

Full Length Research Paper

Isolation and cloning of *ech36* gene from *Trichoderma harzanium*

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A lab experiment was conducted to screen for the presence of *ech36* gene in 80 isolates of *Trichoderma*. Further, using gene specific primers *ech36* gene were cloned into pTZ57R/T from *Trichoderma harzanium* IABT1042. The clone was confirmed through PCR amplification and restriction analysis. The clone were sequenced and analyzed for homology at nucleotide and protein level to find out conserved domains of the putative gene and protein. The gene encoding endochitinase from the species have 99 and 100% homology with reported sequences both at nucleotide and protein level. The cloned *ech36* has a size of 1212 bp, of which 12 bp corresponds to the 3' untranslated region, with a 1030 bp open reading frame. The amino acid sequence of gene has signal peptide sequence ranges from 1 to 25. The nucleotide sequence analysis using GENETOOL software revealed presence of three exon and introns and has unique restriction sites for *HindIII*, *BglIII*, and *HaeIII* at 908, 387 and 672 positions respectively

Key words: *Trichoderma harzanium*, *ech36*, protein.

INTRODUCTION

The present day world is facing various problems regarding food security. The traditional agriculture is affected by various problems such as drought, pest and diseases, reduced availability of the land, increase in population. Among them, pests and diseases cause major losses. Indiscriminate use of pesticide and 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. Therefore, efforts have been made to breed resistant cultivars and to develop biocontrol agents (BCA) for the control of various fungal plant pathogens. *Trichoderma* spp. is a well known BCA against plant diseases. One of the most promising means

to achieve this goal is by the use of new tools based on BCA 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). Species of the genus *Trichoderma* are widely known for their biotechnological interest; however their use as bio-control agents requires a comprehensive analysis of the biological principles of their action. Their antagonistic abilities are described as a combination of several mechanisms, including nutrient competition and direct mycoparasitism, which involves the production of antifungal metabolites and cell wall-degrading enzymes (Vieira et al., 2013). These fungi are opportunistic, avirulent plant symbionts, and function as parasites and antagonists of many phytopathogenic

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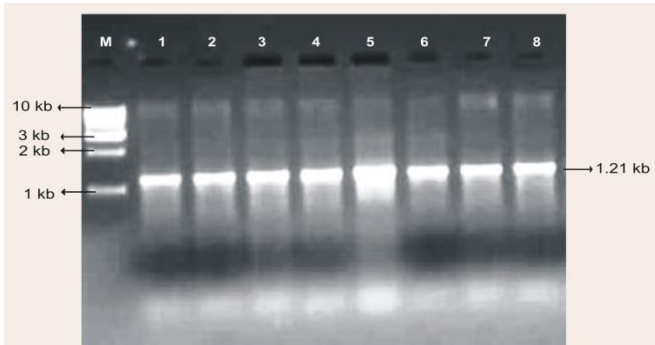


Figure 1. PCR amplification of *ech36* gene. M, 1 kb ladder; 1, *T. virens* (IABT 1010); 2, *T. viride* (IABT 1012); 3, *T. viride* (IABT 1013) 4 – *T. viride* (IABT 1014); 5, *T. virens* (IABT 1017); 6, *T. harzianum* (IABT 1042); 7, *T. viride* (IABT 1021); 8, *T. viride* (IABT 1022).

fungi, thus protecting plants from diseases. So far, *Trichoderma* spp. are the most studied fungal BCA and commercially marketed as biopesticides, biofertilizers and soil amendments (Harman et al., 2004; Lorito et al., 2004). Depending upon the strain, *Trichoderma* spp. (notably *H. lixii*/*T. harzianum*, *H. virens*/*T. virens*, *T. atroviridis*/*T. atroviride* and *T. 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., 1996), 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 glucanases (Lorito, 1998) and is being exploited to control a variety of plant pathogens. Several chitinases and glucanases are isolated from *Trichoderma* and transferred to plants to impart 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 β -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., 1996). 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 (*ech33*) from the biocontrol fungus *T.*

atroviride increased the resistance to sheath blight caused by *Rhizoctonia solani* and rice blast caused by *Magnaporthe grisea* (Liu et al., 2004). The identification of the *Trichoderma* genes involved in these mechanisms and analysis of their expression profiles can provide researchers with biotechnological tools that exhibit anti-fungal activity and that could potentially be used as transgenes capable of inducing resistance to pathogens in economically valuable plants (Vieira et al., 2013). The present study is aim 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 defenses.

MATERIALS AND METHODS

Isolation of genomic DNA from fungus

For isolation and cloning of gene 80 *Trichoderma* isolates were inoculated in 100 ml potato dextrose broths at 30°C in incubator. The complete growth occurred in 2 to 5 days depending on the species. About 100 mg of fungus mycelium was taken in 1.5 ml micro centrifuge tube and 500 μ l of lysis buffer (400 mM Tris-HCl [pH 8.0], 60 mM EDTA [pH 8.0], 150 mM NaCl, 1% sodium dodecyl sulfate), was added. Mycelium was finely macerated using micro-pestle and vortexed for 5 min. The suspension was extracted with equal volume of phenol: chloroform: indole acetic acid (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 μ g 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 50 μ l of TE (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).

PCR amplification

PCR was carried out from *Trichoderma* genomic DNA. The *ech36* gene sequence was downloaded from NCBI gene bank and primers were designed using online software primer-3. PCR amplification using two gene specific primer were used- 5'ATGACACGCCTCCTCGAC3' and RP 3'TACCCATATTCATTAAGGCTATCAA5'. Reaction mixture for PCR (20 μ l) 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 μ l 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 ~1.12 kb amplicon (Figure 1).

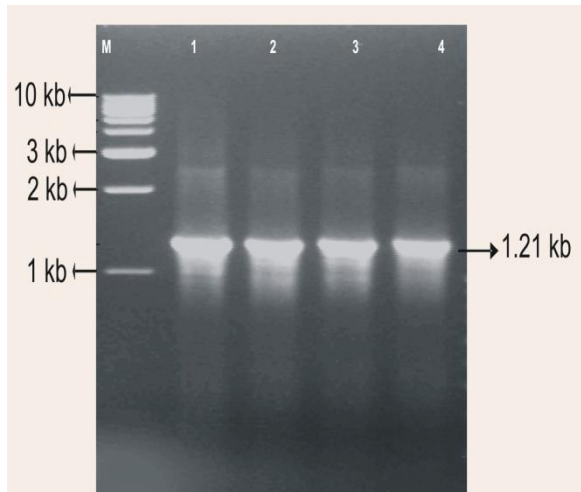


Figure 2. PCR confirmation of *ech36* cloned gene. M = 1KB DNA Ladder; 1. *T. Harzianum*; 2. *T. Harzianum*; 3. *T. Harzianum*.

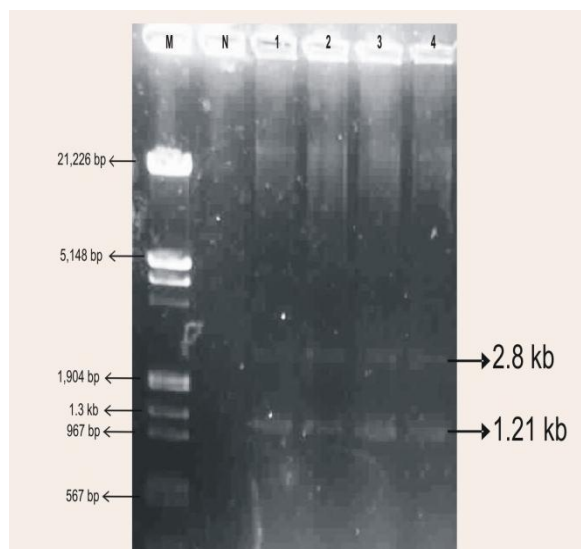


Figure 3. Restriction confirmation of *ech36* gene. M = λ EcoRI/HindIII double digest Ladder; 1. *T. Harzianum*; 2. *T. Harzianum*; 3. *T. Harzianum*; 4. *T. Harzianum*

Cloning of endochitinase gene

The specific size of band was eluted using elution kit (Bangore geni India). The bands (~1.12 kb) corresponding to *ech36* from *T. harzianum* IABT1042, were ligated to pTZ57R/T vector (2886 bp) as described in Inst/A clone™ PCR product cloning kit (#k1214) from MBI Fermentas USA. The ligation products were used to transform *E. coli* DH5 α .

Transformation of *E. coli* DH5 α with recombinant construct

The competent cells of *E. coli* DH5 α were prepared following the

protocol mentioned by Sambrook and Russell (2001). About 100 μ l of freshly prepared competent cells were taken in a chilled centrifuge tube and 10 μ l 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 μ l 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 the plasmid. The culture was centrifuged at 13,000 rpm for 1 min and about 700 μ l of supernatant was discarded and the pellet was dissolved in remaining supernatant and spread on the plates having Luria agar with Amp¹⁰⁰, X-gal IPTG and incubated overnight at 37°C.

The recombinant clones were identified by blue/white assay (Sambrook and Russel, 2001). After incubation only white colonies, were picked up and streaked on plates having Luria agar with Amp¹⁰⁰, 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 plasmids were sequenced using M13 universal forward and reverse 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 silico translation was done using GENETOOL software. Dual and multiple alignments for homology search were performed using the Clustal 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, 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 Apweiler2001; <http://www.ebi.ac.uk/InterProScan/>).

RESULTS

Among the 80 *Trichoderma* isolates only 72 isolates gave amplification of *ech36* gene. Based on previous pathogenicity bioassay in our laboratory on *Rhizoctani solani* the efficient strain *T. harzianum* (IABT1042) was selected for cloning. The cloned *ech36* gene from *T. harzianum* (IABT1042) 12 colonies were observed on selection medium of which 4 were white. Further, these colonies were screened for the presence of *ech36* and only three clones showed the presence of ~1.12 kb insert when checked through PCR with specific primers and restriction analysis (Figures 2 and 3).

One of the clones corresponding to *ech36* was named as pBRS-21. The clone was sequenced using M13 forward and reverse primers at Bangalore Genie Pvt. Ltd. The complete sequence of nucleotides (Figure 4) was found after removing vector sequence through vecscreen service of the NCBI website. The available sequence information from cloned gene was subjected to analysis

ATGACACGCCTCCTCGACGCCAGCTTTCTGCTGCTGCCTGCTATCGCATCGACGCTAT
 TTGGCACCGCCTCTGCACAGAATGCGATATGCGCACTTAAGGGAAAAGCCGGCTGGCA
 AAGTCTTGATGGGATATTGGGAAAATTGGGATGGAGCAGCCAACGGTGTTCACCTG
 GATTTGGTTGGACACCAATCGAAAACCCCATCATTAAACAGAATGGCTACAATGTGA
 TCAACGCCGCCTTCCCCGTTATTCTGTCAGATGGCACAGCGTTATGGGAAAACGACA
 TGGCTCCTGACACACAAGTCGCAACTCCAGCTGAAATGTGTGAGGCTAAAGCAGCTG
 GAGCCACAATTCTGCTGTCAATTGGAGGTGCTACTGCTGGCATAGATCTCAGCTCCA
 GTGCAGTCGCTGACAAGTTCATCGCCACCATTGTACCAATCTTGAAGCAGTACAATTI
 TGACGGCATTGATATAGACATTGAGACGGGGTTGACCAACAGCGGTAATATCAACAC
 ACTCTCCACATCCCAGACCAACTTGATTCGCATCATTGATGGTGTCTTGCTCAGATG
 CCTTCCAACCTTCGGCTTGAATGACCTGAGACAGCGTACGTTACAGGCGGTAGC
 ATCACGTATGGCTCTATTTGGGGAGCGTACCTACCTATCATCCAGAAATATGTTCAA
 ACGGCCGGCTGTGGTGGCTAAACATGCAATATTACAACGGCGACATGTACGGTTGCT
 CTGGCGACTCTTACGCAGCTGGCACTGTCCAAGGATTCATCGCTCAGACTGACTGCC
 AAATGCAGGACTTACCGTCCAAGGCACCACAATCAAGGTTCCGTACGACATGCAAGT
 ACCAGGTCTACCTGCGCAATCAGGAGCTGGCGGTGGTTATATGAATCCAAGCTTGGT
 TGGACAAGCATGGGATCACTACAACGGTGCTCTGAAAGGCTTGATGACGTGGTCAAT
 CAACTGGGATGGAGCGGGTAACTGGACATTTGGCGACAATTTGCTCACTCGCATTGG
 CTAGAAGAAGATGACGGGAGGAAGATTAGGCGTTGAAGTCAATATATTTCTTGATTT
 CATACCAGCGATATGCCATGAATTAGTCAATGATATATATACCTTCCAGCAGTATAT
 GTTGGACTGACTATTTAGTCTATGCACATAAAAACTAGCTTGATAGCCTTTAATGAA
 TATGGGTA

Figure 4. Complete nucleotide sequence of cloned endochitinase gene (*ech36*) from *T. harzanium* (IABT1042). 5'UTR- 0; 3'UTR- 12; Exon-----1-1035; Introns----1036-1112.

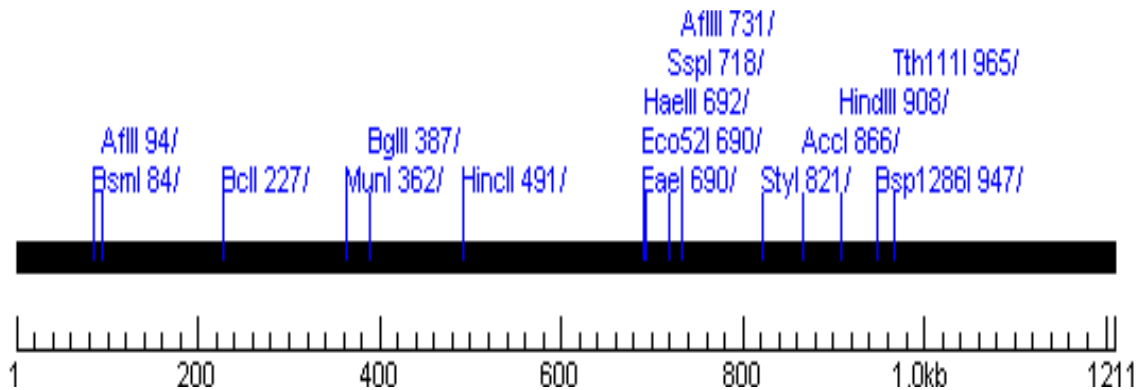


Figure 5. Restriction map of cloned endochitinase gene (*ech36*) sequences from *T. harzanium* (IABT1042) with common enzymes.

using BLAST algorithm available at <http://www.ncbi.nlm.nih.gov>. It showed homology with conserved domain of CHI-18, chitinase like superfamily (Figure 7). The nucleotide sequence of *ech36* showed 99% homology with the published *Trichoderma harzianum* endochitinase Chit36Y (chit36Y) gene, complete cds (AF406791.1), and 96% with *Trichoderma asperellum* chitinase mRNA, complete cds (DQ663089.1). The cloned *ech36* has a size of 1212 bp, of which 12 bp corresponds to the 3' untranslated region, with open reading frame present in the DNA (Figure 10). The nucleotide sequence

was translated to amino acid using GENETOOL software and code for 258 amino acids. The amino acid sequence of gene is shown in Figure 8. It has signal peptide sequence ranges from 1 to 25. The nucleotide sequence analysis using GENETOOL software revealed presence of three exon and introns (Figure 9). Nucleotide alignment of *ech36* reference with cloned endochitinase gene (*ech36*) sequences from *T. harzanium* (IABT1042) was done and shown in Figure 11. Similarly the amino acid alignment was also done and sequence is shown in Figure 12.

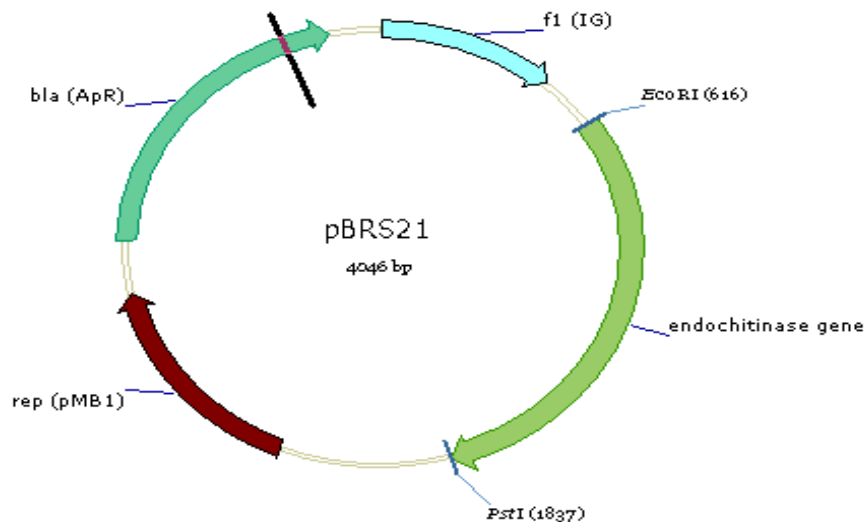


Figure 6. Construct map of pBRS-21 clone.

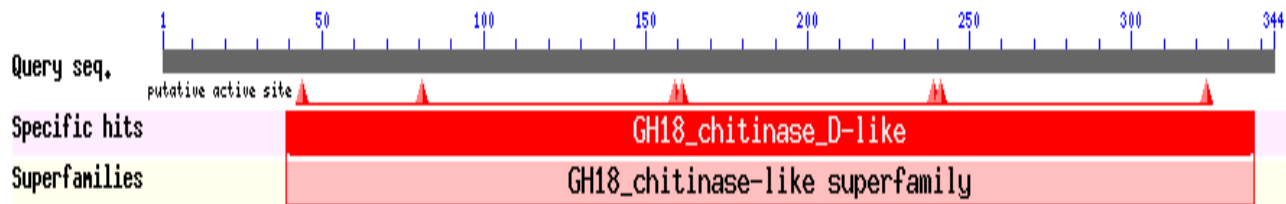


Figure 7. rps BLAST results of cloned endochitinase gene (*ech36*) sequences from *T. harzanium* (IABT1042) showing conserved domain. Cd02871, GH18_chitinase_D-like, GH18 domain of Chitinase D (ChiD). ChiD, a chitinase found in *Bacillus circulans* hydrolyzes the 1, 4-beta-linkages of N-acetylglucosamine.

MTRLLDASFLLLPAAIASTLFGTASAQNAICALKKGKPAQKVLMDGYWENWDGAANGVHPGFGWTPIE
 NPPIKQNGYNVINAAFPVILSDGTALWENDMAPDTQVATPAEMCEAKAAGAT**TILLSIGG**ATAGID
 LSSSAVADKFIATIVPILKQYN**FDGIDIDIE**TGLTNSGNINTLSTSQTNLIRIIDGVLAQMPSNF
 GLTMAPETAYVTGGSITYGSIWGAYLPPIIQKYVQNGRLWNLNMQYYNGDMYGCSDSYAAGTVQG
 FIAQTDCLNAGLTVQGTTIKVPYDMQVPGLPAQSGAGGGYMNPSLVGQAWDHYNGALKGLMTWSI
 NWDGAGNWTFGDNLLTRIGKMTGGRLLGVEVNIFLDFIPAICHELVNDIYTFQOYIVGLTISMHI
 KNLDSLIWV

Figure 8. Deduced amino acid sequences of cloned endochitinase gene (*ech36*) from *T. harzanium* (IABT1042). Signal peptide.....1-25; Chitinase family active site-79-87; Chitin binding domain.....97-106

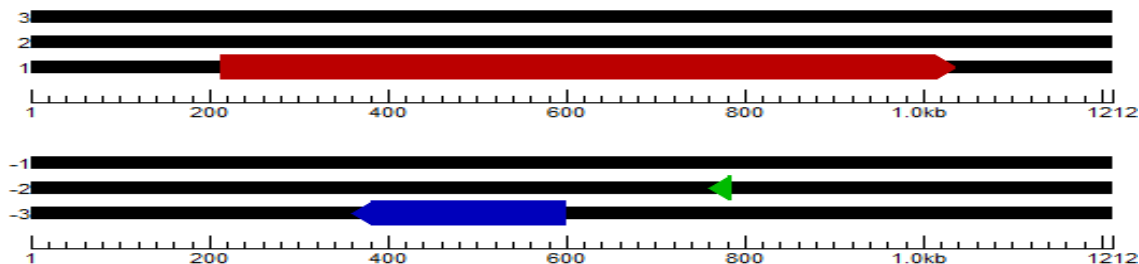


Figure 9. Exon map of cloned endochitinase gene (*ech36*) from *T. harzanium* (IABT1042).

		1		50
Translation of AF406791	(1)	TRLLDASFLLLPAIASTLFGTASAQNA	T	CALKGKPKAGKVLMGYWENWDG
Translation of Assembled	(1)	TRLLDASFLLLPAIASTLFGTASAQNA	I	CALKGKPKAGKVLMGYWENWDG
Consensus	(1)	MTRLLDASFLLLPAIASTLFGTASAQNA		ALKGKPKAGKVLMGYWENWDG
		51		100
Translation of AF406791	(51)	ANGVHPGFGWTPPIENPIIKQNGYNVINAAPVILSDGTALWENDMAPDT		
Translation of Assembled	(51)	ANGVHPGFGWTPPIENPIIKQNGYNVINAAPVILSDGTALWENDMAPDT		
Consensus	(51)	ANGVHPGFGWTPPIENPIIKQNGYNVINAAPVILSDGTALWENDMAPDT		
		101		150
Translation of AF406791	(101)	VATPAEMCEAKAAGATILLSIGGATAGIDLSSAVADKFIATIVPILKQ		
Translation of Assembled	(101)	VATPAEMCEAKAAGATILLSIGGATAGIDLSSAVADKFIATIVPILKQ		
Consensus	(101)	VATPAEMCEAKAAGATILLSIGGATAGIDLSSAVADKFIATIVPILKQ		
		151		200
Translation of AF406791	(151)	NFDGIDIDIETGLTNSGNINTLSTSQTNLIRIIDGVLAQMPNFGTMA		
Translation of Assembled	(151)	NFDGIDIDIETGLTNSGNINTLSTSQTNLIRIIDGVLAQMPNFGTMA		
Consensus	(151)	YNFDGIDIDIETGLTNSGNINTLSTSQTNLIRIIDGVLAQMPNFGTMA		
		201		250
Translation of AF406791	(201)	ETAYVTGGSITYGSIWGAYLPPIQKYVQNGRLWLNMQYYNGDMYGCSG		
Translation of Assembled	(201)	ETAYVTGGSITYGSIWGAYLPPIQKYVQNGRLWLNMQYYNGDMYGCSG		
Consensus	(201)	ETAYVTGGSITYGSIWGAYLPPIQKYVQNGRLWLNMQYYNGDMYGCSG		
		251		300
Translation of AF406791	(251)	SYAAGTVQGFIAQTDCLNAGLTVQGTTIKVPYDMQVPLPAQSGAGGGY		
Translation of Assembled	(251)	DSYAAGTVQDSSLRLTA-----		
Consensus	(251)	DSYAAGTVQ		
		301		345
Translation of AF406791	(301)	MNPSLVQAWDHYNGALKGLMTWSINWDGAGNWTLDGNLLTRIG-		
Translation of Assembled	(268)	-----		
Consensus	(301)			

Figure 12. Amino acid alignment of *ech36* reference with cloned endochitinase gene (*ech36*) sequences from *T. harzianum* (IABT1042).

The cloned *ech36* in Pbrs-21 has unique restriction sites for *Hind*III, *Bgl*II, and *Hae*III at 908, 387, and 692 positions, respectively. The restriction map of the sequence (pBRS-21) is presented in Figure 5. The vector map of pBRS-21 was constructed using the software VECTOR NTI and is presented in Figure 6. The nucleotide sequence of the cloned *ech36* was subjected for BLASTx, and showed 100% homology with the published *T. harzianum* endochitinase Chit36Y (chit36Y) gene, complete cds (AF406791.1) gene at amino acid levels.

DISCUSSION

The development of resistant traits against pathogen become an important target in plant biotechnology, since the traditional approaches to control of epidemic spread of diseases are no longer sufficient. Successful approaches

for enhancing disease resistance of plant were based on the over expression of genes encoding protein that are produced during the natural defense responses of plants. This includes PR proteins of which chitinase is the most important and widely studied one. Thus cloning and characterization of chitinase genes is the first step in development of transgenics resistant to fungal diseases. Therefore, cloning of endochitinase genes and transferring it to plants is a major steps for development of transgenic against resistant to plant pathogen. So in the present study, for the cloning of endochitinase gene from *ech36* from *T. harzianum* (IABT1042) and sequenced functional analysis were done. The *ech36* gene had 99% homology with reported endochitinase gene from *T. harzianum* endochitinase Chit36Y (chit36Y) gene, complete cds (AF406791.1) at nucleotide level, and 100% at amino acid level respectively. Similarly 90% homology was observed in *T. asperellum* and *T. viridae* cloned endochitinase gene (Saiprasad et al., 2009).

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