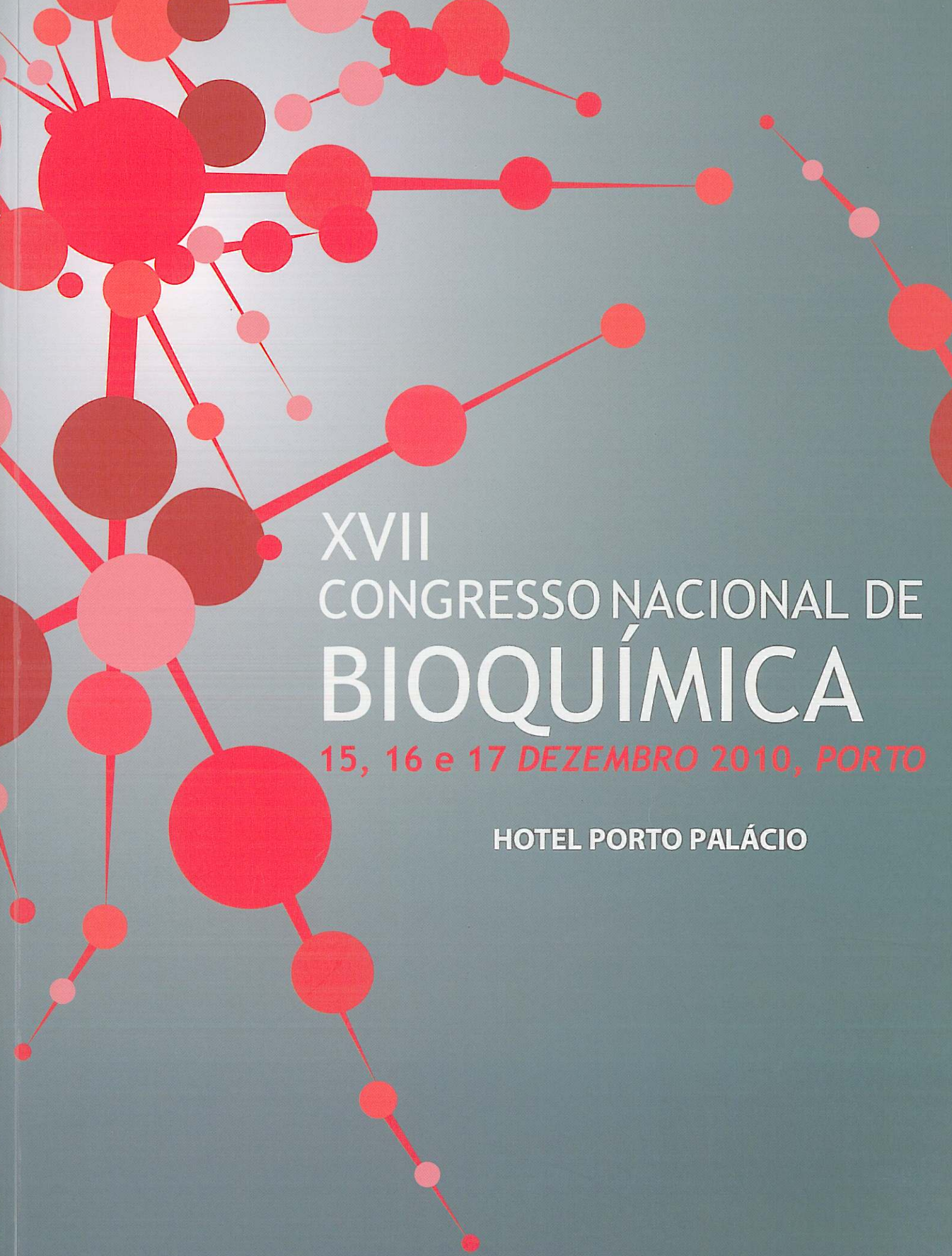

XVII CONGRESSO NACIONAL DE BIOQUÍMICA

15, 16 e 17 *DEZEMBRO* 2010, *PORTO*

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IDENTIFICATION OF *Trichoderma harzianum lip2* GENE BY HE-TAIL PCR

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INTRODUCTION

Trichoderma harzianum **is a widespread soil fungus, known as a biocontrol agent against soilborne plant pathogens. Its biological control against plant fungi pathogens is based**

on different mechanisms, namely the production of several lytic enzymes. In *T. harzianum* several glucanases, celulases, chitinases and proteases has been identified but little is known about its lipolytic system. The aim of this work was to achieve the complete elucidation of *T. harzianum lip2* by HE-TAIL PCR (High-Efficiency Thermal Asymetric Interlaced PCR), a method described as efficient to identify flanking regions from short known DNA sequences.

MATERIALS AND METHODS

From a cDNA library of *T. harzianum* CECT 2413, obtained by NewBiotechnic, it has been selected an EST who showed lipase homology in agreement with the program FASTA: EST-1279.

DNA sequencing was performed using an ABI 373 automated sequencer. After two sequencing rounds, EST-1279 had 1168bp, and a great homology (1,8e-^{80}) with a hypothetical lipase of *Fusarium graminearum*. Comparison between two sequences, suggested that still are lacking 300bp of the beginning of the ORF. In order to determine the lacking bases, it was used an HE-TAIL PCR, that seemed suitable to the problem resolution.

Degenerated 16bp primers R1 (5'-NGTGASWGAMAWGAA-3'), R2 (5'-GTNCGASWCANAWGTT-3'), R3 (5'-WGTGNAGWANCANAGA-3') and R4 (5'-NCAGCTWSCTMTSCTT-3'), as described by Michiels *et al.* (2003) were used. Gene-specific primers, lip2a (5'-CTGGCAGAACCGATTCCCGAGCGCTT-3'), lip2b (5'-ACGCAACTACGATGGCGCCTTGCTCG-3'), lip2c (5'-TGCGATGAACCCACAGCTATCGCCGA-3') and lip2d (5'-GAGAAAGCCTGTACTCCACGTAGAGG-3'), with 26bp and melting temperatures of 70-72ºC were designed in the incomplete ORF of *lip2* at positions referred in Figure 1.

From genomic DNA three rounds of PCR were performed on a MyCycler Thermal Cycler (BIORAD), using the product of the previous PCR as a template for the next. In primary and secondary PCR reactions, a single-step annealing extension at 62ºC-66ºC was used. A detailed cycler program and conditions are given in Table1. Separation and identification of DNA products of the tertiary reaction, including the controls, were made by agarose gel electrophoresis. Bands of about same size in reactions Ri-lip2d and Ri-lip2c, and that didn't occur in reactions Ri-Ri used as control of non specific amplification are possible specific products. Product of tertiary PCR was purified by geneclean and sequenced.

RESULTS AND DISCUSSION

At tertiary PCR reaction only faint amplification products were obtained with degenerate primers R1, R2 or R3. Intense bands only appear in the combinations R4+lip2c and R4+lip2d, as shown in Figure 2. A 2000bp band was selected, purified by geneclean and partially sequenced, who allowed the elucidation of *lip2* ORF beginning and part of the promoter region. At the end, *lip2* sequence had a total of 1992bp, including 548bp of the promoter, 1215bp of ORF and 227bp of terminator. The complete gene sequence was submitted to EMBL databases (Accession number AM774154). Nucleotide and deduced amino acid sequences were analyzed using FASTA programs from EMBL databases.

***Lip2* codifies a 404 amino acids protein, with 44.6KDa. The UniProt accession number for the amino-acid sequence reported in this paper is B7ZET5_TRIHA. Elucidation of complete gene nucleotide sequence of *lip2*, including part of the open reading frame at the N-terminal region and 548bp of the promoter region was achieved by HE-TAIL PCR, which confirmed to be a powerful tool to identify flanking regions from previous known ones.**

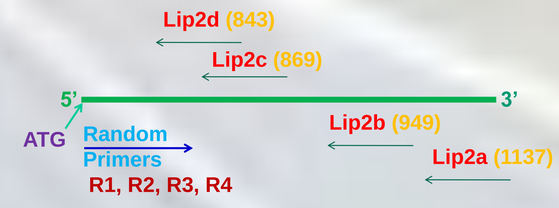

FIGURE 1 - Schematic representation of TAIL-PCR amplification.

TABLE 1 - HE-TAIL PCR cycle settings and conditions*

Reaction	Number of cycles	Thermal settings
Primary	1	93ºC (1min); 95ºC (5min)
	5	94ºC (0,5min); 62ºC (1min) ; 72ºC (2,5min)
	1	94ºC (0,5min); 25ºC ramping at 72ºC en 3min; 72ºC (2,5min)
	15	94ºC (20s) ; 66ºC (3,5min); 94ºC (20s) ; 66ºC (3,5min); 94ºC (0,5min); 42ºC (1min); 72ºC (2,5min)
	1	72ºC (5min); 4ºC. Hold.
Secondary	12	94ºC (20s) ; 65ºC (3,5min); 94ºC (20s) ; 65ºC (3,5min); 94ºC (0,5min); 42ºC (1min); 72ºC (2,5min)
	1	72ºC (5min); 4ºC. Hold
Tertiary	30	94ºC (0,5min); 42ºC (1min); 72ºC (2,5min)
	1	72ºC (5min); 4ºC Hold.

*The primary PCR was performed in a 15µl volume containing 80ng of genomic DNA; 0.2 µM of primer lip2a; 2 µM of a random primer (R1, R2, R3 or R4); 0,2mM of each dNTP. The secondary PCR was performed with primer lip2b (0.2 µM) and the same random primer R (2 µM) as used in the primary reaction. 1µl of 1/50 dilution of the primary PCR was used as a template. The tertiary reaction was carried out with 1 µl of 1/10 dilution of the secondary reaction, 0.2 µM of primers lip2c and lip2d, 0.2 µM of random primer Ri, 0.2 mM of each dNTP. All PCR reactions were performed with 1U Taq DNA polymerase (Promega) and 10X amplification buffer supplied with the enzyme. To exclude nonspecific amplification, a tertiary control reaction R-R was set up without adding gene-specific primers.

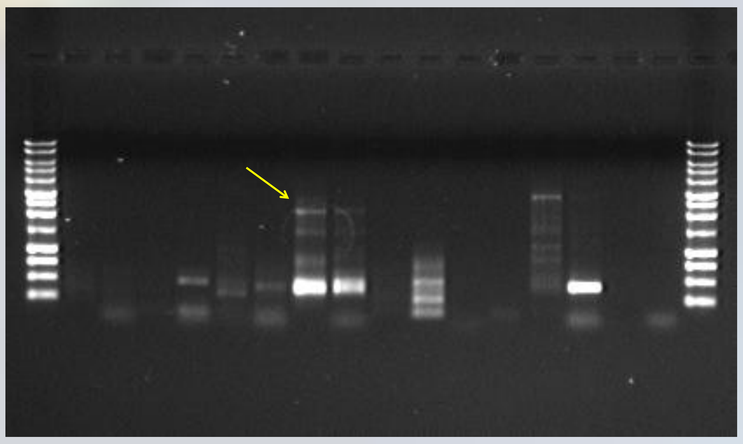

FIGURE 2 - Agarose gel analysis of tertiary HE-TAIL PCR products

Lane: 1,18 – 1Kb molecular weight marker; 2 – R1+lip2c; 3 – R1+lip2d; 4 – R2+lip2c; 5 – R2+lip2d; 6 – R3+lip2c; 7 – R3+lip2d; 8 – R4+lip2c; 9 – R4+lip2d; 10 – R1+R1, 11 – R2+R2; 12 – R3+R3, 13 – R4+R4; 14 – lip2c + lip2c; 15 – lip2d + lip2d; 16, 17 – negative control

ACKNOWLEDGEMENTS

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