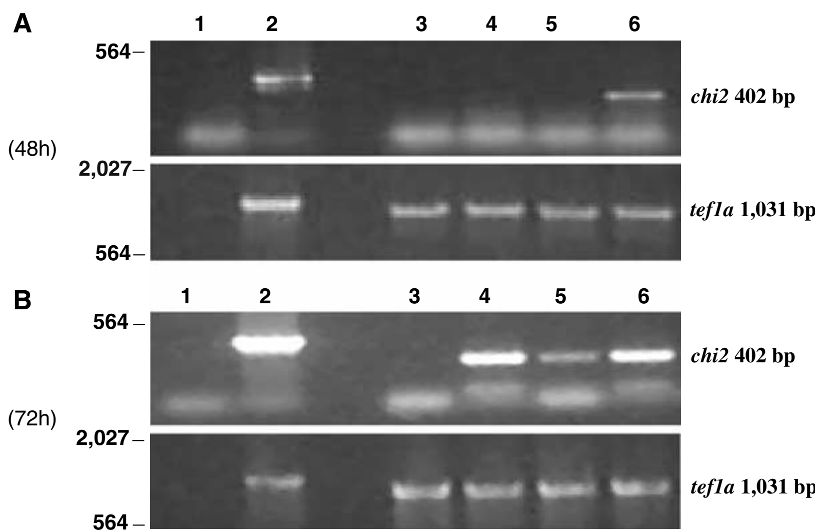

Fig. 3. RT-PCR of *chi2* gene transcripts in different culture conditions. *M. anisopliae* var. *anisopliae* strain E6 was cultured for 48 h (**A**) or 72 h (**B**). Upper gel in each panel represents *chi2* gene transcripts. Lower gel in each panel represents *tef1α* gene transcripts, used to normalize RNA quantity. Numbers at the left represent size in bp. Numbers at the right represent the amplicon size in bp. Lanes: (1) control with no template added; (2) genomic DNA from gene *chi2* template, resulting in a 615-bp product; RNA from cultures in minimal medium added of: (3) 1% glucose; (4) 0.1% GlcNAc; (5) 1% tick cuticle; (6) 0.8% chitin. cDNA amplicon from *chi2* gene is 402 bp long.

fungal growth, when the amino sugar was exhausted. This suggests that the expression of *chi2* gene may be triggered by autolysis. Similar results were described for the *ech42* gene that encodes chitinase ECH42 in *T. harzianum*, in which significant *ech42* expression was detected only after prolonged carbon starvation [6]. In tick cuticle and chitin, the *M. anisopliae chi2* gene transcription was induced, indicating that synthesis is subject to regulation by the substrate.

In fungi, chitinases have a physiological role in hyphal growth and morphogenesis. The relevance of chitinase production and secretion during the penetration of host cuticle by fungal pathogens is not fully understood. To date the exo/endochitinase CHIT30 of *M. anisopliae* strain E6 was shown to be present during *B. microplus* infection [8] and only the *chit1* and *Bbchit1* chitinase genes, from *M. anisopliae* and *B. bassiana*, respectively, have been investigated in the insect fungus pathogenic context. The CHIT42 (*chit1* gene) chitinase from *M. anisopliae* was shown to have no effect on virulence to insects [26], while overproduction of *Bbchit1* did increase the virulence of *B. bassiana* for aphids [12].

Seidl et al. [27] showed that both *chi2* and *chi3* genes from *Metarhizium* are related to chitinase genes from mycoparasites (*Trichoderma*) and to no other chitinases described in all other ascomycetous genomes. The authors suggest that these chitinases probably have special functions in host chitin degradation during parasitism. Indeed, the related *Hypocrea jecorina* (anamorph: *Trichoderma reesei*) chitinase gene *chi18-13* is up-regulated in the presence of host cell wall [27] as is the *Metarhizium chi2* gene in the presence of host cuticle (Fig. 3B).

The cloning and characterization of the chitinase genes is important to elucidate the relationships between chitinases and virulence in insects/ticks or in the fungus morphogenesis. In situ immunodetection of the protein and overexpression and gene silencing experiments are necessary to elucidate its biological role in *Metarhizium*.

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