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Full Length Research Paper

Molecular cloning of endochitinase 33 (*ECH*33) gene from *Trichoderma* harzanium

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This study was conducted to screen for the presence of *ech*33 gene in 80 isolates of *Trichoderma.* Furthermore, using gene specific primers, *ech*33 gene were cloned into pTZ57R/T from *T. harzanium* IABT1068. The clone was confirmed through PCR amplification and restriction analysis. The clones were sequenced and analyzed for homology at nucleotide and protein level to find out conserved domain of protein. Gene encoding endochitinase from both species have 96 and 95% homology with reported sequence both at nucleotide and protein level. The cloned *ech*33 has a size of 1159 bp, of which 9 bp corresponds to the 5' untranslated region, with a 650 bp open reading frame. The amino acid sequence of gene has signal peptide sequence ranges from 1 to 19. The nucleotide sequence analysis using GENETOOL software revealed presence of three exon and four introns, and has unique restriction sites for *HindIII, BamHI* and *SalI,* at 881, 308 and 485 positions, respectively.

Key words: *Trichoderma harzanium, ech*33, signal peptide.

INTRODUCTION

Traditional methods used to protect crops from diseases have been largely based on the use of chemical pesticides. Applications of fungicides can have drastic effects on the environment and the consumers. Chemical methods with repeated use are not economical in the long run because they pollute the atmosphere, damage the environment, leave harmful residues and lead to the development of resistant strains among the target organisms (Naseby et al., 2000). A reduction or elimination of synthetic pesticide applications in agriculture is highly desirable. One of the most promising means to achieve this goal is by the use of new tools based on biocontrol agents (BCAs) for disease control alone or to integrate with reduced doses of chemicals in the control of plant pathogens resulting in minimal impact of the chemicals on the environment (Chet and Inbar, 1994; Harman and Kubicek, 1998). *Trichoderma* spp. is among the most frequently isolated soil fungi present in plant root ecosystems (Harman et al., 2004). These fungi are opportunistic, avirulent plant symbionts and function as parasites and antagonists of many phytopathogenic fungi, thus protecting plants from disease. So far, *Trichoderma* spp. are the most studied fungal BCAs and commercially marketed as biopesticides, biofertilizers and soil amendments (Harman et al., 2004; Lorito et al., 2004). Depending on the strain, *Trichoderma* spp. (notably *Hypocrea lixii/Trichoderma harzianum, Hypocrea virens/Trichoderma virens, Trichoderma atroviridis/ Trichoderma atroviride* and *Trichoderma asperellum)* are used as biocontrol agents against various diseases of crops, vegetables and fruits (Harman et al., 2004). They have evolved numerous mechanisms that are involved in attacking other fungi. These mechanisms include competition for space and nutrients (Elad et al., 1999), mycoparasitism (Haran et al., 1996; Lorito et al., 1996a), production of inhibitory compounds (Sivasithamparam and Ghisalberti, 1998), inactivation of the pathogen's enzymes (Roco and Perez, 2001) and induced resistance (Kapulnik and Chet, 2000).

The antifungal mechanism of *Trichoderma*, an extensively studied and widely used biocontrol fungus, mainly relies on cell wall degrading enzymes such as chitinases and glucanacses (Lorito et al., 1998) and is being exploited to control a variety of plant pathogens. The genes encoding chitinases and glucanacse are

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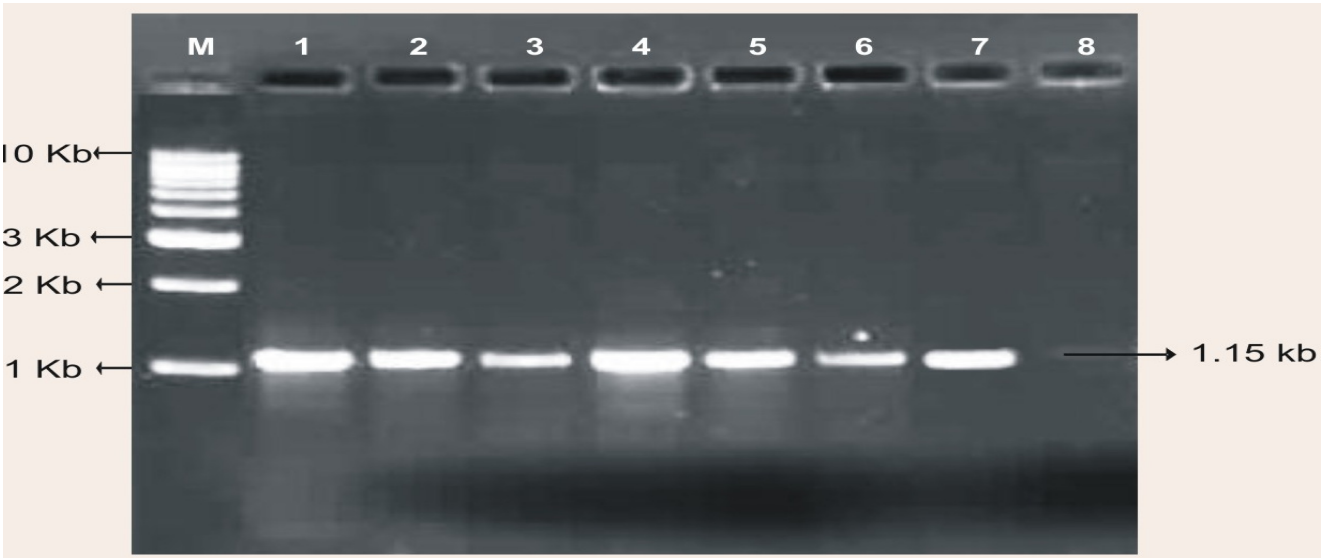

Plate 1. PCR amplification of *ech33* gene (1.15 kb). M = 1 KB DNA ladder; 1. *T. atroviride;* 2 *T. harzianum;* 3. *H. virens;* 4. *T. harzinum;* 5. *T. harzinum;* 6. *T. harzinum;* 7.*T. harzinum.*

isolated from *Trichoderma* and transferred to plants to impact resistance to several fungal plant pathogens. Chitinase encoding genes are being used to improve plant defense against fungal pathogens. These enzymes are capable of degrading the linear homopolymer of ß-1, 4-N-acetyl- D-glucosamine, the main cell wall component of most phytopathogenic fungi, showing strong inhibitory activity *in vitro* on germination and hyphal growth (Lorito et al., 1996a). Plants do have chitinases, but are not as effective as microbial chitinases. Therefore, cloning and characterization of genes from biocontrol microbes such as *Trichoderma* is very important. There are many evidences to show that fungal chitinases alone has increased the resistance of transformed plants against pathogenic fungus. The rice plant transformed with an endochitinase gene (*ech*33) from the biocontrol fungus *T. atroviride* increased the resistance to sheath blight caused by *Rhizoctonia solani* and rice blast caused by *Magnaporthe grisea* (Liu Mei et al., 2004).

Efforts have also been made to produce transgenic plants expressing either plant or microbial chitinase. In recent years, considerable progress has been made in producing disease-resistant and high-yielding transgenic plants. It may be necessary to integrate different resistance genes together in order to extend the host defense.

MATERIALS AND METHODS

Isolation of genomic DNA from fungus

The *T. harzanium* IABT1068 strain were inoculated in100 ml potato dextrose broth at 30°C in room temperature. The complete growth occurred within 2 to 5 days depending upon the species. About 100 mg of fungus mycelium was taken in 1.5 ml micro centrifuge tube and 500 µl of lysis buffer was added. Mycelium was finely macerated using micro-pestle and vortexed for 5 min. The suspension was extracted with equal volume of phenol: chloroform: IAA (25:24:1) and centrifuged at 10,000 rpm for 10 min. The supernatant was taken into a fresh tube and RNase at the rate of 100 µg per ml was added and this solution was incubated for 20 min at 55°C on water bath and then equal volume of isopropanol was added at room temperature, mixed by gentle inversion and kept for 10 min at room temperature. The DNA was recovered by centrifugation at 10,000 rpm for 10 min at 4°C. The DNA pellet was washed with 70% ethanol, air dried and resuspended in 50µl of $T_{10}E_1$ (10 mM Tris-Cl and 1 mM EDTA, pH 7.5). Concentration of DNA was estimated using ethidium bromide spotting method as described by Sambrook and Russel (2001).

Polymerase chain reaction amplification (PCR) amplification

PCR was carried out from *T. harzanium genomic* DNA. For PCR amplification, two gene specific primer were used- FP 5'ATGCCTTCATTGACTGCTCTT 3' and RP 3'TTACCTCAAA GCATTGACAACC5'.

Reaction mixture for PCR (20 µl) contained 10 mM Tris-HCl (PH 8.3), 50 mM KCl, 1.5 mM MgCl2, 10 mM each of dATP, dCTP, dGTP and dTTP, 5 pM primer, 1 µl genomic DNA (100 ng) and one unit of Taq polymerase. Amplification was performed in 0.2 ml tube using thermocycler (Eppendorf 2231, Hamburg, Germany). Initial denaturation was carried out at 94°C for 5 min. Thirty five cycles of the following programme were used for amplification; denaturation at 94°C for 2 min, annealing at 41°C for 2 min and extension at 72°C for 10 min. The amplified products were separated by electrophoresis on 1.2% gel stained in ethidium bromide. The gel was observed and photographed using UV transilluminator. The amplification showed ~1.15 kb amplicon (Plate 1).

Cloning of endochitinase gene

The specific eluted bands (~1.15 kb) corresponding to *ech33* from *T. harzanium* IABT1068, were ligated to pTZ57R/T vector (2886 bp)

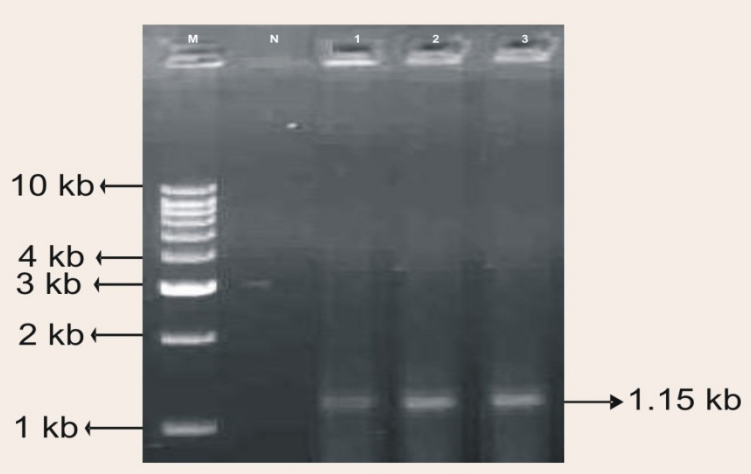

Plate 2. PCR confirmation of *ech*33 cloned gene. M = 1 KB DNA ladder. 1. *T. Harzianum;* 2 *T. harzianum*; 3. *H. harzianum.*

as described in InsT/A clone™ PCR product cloning kit (#k1214) from MBI Fermentas USA. The ligation products were used to transform *Escherichia coli* DH5α.

Transformation of *Escherichia coli* DH5α with recombinant construct

The competent cells of *E. coli* DH5α were prepared following the protocol mentioned by Sambrook and Russell (2001). About 100 µl of freshly prepared competent cells were taken in a chilled centrifuge tube and 10 µl of ligated mixture was added into the tube and was mixed gently. The mixture was chilled in ice for 45 min. Later, heat shock was given by shifting the chilled mixture to preheated 42°C water bath for exactly 2 min. Immediately, it was transferred onto ice to chill for 5 min. To this, 800 µl of Luria broth was added and incubated at 37°C at 200 rpm for 45 min, to allow bacteria to recover and express the antibiotic marker encoded by plasmid. The culture was centrifuged at 13,000 rpm for 1 min and about 700 µl of supernatant was discarded and the pellet was dissolved in the remaining supernatant and spread on the plates having Luria agar with Amp_{100}, X-gal IPTG and incubated overnight at 37°C.

The recombinant clones were identified by blue/white assay. After incubation, only white colonies were picked up and streaked on plates having Luria agar with Amp_{100}, X-gal, IPTG and incubated at 37°C overnight and checked further for the presence of construct through PCR and restriction confirmation.

Sequencing and *in silico* analysis of the clones

The recombinant plasmid was sequenced using M13 universal forward and reverses primers at Bangalore Genei Private Ltd., Bangalore. The sequence was subjected for analysis after removing vector sequence, through vecscreen service available in NCBI website. The available sequence information from cloned fragments was subjected to analysis using BLAST algorithm available at http://www.ncbi.nlm.nih.gov. *In silco* translation was done using GENETOOL software. Dual and multiple alignments for homology search were performed using the Clustral W algorithm in BioEdit software (Hall, 1999). The general features of the protein (amino acid composition) were assessed using the GENETOOL and the presence of a putative signal sequences was predicted using Signal P 3.0 Verson (Bendtsen et al., 2004; http://www.cbs.dtu.dk/services/SignalP/). All other bioinformatics like searching domain and catalytic active sites were performed using tools that are accessible via different links on the proteomics service of the Swiss Institute of Bioinformatics (Zdobnov and Apweiler, 2001; http://www.ebi.ac.uk/InterProScan/).

RESULTS

The cloned *ech*33 gene from *T. harzanium* (IABT1068), 17 colonies were observed on selection medium of which 15 were white. Further, these colonies were screened for the presence of *ech*33 and only three clones showed the presence of ~1.15 kb insert when checked through PCR with specific primers and restriction analysis(Plates 2 and 3).

One of the clones corresponding to *ech*33 was named as pBRS-20. The clone was sequenced using M13 forward and reverse primers at Bangalore Genie Pvt. Ltd. The complete sequence of nucleotides (Figure 1) was found after removing vector sequence through vecscreen service of the NCBI website. The available sequence information from cloned gene was subjected to analysis using BLAST algorithm available at http://www.ncbi.nlm.nih.gov. It showed homology with conserved domain of CHI-18, chitinase like superfamily (Figure 4). The nucleotide sequence of *ech*33 showed 96% homology with the published *H. virens* chitinase 33 (*chi*33) (FJ358733.1), 86% with *T. viriens* chitinase 2 (GQ303455.1), 86% with *H. virens* class III chitinase precursor (*cht*2) (AF395754.1). The cloned *ech*33 has a size of 1159 bp, of which 9 bp corresponds to the 5' untranslated region, with open reading frame present in the DNA (Figure 7). The nucleotide sequence was translated to amino acid using GENETOOL software and code for 258 amino acids containing stop codon. The

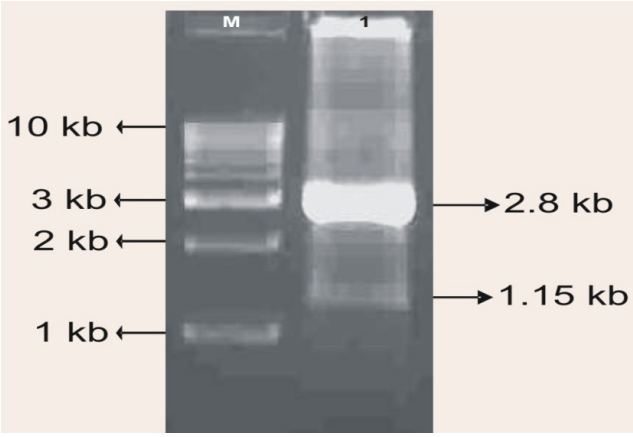

Plate 3. Restriction confirmation of *ech*33 gene. M =1KB DNA ladder; 1. *T. Harzinum.*

Contig

ATGCCTTCATTGACTGCTCTTGCGAGCGCGCTCGCTCTTGTTCCTTCCGTCTTTGCTGGCTGGAATGTTAACTCGAAGCAAAA
CATTGCTGTGTACTGGGGTAAGGCCCTTCTTATGAGCTTTTACACAATTTATATAAGGGAAAATAGCTATCTAACATGACATCTG
TATAGGACAAAACTCGGCCGGCCAGCAAAGCACGCAACAGCGTCTTTCAACCTACTGCAGCGGTACGTTTCTGCTTTATTTCC
CAACACTGCAAGCTTTGATCGGCCAAAATGAGAAAAAGAGCATGTTGAATCTGACAGCTGCCATGTAGATGCCAATATTAATGT
TATTGACATTGCTTTCTTGAACGGAATTACTCCTCCCATGACCAACTTTGCCAATGCCGGTGACCGATGCACGCCCTTTTCAGA
CAACCCTTGGCTCTCGCAATGCCCCGAAATTGAGTAAGTTTCTCTATATGAAGAAATGGGTGTCTGATGTGTATGCAAATATGG
ACTAACTTTAATTCATTTTAAAGGGCGGATATCAAGACTTGCCAGGCTAACGGCAAGACCATCCTCCTTTCTCTTGGTGGTGAC
TCTTACACCCAAGGTGGCTGGAGCTCTGCCAGCGCTGCTCAAGCCGCAGCCAACCAGGTCTGGGCCATGTTCGGTCCCGTTC
AGTCCGGCAGCTCTGCCGAGCGTCCGTTTGGCAGTGCAATCGTGGACGGCTTTGATTTCGACTTTGAGGCCACGACCAACAA
CCTCGCTGCTTTCGGCGCCCAGCTCAAGAGCCTCTCCAACGCTGCCCGGCGGCAAGAAGTACTACTTCTCTGCTGCTCCTCA
GTGCTTCTTCCCAGACGCCGCTGTCGGTGCGCTGATCAACGCCGTCCCCATGGACTGGATCCAGATTCAGTTCTACAACAATC
CTTGCGGCGTCAGTGGCTACACGCCCGGCACCAGCAGCCAGAACAACTACAACTACCAGACCTGGGATACCTGGGCCAAGA
CGAGCCCCAACCCCAACGTCAAGCTTCTTGTCGGCATTCCCGCTGGCCCAGGTGCTGGTCGCGGCTACGTCTCTGGCTCTCA
GCTCACTTCAGTCTTCCAGTACTCGAAGGGGTTCAGCAGCACCTTTGCCGGTGCCATGATGTGGGATATGTCCCAGCTTTACC
AGAACACTGGCTTTGAGGCCCAGGTTGTCAATGCTTTGAGGTAA

Figure 1. Complete nucleotide sequence of cloned endochitinase gene (*ech33*) from *T. harzanium* (IABT1068).

amino acid sequence of gene is shown in Figure 5. It has signal peptide sequence ranges from 1 to 19. The nucleotide sequence analysis using GENETOOL software revealed presence of three exon and four introns (Figure 6).

The cloned *ech*33 in Pbrs-20 has unique restriction sites for *HindIII, BamHI* and *SalI,* at 881, 308 and 485 positions, respectively. The restriction map of the sequence (pBRS-20) is presented in Figure 2. The vector map of pBRS-20 was constructed using the software VECTOR NTI and is presented in Figure 3. The gene is in reverse orientation in the pTZ vector.

The nucleotide sequence of the cloned *ech*33 was subjected for BLASTx and the homology results are presented in Figure 8. It showed 95% homology with the published *H. virens* chitinase 33 (*chi*33) (FJ358733.1), 95% with *H. lixii* chitinase (CAA56315.1), 90% with *H. virens* chitinase (ABP96986.1) and 90% with chitinase-2 of *H. virens* (AAL78811.1) with published sequence of *T. harzanium* (accession no. FJ358733.1) endochitinase gene at amino acid levels (Figure 9).

DISCUSSION

Cloning of chitinase genes is the first step in development of transgenic resistant to fungal diseases. Therefore, cloning of endochitinase genes and transferring it toplants is a major step for development of transgenic against resistant to plant pathogen. So, in this study, for the cloning of endochitinase gene from *ech*33 from *T. harzianum* (IABT1068), sequenced functional analysis were done. The *ech*33 gene had 96% homology with reported endochitinase gene from *H. virens* chitinase 33

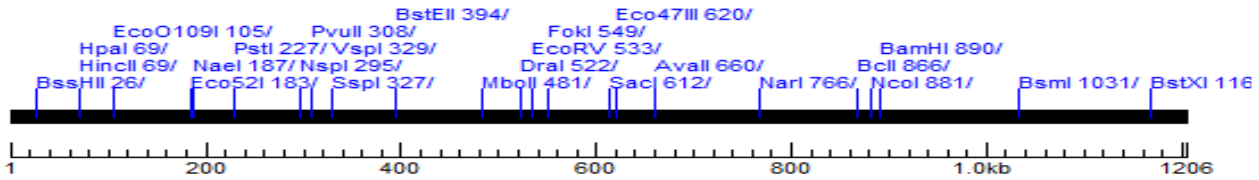

Figure 2. Restriction map of cloned endochitinase gene (*ech33*) sequences from *T. harzanium* (IABT1068) with common enzymes.

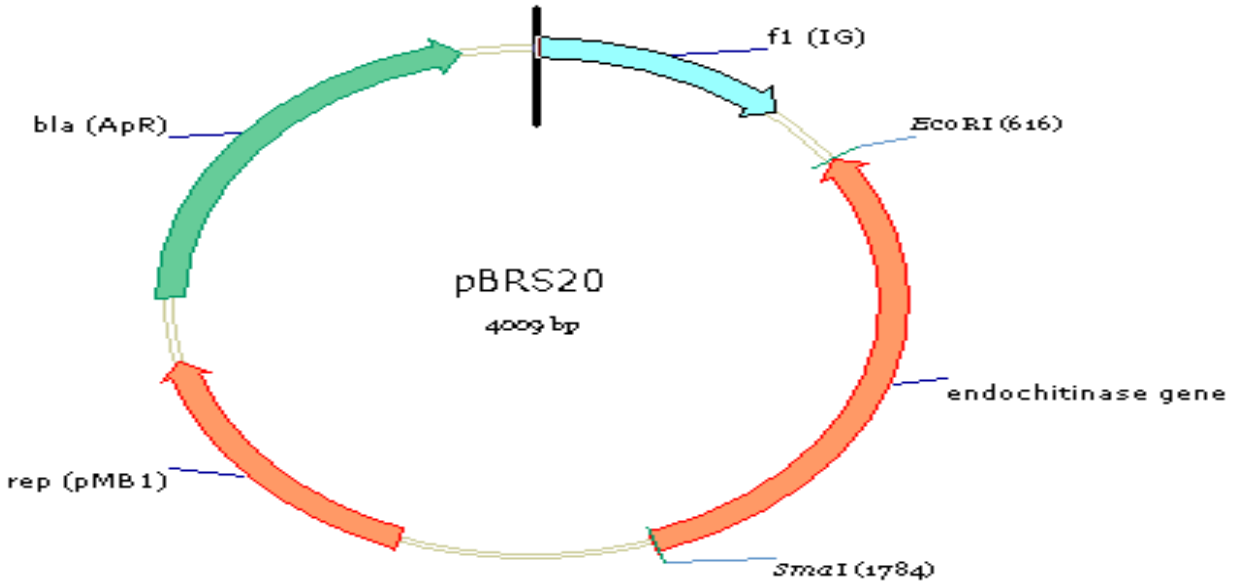

Figure 3. Construct map of pBRS-20 clone.

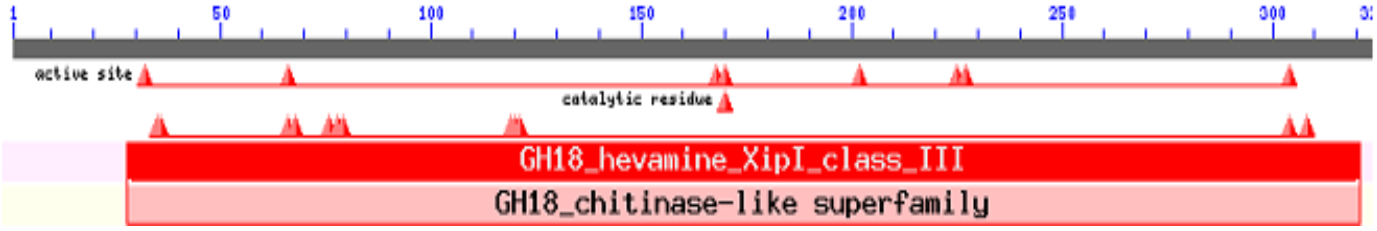

Figure 4. rps BLAST results of cloned endochitinase gene (*ech33*) sequences from *T. harzanium* (IABT1068) showing conserved domain. Description: Cd02877, GH8_hevamine_Ipl_III, this conserved domain family include xylanase inhibitor Ip-I and the class III plant 119356 no.

MPSLTALASALALVPSVFAGWNVNSKQNIAVYWGKALLMSFYTIYIRENSYLT*HLYRTKLGRPAKHATASFNLLQRYVSALFPNTASF
DRPK*EKEHVESDSCHVDANINVIDIAFLNGITPPMTNFANAGDRCTPFSDNPWLSQCPEIE*VSLYEEMGV*CVCKYGLTLIHFKGRISR
LARLTARPSSFLLVVTLTPKVAGALPALLKPQPTRSGPCSVPFSPAALPSVRLAVQSWTALISTLRPRPTTSLLSAPSSRASPTLPGG
KKYYFSAAPQCFFPDAAVGALINAVPMDWIQIQFYNNPCGVSGYTPGTSSQNNYNYQTWDTWAKTSPNPNVKLLVGIPAGPGAGRG
YVSGSQLTSVFQYSKGFSSTFAGAMMWDMSQLYQNTGFEAQVVNALR*

Legend
Signal peptide.....1-19

Figure 5. Deduced amino acid sequences of cloned endochitinase gene (*ech33*) from *T. harzanium* (IABT1068).

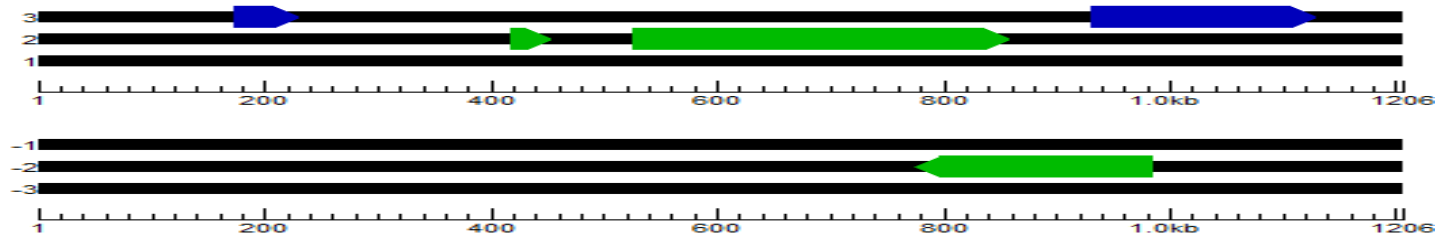

Figure 6. Exon map of cloned endochitinase gene (*ech33*) from *T. harzanium (*IABT1068).

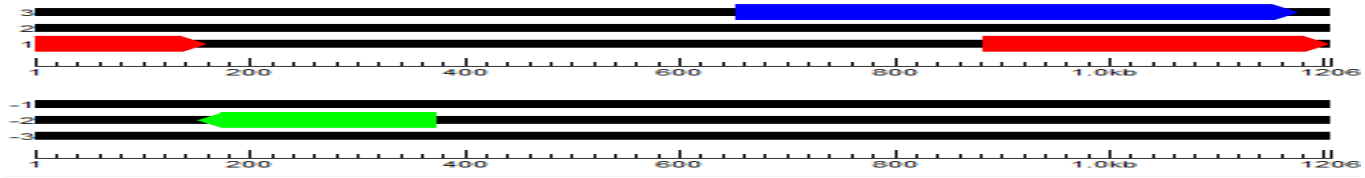

Figure 7. ORF of cloned endochitinase gene (*ech33*) from *T. harzanium* (IABT1068).

```
                                    1                                                  50
Test_Chi_33_Assembled         (1)   ATGCCTTCATTGACTGCTCTTGCGAGCGCGCTCGCTCTTGTTCCTTCCGT
          Test_Chi_Ref        (1)   ATGCCTTCATTGACTGCTCTTGCGAGCGCGCTCGCTCTTGTTCCTTCCGT
             Consensus        (1)   ATGCCTTCATTGACTGCTCTTGCGAGCGCGCTCGCTCTTGTTCCTTCCGT
                                    51                                                 100
Test_Chi_33_Assembled        (51)   CTTTGCTGGCTGGAATGTTAACTCGAAGCAAAACATTGCTGTGTACTGGG
          Test_Chi_Ref        (51)   CTTTGCTGGCTGGAATGTTAACTCGAAGCAAAACATTGCTGTGTACTGGG
             Consensus        (51)   CTTTGCTGGCTGGAATGTTAACTCGAAGCAAAACATTGCTGTGTACTGGG
                                    101                                                150
Test_Chi_33_Assembled       (101)   GTAAGGCCCTTCTTATGAGCTTTTACACAATTTATATAAGGGAAAATAGC
          Test_Chi_Ref      (101)   GTAAGGCCCTTCTTATGAGCTTTTACACAATTTATATAAGGGAAAATAGC
             Consensus      (101)   GTAAGGCCCTTCTTATGAGCTTTTACACAATTTATATAAGGGAAAATAGC
                                    151                                                200
Test_Chi_33_Assembled       (151)   TATCTAACATGACATCTGTATAGGACAAAACTCGGCCGGCCAGCAAAGCA
          Test_Chi_Ref      (151)   TATCTAACATGACATCTGTATAGGACAAAACTCGGCCGGCCAGCAAAGCA
             Consensus      (151)   TATCTAACATGACATCTGTATAGGACAAAACTCGGCCGGCCAGCAAAGCA
                                    201                                                250
Test_Chi_33_Assembled       (201)   CGCAACAGCGTCTTTCAACCTACTGCAGCGGTACGTTTCTGCTTTATTTC
          Test_Chi_Ref      (201)   CGCAACAGCGTCTTTCAACCTACTGCAGCGGTACGTTTCTGCTTTATTTC
             Consensus      (201)   CGCAACAGCGTCTTTCAACCTACTGCAGCGGTACGTTTCTGCTTTATTTC
                                    251                                                300
Test_Chi_33_Assembled       (251)   CCAACACTGCAAGCTTTGATCGGCCAAAATGAGAAAAAGAGCATGTTGAA
          Test_Chi_Ref      (251)   CCAACACTGCAAGCTTTGATCGGCCAAAATGAGAAAAAGAGCATGTTGAA
             Consensus      (251)   CCAACACTGCAAGCTTTGATCGGCCAAAATGAGAAAAAGAGCATGTTGAA
                                    301                                                350
Test_Chi_33_Assembled       (301)   TCTGACAGCTGCCATGTAGATGCCAATATTAATGTTATTGACATTGCTTT
          Test_Chi_Ref      (301)   TCTGACAGCTGCCATGTAGATGCCAATATTAATGTTATTGACATTGCTT-
             Consensus      (301)   TCTGACAGCTGCCATGTAGATGCCAATATTAATGTTATTGACATTGCTT
                                    351                                                400
Test_Chi_33_Assembled       (351)   CTTGAACGGAATTACTCCTCCCATGACCAACTTTGCCAATGCCGGTGACC
          Test_Chi_Ref      (350)   --TAAACGGAATCACTCCTCCCATGACCAACTTTGCCAATGCTGGTGACC
             Consensus      (351)     T AACGGAAT ACTCCTCCCATGACCAACTTTGCCAATGC GGTGACC
                                    401                                                450
Test_Chi_33_Assembled       (401)   GATGCACGCCCTTTTCAGACAACCCTTGGCTCTCGCAATGCCCCGAAATT
          Test_Chi_Ref      (398)   GATGCACTCCCTTCTCCGACAACCCTTGGCTCCTGAGCTGCCCTGAAATT
             Consensus      (401)   GATGCAC CCCTT TC GACAACCCTTGGCTC  G   TGCCC GAAATT
                                    451                                                500
Test_Chi_33_Assembled       (451)   GAGTAAGTTTCTCTATATGAAGAAATGGGTGTCTGATGTGTATGCAAATA
          Test_Chi_Ref      (448)   GAGTAAGTTTCTCTATATGAAGAAATGGGTGTCTGATGTGTATGCAAATA
             Consensus      (451)   GAGTAAGTTTCTCTATATGAAGAAATGGGTGTCTGATGTGTATGCAAATA
                                    501                                                550
Test_Chi_33_Assembled       (501)   TGGACTAACTTTAATTCATTTTAAAGGGCGGATATCAAGACTTGCCAGGC
          Test_Chi_Ref      (498)   TGGACTAACTTTAATTCATTTTAAAGGGCGGATATCAAGACTTGCCAGGC
             Consensus      (501)   TGGACTAACTTTAATTCATTTTAAAGGGCGGATATCAAGACTTGCCAGGC
                                    551                                                600
Test_Chi_33_Assembled       (551)   TAACGGCAAGACCATCCTCCTTTCTCTTGGTGGTGACTCTTACACCCAAG
          Test_Chi_Ref      (548)   TAACGGCAAGACCATCCTCCTTTCTCTTGGTGGTGACTCTTACACCCAAG
             Consensus      (551)   TAACGGCAAGACCATCCTCCTTTCTCTTGGTGGTGACTCTTACACCCAAG
                                    601                                                650
Test_Chi_33_Assembled       (601)   GTGGCTGGAGCTCTGCCAGCGCTGCTCAAGCCGCAGCCAACCAGGTCTGG
          Test_Chi_Ref      (598)   GTGGCTGGAGCTCTGCCAGCGCTGCTCAAGCCGCAGCCAACCAGGTCTGG
             Consensus      (601)   GTGGCTGGAGCTCTGCCAGCGCTGCTCAAGCCGCAGCCAACCAGGTCTGG
                                    651                                                700
Test_Chi_33_Assembled       (651)   GCCATGTTCGGTCCCGTTCAGTCCGGCAGCTCTGCCGAGCGTCCGTTTGG
          Test_Chi_Ref      (648)   GCCATGTTCGGTCCCGTTCAGTCCGGCAGCTCTGCCGAGCGTCCGTTTGG
             Consensus      (651)   GCCATGTTCGGTCCCGTTCAGTCCGGCAGCTCTGCCGAGCGTCCGTTTGG
```

Figure 8. Nucleotide alignment of *ech*33 reference with cloned endochitinase gene (*ech*33) sequences from *T. harzanium* (IABT1068).

```
                                    701                                              750
Test_Chi_33_Assembled     (701)     CAGTGCAATCGTGGACGGCTTTGATTTCGACTTTGAGGCCACGACCAACA
          Test_Chi_Ref    (698)     CAGTGCAATCGTGGACGGCTTTGATTTCGACTTTGAGGCCACGACCAACA
             Consensus    (701)     CAGTGCAATCGTGGACGGCTTTGATTTCGACTTTGAGGCCACGACCAACA
                                    751                                              800
Test_Chi_33_Assembled     (751)     ACCTCGCTGCTTTCGGCGCCCAGCTCAAGAGCCTCTCCAACGCTGCCCGG
          Test_Chi_Ref    (748)     ACCTCGCTGCTTTCGGCGCCCAGCTCAAGAGCCTCTCCAACGCTGCC-GG
             Consensus    (751)     ACCTCGCTGCTTTCGGCGCCCAGCTCAAGAGCCTCTCCAACGCTGCC GG
                                    801                                              850
Test_Chi_33_Assembled     (801)     CGGCAAGAAGTACTACTTCTCTGCTGCTCCTCAGTGCTTCTTCCCAGACG
          Test_Chi_Ref    (797)     CGGCAAGAAGTACTACTTCTCTGCTGCTCCTCAGTGCTTCTTCCCAGACG
             Consensus    (801)     CGGCAAGAAGTACTACTTCTCTGCTGCTCCTCAGTGCTTCTTCCCAGACG
                                    851                                              900
Test_Chi_33_Assembled     (851)     CCGCTGTCGGTGCGCTGATCAACGCCGTCCCCATGGACTGGATCCAGATT
          Test_Chi_Ref    (847)     CCGCTGTCGGTGCGCTGATCAACGCCGTCCCCATGGACTGGATCCAGATT
             Consensus    (851)     CCGCTGTCGGTGCGCTGATCAACGCCGTCCCCATGGACTGGATCCAGATT
                                    901                                              950
Test_Chi_33_Assembled     (901)     CAGTTCTACAACAATCCTTGCGGCGTCAGTGGCTACACGCCCGGCACCAG
          Test_Chi_Ref    (897)     CAGTTCTACAACAATCCTTGCGGCGTCAGTGGCTACACGCCCGGCACCAG
             Consensus    (901)     CAGTTCTACAACAATCCTTGCGGCGTCAGTGGCTACACGCCCGGCACCAG
                                    951                                             1000
Test_Chi_33_Assembled     (951)     CAGCCAGAACAACTACAACTACCAGACCTGGGATACCTGGGCCAAGACGA
          Test_Chi_Ref    (947)     CAGCCAGAACAACTACAACTACCAGACCTGGGATACCTGGGCCAAGACGA
             Consensus    (951)     CAGCCAGAACAACTACAACTACCAGACCTGGGATACCTGGGCCAAGACGA
                                    1001                                            1050
Test_Chi_33_Assembled     (1001)    GCCCCAACCCCAACGTCAAGCTTCTTGTCGGCATTCCCGCTGGCCCAGGT
          Test_Chi_Ref    (997)     GCCCCAACCCCAACGTCAAGCTTCTTGTCGGCATTCCCGCTGGCCCAGGT
             Consensus    (1001)    GCCCCAACCCCAACGTCAAGCTTCTTGTCGGCATTCCCGCTGGCCCAGGT
                                    1051                                            1100
Test_Chi_33_Assembled     (1051)    GCTGGTCGCGGCTACGTCTCTGGCTCTCAGCTCACTTCAGTCTTCCAGTA
          Test_Chi_Ref    (1047)    GCTGGTCGCGGCTACGTCTCTGGCTCTCAGCTCACTTCAGTCTTCCAGTA
             Consensus    (1051)    GCTGGTCGCGGCTACGTCTCTGGCTCTCAGCTCACTTCAGTCTTCCAGTA
                                    1101                                            1150
Test_Chi_33_Assembled     (1101)    CTCGAAGGGGTTCAGCAGCACCTTTGCCGGTGCCATGATGTGGGATATGT
          Test_Chi_Ref    (1097)    CTCGAAGGGGTTCAGCAGCACCTTTGCCGGTGCCATGATGTGGGATATGT
             Consensus    (1101)    CTCGAAGGGGTTCAGCAGCACCTTTGCCGGTGCCATGATGTGGGATATGT
                                    1151                                            1200
Test_Chi_33_Assembled     (1151)    CCCAGCTTTACCAGAACACTGGCTTTGAGGCCCAGGTTGTCAATGCTTTG
          Test_Chi_Ref    (1147)    CCCAGCTTTACCAGAACACTGGCTTTGAGGCCCAGGTTGTCAATGCTTTG
             Consensus    (1151)    CCCAGCTTTACCAGAACACTGGCTTTGAGGCCCAGGTTGTCAATGCTTTG
                                    1201
Test_Chi_33_Assembled     (1201)    AGGTAA
          Test_Chi_Ref    (1197)    AGGTAA
             Consensus    (1201)    AGGTAA
```

Figure 8. Continues.

```
                                    1                                                50
Test_Chi_33_Assembled     (1)       MTNFANAGDRCTPFSDNPWLSQCPEIDSRASPTLP---------------
     Test_Chi_33_Ref      (1)       MTNFANAGDRCTPFSDNPWLLSCPEIEADIKTCQANGKTILLSLGGDSYT
           Consensus      (1)       MTNFANAGDRCTPFSDNPWL  CPEIDA
                                    51                                              100
Test_Chi_33_Assembled     (36)      --------------------------------------------------
     Test_Chi_33_Ref      (51)      QGGWSSASAAQAAANQVWAMFGPVQSGSSAERPFGSAIVDGFDFDFEATT
           Consensus      (51)
                                    101                                             150
Test_Chi_33_Assembled     (36)      -----------------GGKKYYFSAAPQCFFPDAAVGALINAVPMDWIQ
     Test_Chi_33_Ref      (101)     NNLAAFGAQLKSLSNAAGGKKYYFSAAPQCFFPDAAVGALINAVPMDWIQ
           Consensus      (101)                      GGKKYYFSAAPQCFFPDAAVGALINAVPMDWIQ
                                    151                                             200
Test_Chi_33_Assembled     (69)      IQFYNNPCGVSGYTPGTSSQNNYNYQTWDTWAKTSPNPNVKLLVGIPAGP
     Test_Chi_33_Ref      (151)     IQFYNNPCGVSGYTPGTSSQNNYNYQTWDTWAKTSPNPNVKLLVGIPAGP
           Consensus      (151)     IQFYNNPCGVSGYTPGTSSQNNYNYQTWDTWAKTSPNPNVKLLVGIPAGP
                                    201                                             250
Test_Chi_33_Assembled     (119)     GAGRGYVSGSQLTSVFQYSKGFSSTFAGAMMWDMSQLYQNTGFEAQVVNA
     Test_Chi_33_Ref      (201)     GAGRGYVSGSQLTSVFQYSKGFSSTFAGAMMWDMSQLYQNTGFEAQVVNA
           Consensus      (201)     GAGRGYVSGSQLTSVFQYSKGFSSTFAGAMMWDMSQLYQNTGFEAQVVNA
                                    251
Test_Chi_33_Assembled     (169)     LR
     Test_Chi_33_Ref      (251)     LR
           Consensus      (251)     LR
```

Figure 9. Amino acid alignment of *ech*33 reference with cloned endochitinase gene (*ech*33) sequences from *T. harzanium* (IABT1068).

(*chi*33) (FJ358733.1) at nucleotide level and 95% at amino acid level, respectively. Similarly, 95% homology was observed in novel cloned *cry1le1* gene in *B. thuringiensis* (Song et al., 2003). The amino acid sequence of cloned *ech*33 differed from the amino acid sequences of *ech*33 used as a reference at two position including, 185th (D changed to R) and 187th (S changed to A) positions and the changes were observed in critical regions. Similarly, remarkable changes in two positions were found in *T. harzanium chi*42 gene (Kuranda and Robbins, 1991).

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