Pak. J. Bot., 40(1): 421-426, 2008.

ISOLATION AND CLONING OF *CRE1* GENE FROM A FILAMENTOUS FUNGUS *TRICHODERMA HARZIANUM*

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Abstract

Cellulases and hemicellulases are two important classes of enzymes produced by filamentous fungi and secreted into the cultivation medium. The production of these enzymes is under carbon catabolite repression (CCR), a general mechanism that prevents their synthesis in the presence of a preferred carbon source such as glucose. CRE1 causes the repression of transcription of cellulase and xylanase encoding genes. This study describes the isolation and cloning of a partial sequence of glucose repressor *creI* gene from *Trichoderma harzianum* E-58. The fungus was grown in Vogel's medium at 28°C, and pH 5.5 with glucose as a carbon source. Genomic DNA was subjected to the polymerase chain reaction (PCR) for amplification of *cre1* gene by using degenerate primers. The PCR product was purified through agarose gel electrophoresis and ligated into pTZ57R/T vector. The ligation mixture was then transformed into *E. coli* DH10B and spread on the LB agar plate containing ampicillin. Clone analysis by PCR was done to confirm the positive transformants. These results will help study the regulation of cellulase and xylanase gene expression in the fungus in future.

Introduction

Cellulose comprises long polymers of β-1, 4-linked glucose units forming a higher-ordered fibrillar structure. Hemicelluloses comprise various heteropolysaccharides formed either by xylose (xylans) or mannose and glucose. Complete hydrolysis of these substrates requires a synergistic action of a large number of extracellular enzymes, but their yield is significantly low due to catabolite repression. Glucose and related sugars repress the transcription of genes encoding enzymes required for the utilization of xylan or cellulose as carbon sources. In most fungal species, positive and negative regulatory elements upstream of the genes encoding extracellular enzymes coordinately regulate the expression to adjust the enzyme levels to the prevailing conditions.

The fungi are eukaryotic, achlorophyllous and heterotrophic thallopytes comprising about 1.5 million species, of which only 74, 000 species are described (Hawksworth, 2001; Jamil *et al*., 2007). *Trichoderma harzianum* is a filamentous fungus and is a potential biological control agent against root rot pathogens (Elad *et al*., 1982; Malik & Dawar 2003). Filamentous fungi have been used for more than 50 years in the production of industrial enzymes (Dalboge, 1997). Cloning of regulatory genes involved in the expression of extracellular enzymes is very important. Numerous cellulase and hemicellulase encoding genes have been cloned from *Trichoderma reesei* and other *Trichoderma* spp., but only a few (*cbh*1, *cbh*2, *xyn*1 and *xyn*2) have been investigated in detail concerning their expression and regulation (Stangl *et al*., 1993; Zeilinger *et al*., 2000; Aro *et al*., 2001). Until recently, there has been an emphasis on the role of positive regulators in the induction of xylanase genes. There is a need to understand the mechanism by which negative regulatory factors repress the expression of xylanolytic enzymes and which reduce the cost effective industrial production of xylanases (Rao *et al*., 2003).

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Carbon catabolite repression CCR is a major mechanism controlling metabolic processes of both prokaryotic and eukaryotic microbes. In microorganisms it is a promising mean to control the synthesis of gene expression (Ilmen *et al*., 1996). It is an important regulatory mechanism that leads to the repression of genes encoding enzymes that are involved in the utilization of complex carbon sources when simple sugars like glucose are available (Schmol & Kubicek, 2003).

CCR is exerted by the repressor CreA (Dowzer & Kelly 1991). CreA, carbon catabolite repressor of *Aspergillus niger* is the analogous of *Saccharomyces cerevisiae* MIGI (Nehlen & Ronne, 1990; Nahlin *et al*., 1991). The homologue of CreA has also been found in other filamentous fungi such as *Aspergillus nidulans* and *Trichoderma reseei*.

In filamentous Ascomycetes, glucose repression is mediated by CRE1. In *Trichoderma reesei*, carbon source regulation of cellulase and hemicellulase gene expression at the transcriptional level is mediated by the Cre1 protein which is similar to the glucose repressor CreA of *Aspergillus nidulans* and *Aspergillus niger*, particularly in two zinc fingers of the Cys2 His2 class (Ilmen, 1997). CCR of the expression of the *T. reesei xynI* gene mediated by Cre1, has been investigated (deGraff *et al*., 1994, Mach *et al*., 1996). Similarly glucose repression of *cbh1* expression by Cre1 has also been reported (Ilmen *et al*., 1996).

The present report describes the isolation of the *creA* equivalent, *cre1* gene (partial sequence) of *Trichoderma harzianum* and its cloning in *E. coli.*

Materials and Methods

Chemicals: The chemicals were purchased form Sigma Chemical Co., Missouri USA, unless otherwise stated.

Fungal strain and culture conditions: *Trichoderma harzianum* E58 strain was maintained on agar slants (MYG) (Ahmed *et al*., 2007). The fungus was cultivated in Vogel's medium for 5 days at 28 ^{o}C with shaking at 120 rpm (Qurrat-ul-Ain *et al*., 2003).

Plasmid: The plasmid pTZR57R/T was obtained from Fermentas containing ampicillin resistance gene.

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-Samarrai & Schmid, 2000). DNA concentrations were determined spectrophotometrically.

PCR amplification of *cre1* partial gene sequence: Degenerate primers were designed according to the highly conserved Zn finger domains of the *creA* protein of *A. niger*. The net sequence of the primers was:

Primer 1 (antisense): 5$^{/}$-GGCGGATCCT(C,T)TGGNGT-(G,A)TCNGG-3$^{/}$

Primer 2 (sense): 5$^{/}$-GGCGGATCCTTNGG(G,A)-TT(G,A)TA(A,G)TA(T,G)TTNGG-3$^{/}$

The reaction mixture for PCR contained 50 ng of *T. harzianum* DNA as template, 0.2 m*M* dNTPs (each), $MgCl_2$ (1-4 m*M*), 5.02 *µM* primer 1 and 6.05 *µM* primer 2, 1.25 units/50 *µL* of *Taq* DNA polymerase (Fermentas). Temperature program was as: four cycles of repeated denaturation at 96°C for 1 min., annealing at 37°C for 30 seconds; polymerization at 72°C, followed by 25 cycles of repeated denaturation at 96°C for 1 min., annealing at 55°C for 1 min. and polymerization at 72°C for 1 min.

Ligation and transformation of the gene into plasmid: The PCR amplified *cre1* 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. The ligated products were transformed into *E. coli* competent cells. Colonies from successful transformations were picked up and grown.

Clone Analysis by PCR: From the LB-ampicillin agar plate, single colony with transformed recombinant vector was picked and resuspended in 30 µL of the PCR mixture. The reaction mixture was incubated for 5 min., at 94°C to lyse the cells and inactivate nucleases. Amplification of 30 cycles through PCR with conditions as described above was performed and PCR products were visualized by agarose gel electrophoresis.

Results and Discussion

Effect of glucose on induction of cellulases and hemicellulases in *T. harzianum*: In fungi, the production of cellulolytic and hemi-cellulolytic enzymes is subject to transcriptional regulation by available carbon source. The xylanase and cellulase genes are repressed in the presence of glucose. We have previously reported that xylanase from *T. harzianum* is expressed during growth on xylan but when grown on glucose no xylanase activity was detected (Ahmed *et al*., 2007). Likewise endoglucanase was induced by CMC but repressed by glucose (Qurrat-ul-Ain *et al*., 2003).

Amplification of *cre1* partial gene sequence: DNA was isolated from the fungus grown in Vogel's medium for 5 days at 28°C with shaking at 120 rpm. This is 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 (Ali *et al*., 2003). DNA was isolated from the fungus after Al-Samarrai & Schmid, (2000) (Fig. 1). Genomic DNA was treated with RNases to remove RNA contamination.

Knowledge concerning the DNA binding repressor of filamentous fungi is limited to few-isolated factors including the glucose repressor *cre1/cre*A from various fungi (Drysdale *et al*., 1993, Strauss *et al*., 1995, Takashima *et al*., 1998; Vautard *et al*., 1999).

In order to investigate the mechanism of CCR in the industrially important fungus *T. harzianum*, degenerate PCR primers were designed to amplify *cre1* gene. Several tries were made with different $MgCl_2$ and primers concentrations. Intense band of *creI* gene was found when 2 *mM* $MgCl_2$ concentration and 0.5 u*M* primer concentration was used (Fig. 2). The concentration of PCR purified fragment was determined by performing dot test according to Ausubel *et al*., (1998). The amplified DNA was recovered from agarose gel with the help of DNA extraction kit from Fermentas.

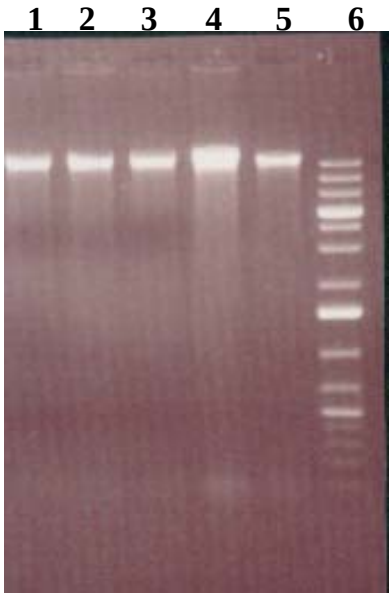

Fig. 1. DNA samples extracted from *Trichoderma harzianum*. Lane 1, 2, 3, 4, 5: *T. harzainum* DNA Lane 6: Marker.

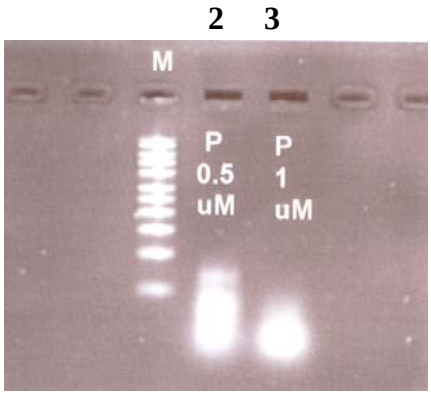

Fig. 2. PCR for amplification of *creI* gene from *Trichoderma harzianum*
Primer concentration was optimized at same $MgCl_2$ concentration. *Cre1* gene amplification (~400 bp) was observed at two different primer concentrations. Lane 1 shows marker (M), 1 kb ladder. Lane 2 shows 0.5 u*M* and lane 3 shows 1.0 u*M* of each primer concentration (P), respectively at 2 m*M* $MgCl_2$ concentration.

Cloning and transformation: The amplified product was ligated into pTZ57R/T plasmid with the help of DNA ligase by using insT/A Clone™ PCR product cloning kit and transformed into *E. coli* DH 10B strain. Colonies from successful amplification were picked up and grown. The recombinant plasmids were selected on LB agar ampicillin plates. Earlier, isolation and characterization of *creA* gene from *Humicola grisea* and *Acremonium chrysogenum* has been reported by Takashima *et al*., (1998) and Jekosch & Kuck (2000), respectively. Ilmen *et al*., (1996) isolated and characterized *cre1* gene from *Trichoderma* spp.

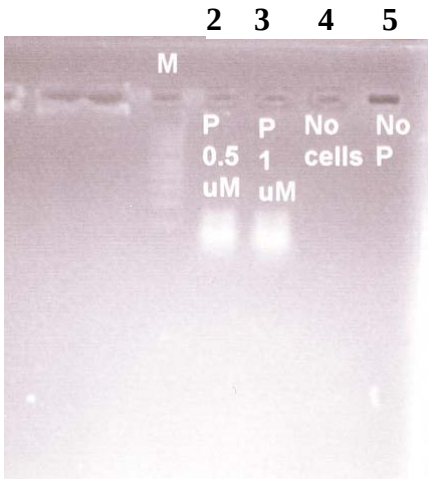

Fig. 3. Clone analysis by PCR
PCR was used for direct analysis of the positive transformants, using 1% agarose gel. Amplification was achieved only in cells containing plasmids with *crel* gene. No amplification was observed in the absence of either primers or *E. coli* cells. Lane 1 shows marker (M), 1 kb ladder. Lane 2 and 3 show amplification at 0.5 u*M* and 1 u*M* primer concentration (P), respectively. Lane 4 shows no *E. coli* cells, while lane 5 shows absence of primers (P).

Clone analysis: PCR was used for direct analysis of the positive transformants. Four samples (S1 to S4) were prepared. S1 with 0.5 u*M* of each primer concentration, S2 with 1 u*M* of each primer concentration, S3 with 0.5 u*M* of each primer concentration but no *E. coli* cells and S4 with *E. coli* cells but no primers. PCR results with 1% agarose gel revealed that successful amplification with 0.5 u*M* and 1 u*M* of each primer concentration was achieved but no amplification was observed in the absence of either primers or *E. coli* cells. These results suggest that ~400 bp fragment of the *cre1* partial gene sequence was successfully cloned in *E. coli*.

Conclusion

Successful amplification of *cre1* partial gene sequence was achieved from *T. harzianum*. The amplified product was cloned into *E. coli*. This study will help to study the role of *cre1* gene under CCR of cellulase and hemicellulase genes in the fungus in future.

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(Received for publication 23 September 2007)