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Evidence of alternative splicing of the *chi2* chitinase gene from *Metarhizium anisopliae*

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ABSTRACT

Metarhizium anisopliae is a filamentous fungus used in the biological control of arthropods and produces several chitinases in order to break the host cuticle chitin fibers. Chitinase function during fungal cell development and/or infection processes is also an important aspect when analyzing the life cycle of entomopathogens. The expression profile analysis of the endochitinase *chi2* gene acquired by RT-PCR experiments indicated the presence of two different transcripts, suggesting the occurrence of alternative splicing in the *chi2* gene. The presence of two transcripts, characterized by the removal or retention of the second 72 bp intron, was further confirmed by DNA sequencing, Northern blot and qRT-PCR. Furthermore, we detected the synthesis of two different proteins from the transcripts by two-dimensional Western blot and mass spectrometry analyses. This is the first reported occurrence of alternative splicing in *M. anisopliae*.

1. Introduction

In general, exons and introns found in fungi share common characteristics to metazoans and other organisms, as conserved acceptor and donor sites (5′GU … AG3′), conserved branch point and the sequence length between the branch point and the 3′ splice site. It also has been shown that the key components of the spliceosome are present in organisms since the last common eukaryotic ancestor, indicating that the mechanism as a whole, including alternative splicing, is conserved in Eukaryotes (Collins and Penny, 2005; Kupfer et al., 2004). However, fungal introns differ in important aspects concerning the splicing process itself, such as smaller size and higher information content, suggesting that splicing in fungi follow the intron definition model. According to this model pre-mRNAs encompassing small introns use the intron sequence, rather than the exon sequence, for the initial pairing of the splice sites (Kupfer et al., 2004; Lim and Burge, 2001). This mechanism implies that splice sites on either side of an intron are recognized as a unit and so the alternative splicing is more likely to occur by intron retention. Indeed, in a thorough survey conducted across 42 eukaryotic genomes, it was demonstrated that the prevalent alternative splicing event in fungi involves intron retention rather than the formation of exon cassettes (McGuire et al., 2008). Several filamentous fungi showed intron retention in a number of gene classes: the lignin degrading fungus *Phanerochaete chrysosporium* presents splicing variations in lignin peroxidase (LiP) genes (Macarena et al., 2005); the *hex-1* gene from *Neurospora crassa* (Leal et al., 2009) and the chitinase genes of *Trichoderma reesei* (Seidl et al., 2005). *Fusarium oxysporum* (Gomez-Gomez et al., 2001), *Trichoderma harzianum* (Dana and Pintor-Toro, 2005) and *Mucor circineloides* (Baba et al., 2005) also present splicing variations.

The entomopathogenic fungus *Metarhizium anisopliae* has been used as a model for studying host–pathogen interactions (Roberts and St Leger, 2004; Schrank and Vainstein, in press). It produces several hydrolases, including chitinases, in order to degrade the exoskeleton and invade its hosts (Pedrini et al., 2007). Chitinases are enzymes that can degrade both exogenous chitin, present in the cuticle of hosts of entomopathogenic fungi, and endogenous chitin, present in the fungal cell wall, allowing its remodeling (Seidl, 2008). The *chi2* gene (GenBank accession number DQ011663), which codes for a 42 kDa chitinase (CHI2), was previously isolated and characterized (Baratto et al., 2006). It comprises 1524 bp, possesses two introns of 210 and 72 bp, a glycosyl-hydrolase family 18 domain and a signal-peptide at the N-terminus. We have shown that the knock-out of this single chitinase gene diminishes the insect infection efficiency (Boldo et al., 2009).

In this work, we show that the *chi2* from *M. anisopliae* produces two transcript forms by alternative splicing to synthesize two

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different proteins, and discuss the possible role of such processing. This is the first report of alternative splicing in entomopathogenic fungi.

2. Material and methods

All enzymes were obtained from Promega (Madison, WI); all chemicals were purchased from Sigma-Aldrich (St. Louis, MO) or Bio-Rad (Hercules, CA), except when specified.

2.1. RT-PCR and sequencing analyses

M. anisopliae strain E6 was grown in 1% crystalline chitin-containing medium for defined periods (16, 24, 32 or 48 h) at 28 °C (Frazzon et al., 2000; Lubeck et al., 2008). Total RNA was extracted from mycelia using a previously reported phenol-chloroform extraction method (Dutra et al., 2004). The samples were treated with DNase and cDNA was synthesized. A portion of the *chi2* gene was amplified using specific primers spanning the 72-base-pair intron (Chi2For and Chi2Rev; Table 1). The PCR was performed as follows: 95 °C for 5 min; 95 °C for 1 min, 60 °C for 45 s, 72 °C for 50 s (26 cycles);and 72 °C for 5 min. The samples were separated in a 1.5% agarose gel, visualized in an UV transilluminator and the results were recorded. Primers chi2cdnaF and chi2cdnaR were used for transcript cloning and sequencing (Table 1). Cloning was carried out using standard procedures (Sambrook and Russel, 2001). Sequencing analyses were conducted using a MegaBACE 1000 DNA sequencer (GE Life Sciences, Uppsala, Sweden) and the reactions were performed according to the manufacturer's instructions.

2.2. Northern blot analysis

M. anisopliae was grown in liquid media containing 1% crystalline chitin (w/v), 1% glucose (w/v), 1% N-acetyl-D-glucosamine (GlcNAc) or 0.25% GlcNAc as the sole carbon source (Boldo et al., 2009). After 24, 48, 72, 96 or 120 h of incubation at 28 °C and 150 RPM, mycelia were collected and ground to a fine powder with the aid of liquid nitrogen. Total RNA was extracted using the previously reported procedure (Dutra et al., 2004). Normalization was performed using the Qubit System (Invitrogen) and by densitometry analysis using ImageJ software v. 1.41o (Abramoff et al., 2004). The samples were separated in a denaturing agarose 1.5% gel for 3 h at 100 V. After electrophoresis, samples were transferred to a nylon membrane and baked at 80 °C for 2 h. The membrane was pre-hybridized for 2 h in the appropriate buffer (Sambrook and Russel, 2001). Probes for the *chi2* (comprising nt 683–1170 (487 bp) of the *chi2* gene ORF—accession number DQ011663.2) and *tub* (comprising nt 347–864 (517 bp) of the β-*tubulin* ORF—accession number DQ393577.1) gene transcripts were hybridized for 16 h at 42 °C after ^{32}P incorporation. The membrane was washed and exposed to Kodak-K Screen and the digital image was obtained using the Pharos system (Bio-Rad, Hercules, CA). Absolute intensity analysis was performed using ImageJ software v. 1.41o (Abramoff et al., 2004).

2.3. qRT-PCR analysis

Total RNA samples from the same conditions used for Northern blot analysis (item 2.2) were treated with DNase and cDNA was synthesized. The samples, normalized as previously described, were subjected to qRT-PCR using specific primers for the *chi2* (chi2F and chi2R) and *tub* (β-tubulin; tubF and tubR) genes (Table 1). Based on the Northern blot results, the *tub* gene was chosen as the reference gene. The completely processed form of *chi2* was used as a reference to assess the expression pattern of the two transcript forms using a specific incompletely processed set of primers (qRT-PCR_chi2_exon2_NP_F and qRT-PCR_chi2_exon2_NP_R; Table 1). The reactions were performed in a 7500 Real-Time PCR System (Applied Biosystems). PCR thermal cycling conditions were as follows: an initial step at 95 °C for 5 min and 40 cycles at 95 °C for 15 s, 50 °C for 30 s and 72 °C for 20 s. Platinum SYBR green qPCR Supermix (Invitrogen) was used as reaction mixture, adding 10 pmol of each primer and 2 μL of template cDNA at a final volume of 25 μL. All experiments were done with two independent cultures and each cDNA sample was analyzed in duplicate with each primer pair. Melting curve analysis was performed at the end of the reaction to confirm a single PCR product. The results were analyzed by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) and the statistical differences between groups were evaluated using ANOVA (Analysis of Variance) and Duncan analysis ($p<0.001$).

2.4. 2D-SDS-PAGE Western blot analysis

M. anisopliae was grown in 1% crystalline chitin-containing medium for 96 h at 28 °C and 150 RPM in a bench incubator. The mycelia were filtered and the supernatant was lyophilized and suspended in 1/10 of the original volume in sterile Milli-Q water. Approximately 2 mg of total protein was precipitated using 10% TCA for 16 h at 4 °C. Samples were centrifuged at 14,000 *g* for 15 min, washed with acetone five times, air-dried and solubilized in isoelectric focusing buffer (IEF buffer) containing 7 M urea, 2 M thio-urea, 4% (w/v) CHAPS, 1% (w/v) dithiothreitol (DTT) and 0.2% (v/v) ampholytes pH 3–10. The proteins were separated according to their isoelectric point in 17 cm IPG strips with pH values ranging from 4 to 7, and actively rehydrated (50 V) at 20 °C for 16 h. Isoelectric focusing was performed in a Protean IEF cell system (Bio-Rad, Hercules, CA) up to 70,000 VH at a maximum voltage of 10,000 V. The strips were equilibrated for 15 min in equilibration buffer I (30%, v/v, glycerol, 6 M urea, 1% DTT, a trace of Bromophenol Blue) and for 15 min in equilibration buffer II (equilibration buffer I with DTT replaced by 4% iodoacetamide). In the second dimension, IPG strips

Table 1
List of primer sets used in this work.

Primer[a,b]	Sequence (5′–3′)	Purpose	Expected length (bp)
chi2cdnaF[a,b]	GGTACCAATGCATCATCTACGCGCTC	*chi2* gene full length cDNA amplification	1258
chi2cdnaR[a,b]	GGATCCTCACCGGCATGACGACCACC	*chi2* gene full length cDNA amplification	
chit2For[a,b]	GCCCCCTTCAACTACAACAA	RT-PCR	487
chit2Rev[a,b]	ACACATTGGTAAGGGGGAAC	RT-PCR	
chi2F[b]	TTTGGAAATGGCGGCAAC	qRT-PCR	117
chi2R[b]	GGACTGGCAGGTGTGTATGG	qRT-PCR	
tubF[n.a.]	CATCTCTGGTGAACACGGC	qRT-PCR	200
tubR[n.a.]	AGTTGTCGGGACGGAAAAG	qRT-PCR	
qRT-PCR_chi2_exon2_NP_F[a]	GCCTCCGAGTTCTACGCC	qRT-PCR	127
qRT-PCR_chi2_exon2_NP_R[a]	CGAGATTCTTTTTTTTTT	qRT-PCR	

n.a.—Not applicable.
[a] Amplifies the intron-containing transcript cDNA.
[b] Amplifies the intron-less transcript cDNA.