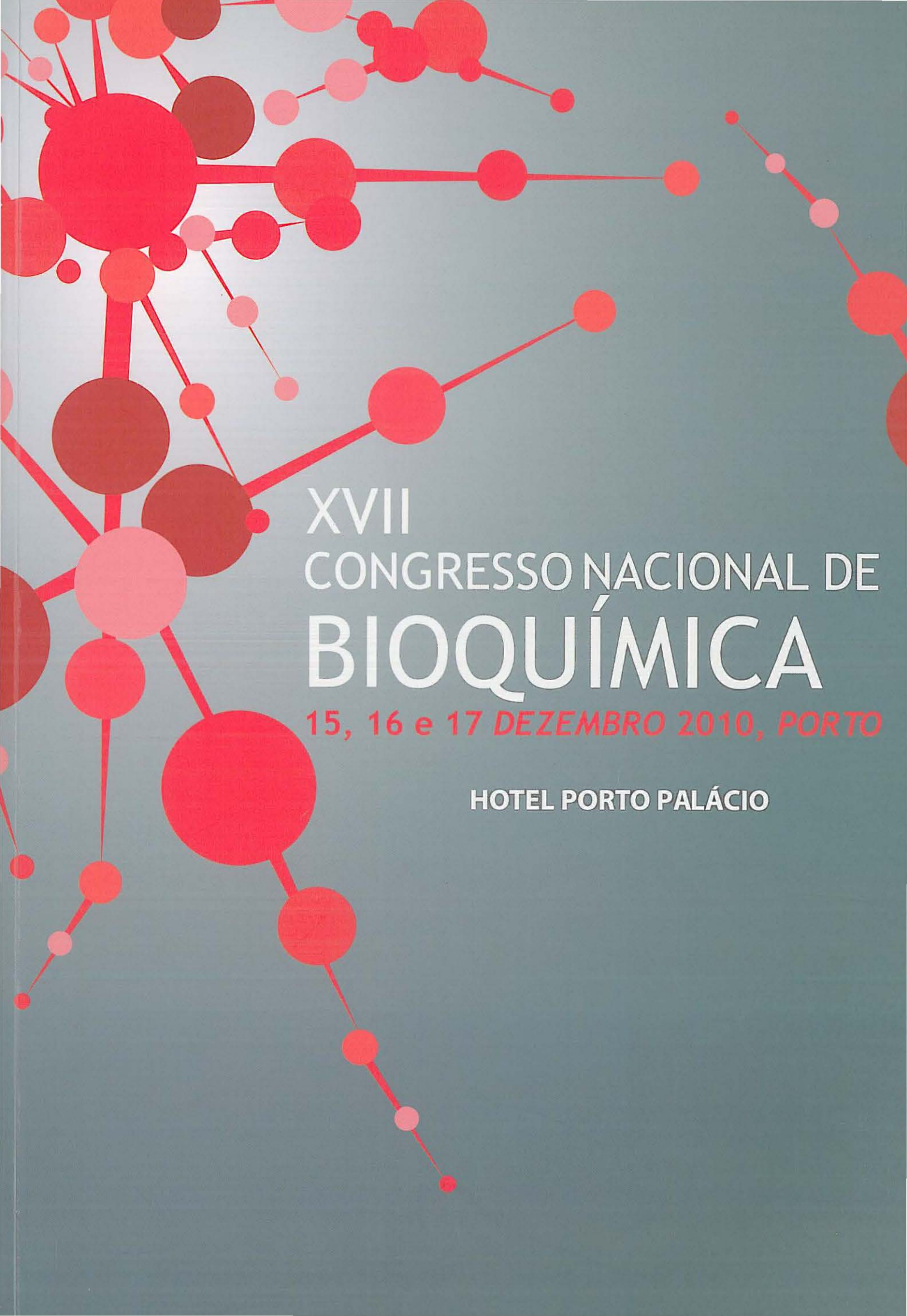

XVII CONGRESSO NACIONAL DE BIOQUÍMICA

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

HOTEL PORTO PALÁCIO

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Identification of *Trichoderma harzianum* lip2 gene by HE-TAIL PCR

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Trichoderma harzianum is a widespread soil fungus, known as a biocontrol agent against soil borne 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 HETAIL PCR (High-Efficiency Thermal Asymetric Interlaced PCR), a method described as efficient to identify flanking regions from short known DNA sequences.

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'-NGTCGASWGAMAWGAA-3'), R2 (5'-GTNCGASWCANAWGTT-3'), R3 (5'-WGTGNAGWANCANAGA-3') and R4 (5'-NCAGCTWSCTMTSCTT-3'), were used. Gene-specific primers, Iip2a (5'-CTGGCAGAACCGATTCCCGAGCGC TT-3'), lip2b (5'-ACGCAACTACGATGGCGCCTTGCTCG-3'), lip2c (5'-TGC GATGAACCCACAGCTATCGCCGA-3') and Iip2d (5'-GAGAAAGCCTGTACTCCACGTAGAGG-3') with 26bp and melting temperatures of 70-72ºC were designed in the incomplete ORF of *Iip2*. 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. Separation and identification of DNA products of the tertiary reaction, including the controls, were made by agarose gel electrophoresis.

At tertiary PCR reaction none amplification products were obtained with degenerate primers R1, R2 or R3. Bands only appear in the combinations R4+lip2c and R4+lip2d. A 2000bp band was selected, purified by geneclean and partially sequenced, who allowed the elucidation of *Iip2* ORF beginning and part of the promoter region. In the end, *Iip2* 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 44.6 KDa. The UniProt accession number for the amino-acid sequence reported in this paper is B7ZET5_TRIHA.

Elucidation of complete gene nucleotide sequence of *Iip2*, 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.

The Project COMBATINTA/SP2.P11/02 Interreg IIIA - Cross-Border Cooperation Spain-Portugal, financed by The European Regional Development Fund, and the Project "Identification, characterization and role of molecular factors associated with the mechanisms of infection of Fagaceae species by *Phytophthora cinnamomi*" (PTDC/AGR-*AAM/67628/2006*) FCT, supported this work.