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Full Length Article

Cloning and Phylogenetic Analysis of an Actin Encoding DNA Fragment from Filamentous Fungus *Trichoderma harzianum*

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Abstract

This study describes the isolation, cloning and phylogenetic studies of a partial sequence of actin gene from the fungus *Trichoderma harzianum* E-58, which was grown in Vogel's medium at 28°C and pH 5.5, with glucose as a carbon source. For the amplification of β-actin gene, the genomic DNA was subjected to the polymerase chain reaction (PCR) by using sequence specific primers. Through agarose gel electrophoresis the amplified PCR product was purified and ligated into pTZ57R/T vector. The ligation mixture was then transformed into *E. coli* DH10B and spread on ampicillin containing LB agar plate. Restriction analysis was done to confirm the positive transformants. Further, the gene was sequenced, and on the basis of this partial sequence, a dendrogram was made to assess homology among different fungi. These results will surely help study the regulation of cellulase gene expression in the fungus in future. © 2013 Friends Science Publishers

Keywords: Actin gene; *Trichoderma harzianum*; Cloning; *E. coli*; Phylogeny

Introduction

Actin is the major cytoskeletal protein and involved in a variety of processes including cellular motility, intracellular transport, cytoskeletal structure, cell-surface mobility, cytokinesis, cytoplasmic streaming, clot retardation, microvillar movement, exocytosis and endocytosis, and probably chromosomal condensation and mitosis (Sheterline *et al*., 1996). Within eukaryotic cells the actin is one of the most conserved protein and plays key roles in various cellular processes like cell growth regulation and motility of the components in the cells (Neveu *et al*., 2007). β-actin gene is very important with regard to gene expression. It is used as an internal control in gene expression studies of various organisms including fungi (Rady and Shearer, 1997). Higher eukaryotic organisms express several actin genes however their primary structure is similar. Only a single copy of the actin-encoding gene has been reported in yeast and some fungi but the genomic structure of actin-encoding genes is variable with respect to introns (Takashima *et al*., 1999).

For more than 50 years fungi have been used for the production of industrial enzymes and microbial biomass proteins (Irshad *et al*., 2008; Athar *et al*., 2009; Ahmed *et al*., 2010; Rajoka *et al*., 2012). *Trichoderma* species are reported to produce enzymes involved in the degradation of cellulose, xylan and pectin to fermentable sugars (Ahmed *at al*., 2009a; Mushtaq *et al*., 2009).

Cellulases production from *Trichoderma harzianum* has been improved since the development of recombinant DNA techniques (Jamil *et al*., 2005) and as promoter regions and intron/exon splicing signals regulate the expression of fungal genes (Ballance, 1986; Diez *et al*., 2000), we are in search of new promoters in order to use those for the studies of gene expression. The present report describes the isolation of actin gene (partial sequence) from *T. harzianum* and its cloning in *E. coli*. In addition, to authenticate the use of actin to study phylogenetic relationships, we have compared the sequence of the gene with those from other fungi.

Materials and Methods

Chemicals

The chemicals were purchased from Sigma Chemical Co., Missouri USA, unless otherwise stated.

Fungal Strain and Culture Conditions

T. harzianum E58 was maintained at 4°C after growing for 7 days in MYG medium (0.2% yeast extract, 2% glucose, 0.2% malt extract and 2% agar) at 28°C (Ahmed *et al*., 2003; Ahmed *et al*., 2005). The fungus was cultivated in Vogel's medium for 5 days at 28°C with shaking at 120 rpm (Ahmed *et al*., 2007; Ahmed *et al*., 2012) with 1% glucose as a carbon source.

Escherichia coli DH10B served as host organism and was grown overnight in Lauria-Bertani (LB) medium containing ampicillin (50 μg/mL) at 37°C. The plasmid pTZ57R/T (Fermentas) containing ampicillin resistance gene and was used for cloning of actin gene.

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Isolation and Manipulation of Nucleic Acids

For DNA isolation, the fungus was grown in Vogel's medium supplemented with 2% glucose. DNA was extracted as described previously (Al-Sammarie and Schmid, 2000; Saadia *et al*., 2008).

PCR Amplification of Actin Partial Gene Sequence

Using DNA as a template, a partial actin gene was amplified by PCR technique with *Taq* polymerase (Fermentas). The net sequence of the primers was:

Forward 5/-
TATCATGATCGGTATGGGTCAGAAGGACTCC
Reverse 5/-
CATCACAATGTTGCCGTAGAGCTCCTTTCGG

The amplification was carried out under the following conditions: 35 cycles of 1 min at 94°C, 1 min at 42°C and 1 min at 72°C, with a final extension of 10 min at 72°C. The PCR products were separated through electrophoresis on agarose gel and visualized by ethidium bromide staining. The amplicon of expected size was gel purified by DNA extraction kit (Qiagen kit).

Cloning of the Actin Gene

The PCR amplified β-actin partial gene sequence was ligated into the pTZ57R/T plasmid with the help of DNA ligase by using Ins T/A Clone™ PCR product cloning kit. *E. coli* competent cells were used for the transformation of ligated products. From successful transformations the colonies were picked up and cultured overnight in LB medium and used for miniprep analysis. Plasmid preparations were carried out using the Qiagen Miniprep Kit. Recombinant vectors were double digested with *Eco*R1 and *Hind*III under optimal buffering conditions at 37°C for 1 h to confirm the presence of insert.

Sequencing and Phylogeny

After restriction analyses of the clone, a 3.6 kb *Eco*R1-*Hind*III fragment suggested that a part of it contained gene encoding an actin. This partial actin gene fragment was sequenced by the dideoxynucleotide method from Centre for Applied Molecular Biology (CAMB), Lahore, Pakistan. The partial nucleotide sequence corresponding to the gene encoding β-actin was deposited in the GenBank and allotted an accession no. HQ222609. The partial β-actin gene sequence was used to study the phylogenetic relationships among different fungi.

Results

Amplification of β-actin Partial Gene Sequence

The fungus was grown in Vogel's medium for 5 days at 28°C with shaking at 120 rpm for the isolation of genomic DNA. To remove RNA contamination the genomic DNA was treated with RNases and subjected to PCR. Several tries were made with different $MgCl_2$ and primer concentrations. Intense band of β-actin gene was found at 2 mM $MgCl_2$ concentration (Fig. 1).

With the help of DNA extraction kit the amplified DNA was recovered from agarose gel and run again on 1% agarose gel for confirmation (Fig. 2).

Cloning and Restriction Analysis

The gel purified PCR product was ligated into the plasmid pTZ57R/T with the help of DNA ligase by using insT/A Clone™ PCR product cloning kit and transformed into *E. coli* DH 10B strain. The recombinant plasmids were selected on LB agar containing ampicillin (50 µg/mL).

Colonies of *E. coli* carrying the recombinant vector were cultured overnight and used for miniprep analysis. Ligation of actin was confirmed through restriction digestion. The recombinant construct was double digested with *Eco*RI and *Hind*III resulting in the separation of β-actin gene from the vector (vector size, 2886 bp) as shown in the Fig. 3. Thus restriction digestion confirmed the cloning of actin gene. The partial actin gene was sequenced and deposited to Gene Bank and assigned Gene Bank accession number HQ222609.

In this report the isolation, cloning and phylogenetic relationship of β-actin is described. BLAST (Basic Local Alignment Search Tool) has shown that this partial actin gene has 92% gene sequence similarity with *Aspergillus terreus* NIH2624 actin (ATEG_06973) partial mRNA and *A. flavus* NRRL3357 actin Act1, mRNA, 91% gene sequence similarity with *T. harzianum* partial actin gene and *T. reesei* (QM9414-Rut C30) gene for actin.

Discussion

The fungus growth conditions were in agreement with earlier work done where it was found that the optimal growth conditions of *T. harzianum* in Vogel's medium were at 28°C, pH 5.5 and shaking at 120 rpm (Saadia *et al*., 2008; Ahmed *et al*., 2009b).

The structural designs of introns are very useful to study phylogenetic relationships. Therefore, different researchers have isolated actin genes from different fungi and studied their intron architectures. But it was unusual not unique, when it was found that *Phytophthora* actin genes did not contain introns and relatively low sequence conservation was found between *P. megasperma* actin and the actins of other fungi, and a large evolutionary distance was found between Oomycetes and other fungi (Dudler, 1990). The actin genes of *Dictyostelium* (Firtel *et al*., 1979), *Oxytricha* (Kaine and Spear, 1982), and *Tetrahymena* (Cupples and Pearlman, 1986; Hirono *et al*., 1987) are also without introns. Matheucci *et al*. (1995) isolated actin gene from *T. reesei* and found that it has five introns located in identical positions as reputed ancestral

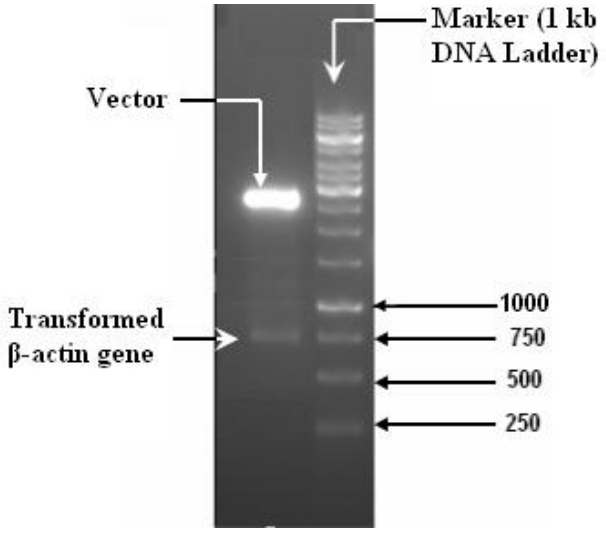

Fig. 3: Digestion of recombinant plasmid by *Eco*R1 and *Hind*III. Lane 1: Recombinant plasmid digested *by Eco*R1 and *Hind*III. Lane 2: DNA Molecular weight marker, Fermentas

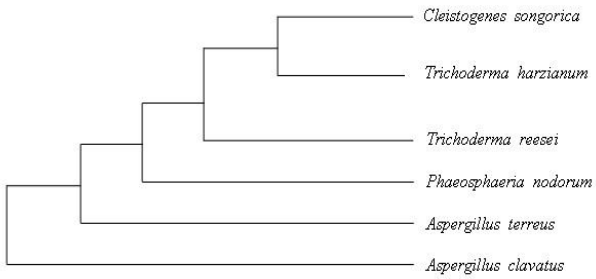

Fig. 4: Phylogenetic tree of fungal actins

actin genes of *Thermomyces lanuginosus* and *A. nidulans*. Similarly, Diez *et al*. (2001) isolated actin gene from *Penicillium chrysogenum* and found that after splicing the predicted protein exposed 90% identity to actins from fungi and grouping of *P. chrysogenum* was found to be very close to those from the bulk of filamentous fungi.

The phylogenetic analysis of highly conserved β-actin gene from *T. harzianum* perfectly agrees with taxonomical classification. In future, this knowledge must be very useful in genetic research on fungi, as β-actin gene can be used as a control in expression analysis. Furthermore, the comparisons of actin genes from different fungi are very useful for phylogenetic studies.

Cellulases are industrially important enzymes and have many important and many potential applications, for example, in the production of bio-fuel and bio-energy, in paper and pulp idustry and in textile industry (Ghori *et al.*, 2011; 2012). Another promising area of the cellulases is the bioconversion of renewable cellulosic biomass to sugars for fermentation of bioethanol and other biobased products also on large scale (Cherry and Fidantsef, 2003; Aygan and

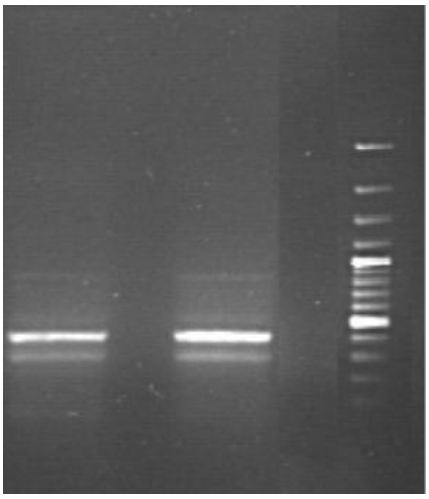

Fig. 1: PCR for amplification of β-actin partial gene from *Trichoderma harzianum*. Lane 1, 2 PCR amplified partial actin gene from *T. harzainum* Lane 3: Marker (1 kb DNA ladder, Fermentas)

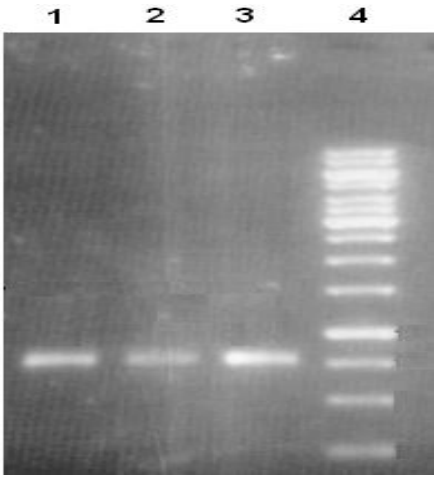

Fig. 2: 1% agarose gel for confirmation of gel purified PCR product. Lane 1, 2, 3 Gel purified actin partial gene (760 bp), Lane 3: Marker (1 kb DNA ladder, Fermentas)

Arikan, 2008). β-actin gene plays a very important role in cellulases expression.

In conclusion, we successfully isolated a PCR fragment of 760 bp partial actin gene from *T. harzianum*, cloned into pTZ57R/T and transformed into *E. coli*. On the basis of sequence similarities, a dendrogram was made to assess homology among different fungi (Fig. 4). Among these fungi, *A. clavatus*, *A. terreus* and *Phaeosphaeria nodorum* actins are thought to be evolutionarily the closest to the ancestral actins. *T. reesei* actin is evolutionarily closer to the ancestral actin among these fungi, but *T. harzianum* and *Cleistogenes songorica* are thought to be more diverged forms of ancestral actin. This study will help in future studies on cellulases expression.

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