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Cloning partial endochitinase cDNA of *Trichoderma harzianum* antagonistic to *Colletotrichum falcatum* causing red rot of sugarcane

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The saprophytic fungus, *Trichoderma harzianum* strain T5 antagonistic to the red rot pathogen of sugarcane, *Colletotrichum falcatum* excreted chitinases, viz. N-acetyl-β-D-glucosaminidase, 1,4-β-D-N-N′ chitobiosidase and 1,4-β-D-N-N′-N″ chitotriase into the culture medium containing pathogenic cell wall or chitin. The protein profile analysis on SDS–PAGE stained proteins is in the range of 20–124 kDa. The fungus produced a 97 kDa N-acetyl glucosaminidase in medium amended with fungal cell wall or colloidal chitin. Chitobiase isoforms of 66, 56, and 50 kDa and 66 and 50 kDa were detected in fungal cell wall and colloidal chitin amended media respectively. The fungus also excreted chitotriase isoforms of 66 and 50 kDa in cell wall and 50 kDa in colloidal chitin amended media respectively. The chitinase enzymes of 66 and 50 kDa, which degraded both chitobiose and chitotriose, are isoforms of both chitobiase and chitotriase that got separated at the same distance in SDS–PAGE. In an attempt to clone the endochitinase gene from *T. harzianum* T5, a partial cDNA of 246 bp (Genbank accession number AY762230) was obtained through RT–PCR and the deduced sequence showed high level of homology with chitinase sequences of the database.

Chitin is a significant component in the cell walls of large groups of fungi except members of Oomycetes. Chitin is made up of molecules of N-acetylglucosamine, which are the building blocks linked together by 1,4-β-glycosidic bonds. Chitinases are enzymes that cleave the bond between the C_1 and C_4 of two consecutive N-acetylglucosamines of chitin. Chitinases produced by fungi have been shown to be involved in a variety of functions such as cell-wall digestion, germination of spores, assimilation of chitin and mycoparasitism. Thus chitinases have the potential as effective antifungal agents[1,2]. The chitinolytic system of *Trichoderma* spp. is much diversified. It is obvious that homologues of a 42 kDa endochitinase[3] have often been purified[4], followed by frequent isolation of an N-acetyl-â-D-glucosamine of 70–73 kDa. The gene encoding the 42 kDa endochitinase has high homology to endochitinases from other fungal species[5] and hence is a useful gene for phylogenetic analysis of *Trichoderma*. *T. harzianum* strain T5 isolated from sugarcane rhizosphere displayed antifungal activities against *Colletotrichum falcatum* Went (perfect state: *Glomerella tucumanensis* (Speg.) Arx & Muller), causing red rot in sugarcane[6]. Mycoparasitic fungi provide rich sources of antifungal genes that can be utilized to genetically engineer important crops for resistance against fungal pathogens[1]. We report here detection of three extracellular chitinases of *T. harzianum* produced on *C. falcatum* cell wall and colloidal chitin amended media and cloning its partial endochitinase gene sequence.

T. harzianum strain T5 that has been isolated from sugarcane rhizosphere was grown in a minimal medium containing K_2HPO_4 – 1.5 g; $MgSO_4 \cdot 2H_2O$ – 1.5 g; and cell-wall chitin or colloidal chitin – 1 g/l. Colloidal chitin was prepared by digesting powdered crab shells (Sigma Aldrich, USA) with concentrated HCl overnight at 4°C. Cell-wall chitin was prepared from mycelial mats of *C. falcatum* as described earlier[7], in which mycelia from eight-day-old cultures were homogenized in a homogenizer for 60 s using 5 ml water/g wet weight of mycelia. The homogenate was

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filtered through a coarse sintered glass funnel and the residue obtained on the filter was saved. The residue was homogenized three more times in water, once in a mixture of chloroform and methanol (1 : 1) and finally in acetone. This preparation when air-dried represents the fraction referred to as the mycelial walls. The culture of *T. harzianum* T5 was grown at 30°C for 8 days to extract proteins from the culture filtrate.

Extracellular proteins were purified from culture filtrates by ammonium sulphate precipitation. The chromogenic derivatives, viz. *p*-nitrophenyl-N-acetyl-β-D-glucosaminide 0.5 mM, *p*-nitrophenyl-β-D-N,N′-diacetylchitobiose 0.3 mM and *p*-nitrophenyl-β-D-N,N′,N″ triacetylchitotriose 0.2 mM (Sigma, USA) were used as substrates for determination of N-acetyl-β-D-glucosaminidase, chitobiosidase and chitotriase respectively. Five µl of the enzyme source was incubated with 45 µl of 0.5, 0.3 and 0.2 mM concentrations of respective substrates in 50 µl KPO$_4$ buffer (pH 6.7) at 37°C for 10 min followed by the addition of 50 µl of 0.1 M NaOH to arrest enzyme reaction. The release of the chromatophore, *p*-nitrophenol from the substrates was determined by measuring the absorbance at 420 nm in a microspectrophotometer (Spectramax190, Molecular Devices, USA). Enzyme activity was expressed as pmol of *p*-nitrophenol released per µg protein per min under the specified condition[8].

For determination of chitinolytic activity, 5 µl of the enzyme was incubated with 5 µl of 1% swollen chitin in phosphate buffer, pH 7.0 at 37°C for 10 min. The reducing sugar released was measured by dinitrosalicylic acid method[9] and the amount of monomer released was extrapolated from the standard graph of N-acetylglucosamine. Chitinase activity was expressed as nmol/µg protein/min.

Proteins prepared in sample buffer without mercaptoethanol and incubated for 10 min at room temperature prior to loading were separated by 12% SDS–PAGE. Each lane was loaded with 30 µg of protein. Molecular weight of proteins was estimated as described by Sambrook *et al.*[10]. Enzymes were reactivated in the replica gel by removing SDS by casein-EDTA procedure developed by McGrew and Green[11], and modified by Haran *et al.*[12]. In this procedure, after electrophoresis, the gel was immersed in Buffer A: 1 M Tris HCl, pH 9.0 (40 ml), 20% sodium azide (1 ml), 0.5 M EDTA, pH 8.0 (4 ml) and casein (10 g) per litre. With gentle shaking, the buffer was changed every 30 min, four times. The gel was washed two times with acetate buffer 0.1 M, pH 4.8. Then the gel was overlayed with 4 ml of 1% low melting agarose (Sigma) in 0.1 M acetate buffer (pH 4.8) containing 1.2 mg of fluorescent substrates[13]. The chitinolytic enzymes appeared as fluorescent bands under UV light because of enzymatic hydrolysis of fluorescent 4-methyl umbelliferone from the GlcNAc oligosaccharides. The substrates used were 4-MU-GlcNAc, 4-MU-(GlcNAc)$_2$ and 4-MU-(GlcNAc)$_3$ (Sigma, USA) for detection of N-acetyl-β-D-glucosaminidase, chitobiosidase and chitotriase respectively.

The molecular weight of chitinases was determined using medium range standard proteins (Bangalore Genei, India).

Total RNA was extracted using the TRI Reagent (Sigma) from *T. harzianum* grown in a medium containing cell wall 1 g, colloidal chitin 1 g, molasses 10 ml, MgSO$_4$.2H$_2$O 1.5 g, K$_2$HPO$_4$ 1.5 g/l water. mRNA has been prepared from the total RNA using Genelute mRNA preparation kit (Sigma). The purified mRNA from *T. harzianum* after DNAase I treatment was used for reverse transcription to obtain cDNA. The cDNA synthesis was performed using QIAGEN One-Step RT–PCR kit (Qiagen, Germany) for 30 min at 50°C. The cDNA obtained was PCR-amplified in 30 cycles consisting of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min in a thermocycler (Mastercycler, Eppendorf, Germany) using the primers 5′TCCCARAYHCCR-TTCTCCCA3′ (forward primer) and 5′AAYYTBATGG-CYTAYGACT3′ (reverse primer). The primer sequence was the same as that used for obtaining the endochitinase cDNA (BQ172872) of *T. harzianum* fungus. The ds DNA obtained from cDNA was analysed by electrophoresis on 1.5% agarose gel.

The RT–PCR product was subcloned into pDrive cloning vector (Qiagen) and recombinant plasmids were transformed into QIAGEN EZ competent cells. Positive clones were detected as white colonies in blue-white screening method using X-gal and IPTG (inducer) (Sigma). Plasmids were isolated from white colonies and checked for inserts with the same primers to confirm their specificity to the cloned cDNA product. The amplified product was analysed on 1.5% agarose gel.

For sequencing the partial cDNA of endochitinase, PCR products were obtained using non-fluorescent primers under the same PCR conditions and after treating them with exonuclease I and shrimp alkaline phosphatase (Amersham) at 37 and 80°C (15 min each). Sequencing of the PCR products was carried out using 50 ng (2 µl) of PCR product and 4 pM (1 µl) of non-fluorescent primer (forward and reverse separately), 4 µl of BigDye Terminator ready reaction kit (Perkin Elmer), and 3 µl of milliQ water to adjust the volume to 10 µl. Cycle sequencing was carried out in a GeneAmp9600 thermal cycler (Perkin Elmer) for 30 cycles at 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. Extended products were purified by alcohol precipitation followed by washing with 70% alcohol. Purified samples were dissolved in 10 µl of 50% Hi-Di formamide and analysed in an ABI 3700 automated DNA Analyzer (Perkin Elmer).

Sequences were aligned with the Clustal X version 1.81 program and adjusted by eye with BioEdit version. 3.0. N-terminal and C-terminal sequences as well as other ambiguous alignment positions were removed. Phylogenetic reconstruction of the partial endichitinase cDNA sequence was performed by the neighbour-joining method as implemented[14] in the MEGA software version 4.0 using poison correction and gamma distance ($\alpha = 1.6$, estimated from the dataset). A bootstrapped neighbour-joining tree

was obtained by feeding the NEIGHBOR program (PHYLIP package version 3.5)[15]. A consensus nucleotide maximum parsimony tree was calculated from 1097 trees obtained from 500 bootstrap resamplings feed to the PROTPARS program (PHYLIP version. 3.5) based on 500 bootstrap resamplings.

The following accessions from the database coding for the chitinase gene were used in phylogenetic analysis: AJ563359 (*T. asperellum*), AY665695 (*T. viride*), AY762230 (*H. lixii*), AF188931, AF188918, AF188962 (*H. rufa*), AAQ79796 (*H. minutispora*), AY258898 (*T. hamatum*), AF400747 (*T. minutisporum*), AF486002 (*T. croceum*), AY240220 (*H. pachybasioides*), AF486003 (*H. pilulifera*), AF399275 (*H. strictipilis*), AF399262 (*T. fertile*), AF399273 (*T. oblongisporum*), AY292527 (*V. fungicola*) and AY240219 (*H. pachybasioide*). Most of the sequences used correspond to microbial endochitinases.

In the first assay of dinitrosalicylic acid method, synthesis of more chitinases was recorded in chitin-amended medium than in *C. falcatum* cell wall-amended medium. In the second assay of using three chromogenic substrates, synthesis of all three types of enzymes was recorded and among the three chitinases, N-acetyl-β-D-glucosaminidase production was the highest followed by chitobiosidase and chitotriase in both media (Table 1). SDS–PAGE separation of extracellular proteins showed proteins in the range of 20–124 kDa (Figure 1) in both media. We have standardized detection of chitinase isoforms in sodium dodecyl sulphate–polyacrylamide gel after reactivation by casein-EDTA procedure. In this procedure, activity of chitinase isoforms was detected as discrete flourescent bands under UV light, which diffused after a few moments.
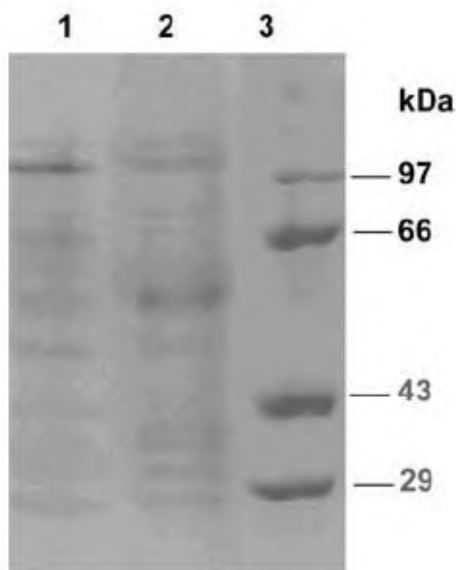

Figure 1. Separation of *Trichoderma harzianum* T5 extracellular proteins on SDS–PAGE. Lane 1, Chitin medium; lane 2, *Colletotrichum falcatum* cell-wall medium; lane 3, Protein marker.

The fungus produced N-acetyl glucosaminidase of 97 kDa both in cell wall and colloidal chitin medium, which was detected in the overlay gel using 4-MU-(GlcNAc) as substrate. Three fluorescent bands with approximate molecular weights of 66, 56 and 50 kDa were detected when grown in cell wall-amended medium and two bands of 66 and 50 kDa in colloidal chitin-amended medium using 4-MU-(GlcNAc)$_2$ as substrate. Also, two fluorescent bands of 66 and 50 kDa in the cell-wall medium and a 50 kDa band in colloidal chitin medium using 4-MU-(GlcNAc)$_3$ as substrate (Figure 2) were detected. These fluorescent bands, on the basis of their molecular weights, overlap to the stained proteins in SDS–PAGE. The chitinase enzymes of 66 and 50 kDa which degraded both chitobiose and chitotriose are supposed to be isoforms of both chitobiase and chitotriase that got separated at the same distance in SDS–PAGE.

Microorganisms synthesize chitinase for utilization of chitin as a source of carbon and nitrogen[16], since chitin can be derived from cellulose by substitution of the hydroxyl group on carbon atom 2 of glucose with an acetylated amino group. Earlier reports[17] indicated that the molecular weight of microbial chitinases ranges from 20 to 120 kDa. The molecular weight of the proteins synthesized by *T. harzianum* T5 on cell-wall chitin or colloidal chitin-amended medium showed proteins in the range of 20–124 kDa. The molecular weight of chitinase of *T. harzianum* Rifai T$_{24}$ was reported[18] as 43 kDa. Alexandre *et al.*[19] have identified N-acetyl-β-D-glucosaminidase of 73 and 68 kDa in two different truncated forms and endochitinase of 45 kDa. Also, N-acetyl-β-D-glucosaminidase of 36 kDa has been purified from *T. harzianum* which controls *Crinipellis perniciosa*[20]. A 33 kDa chitinase Bbchit1 was purified from *Beaveria bassiana*[21]. Hoell *et al.*[22] have isolated a novel endochitinase of 30 kDa from *T. atroviride*. The overlay gel containing fluorescent substrates detected chitinolytic enzymes as fluorescent bands under UV light because of enzymatic hydrolysis of fluorescent 4-methyl umbelliferone from the GlcNAc oligo-
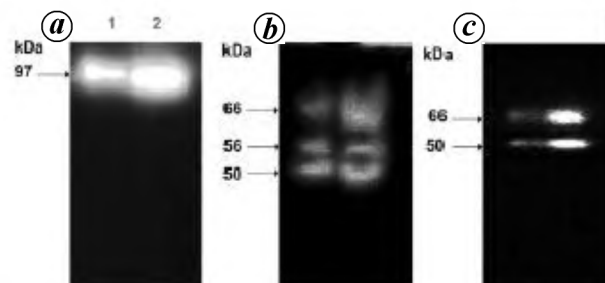

Figure 2. Detection of chitinolytic enzymes in extracellular proteins after separation by SDS–PAGE. Chitinolytic activity was detected with the substrates 4-MU-GlcNAc (*a*), 4-MU-(GlcNAc)$_2$ (*b*) and 4-MU-(GlcNAc)$_3$ (*c*). Lanes 1 and 2, Extracellular proteins from *T. harzianum* T5 grown in chitin medium and *C. falcatum* cell-wall medium respectively.

Table 1. Production of chitinases by *Trichoderma harzianum* T5 on two different media

	Enzyme activity of different chitinases (pmol/µg protein/min)*			
Medium	Chitinase** (nM of GlcNAc/µg protein/min)	N-acetyl β-D-glucosaminidase	Chitin 1,4-β-D-N,N′-chitobiosidase	Chitin-β-N,N′,N″-chitotriase
Cell-wall chitin	1479.00 ± 22	21.82 ± 1.98	20.32 ± 2.42	11.22 ± 1.07
Colloidal chitin	3814.47 ± 34	21.45 ± 1.87	16.87 ± 1.69	9.91 ± 1.02

*Values are mean of three replicates.
**Differences in mean enzyme activity are significant between media at 1% level.

Figure 3. The 246 bp partial cDNA product coding for putative endochitinase gene separated on agarose gel. Lane 1, cDNA product; lane 2, 100 kb DNA ladder.

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gtcacggtctgggacacctgctctgggcaagatgtcaatcttgcttcgactaaaacggtc
 V  T  V  W  D  T  C  S  G  Q  D  V  N  L  A  S  T  K  T  V
caaccccaatcttaccaccatacgggaaaggatcaggctatcaaggcttatatcaacgga
 Q  P  Q  S  Y  H  H  T  G  K  D  Q  A  I  K  A  Y  I  N  G
ggcgttcccgcttcccagatcgttcttggcatgcctatctatggacgatctttcgagagc
 G  V  P  A  S  Q  I  V  L  G  M  P  I  Y  G  R  S  F  E  S
actaacggcattggccaaacctacagtggaattggatctggaagctgggagaacggagtc
 T  N  G  I  G  Q  T  Y  S  G  I  G  S  G  S  W  E  N  G  V
tgggaa
 W  E
```

Figure 4. Partial cDNA sequence of endochitinase (Genbank accession number AY762230) from *T. harzianum* T5 and its deduced amino acid sequence.

saccharides. Our early studies indicated that chitinases of *T. harzianum* T5 produced on different substrates caused significant antifungal activities against *C. falcatum*[6]. The role of *Trichoderma* chitinases in mycoparasitism and biological control of plant pathogenic fungi has been frequently suggested[23,24]. Higher levels of antifungal activity were recorded when more chitinolytic/glucanolytic enzymes of *T. harzianum* are tested together[4,25]. Many *Trichoderma* and *Gliocladium* spp. isolates obtained from natural habitats have been used in biocontrol trials against several soil-borne plant pathogenic fungi under both green-house and field conditions. The antagonists kill the host by direct hyphal contact, causing the affected cells to collapse or disintegrate; vegetative hyphae of all species have been found susceptible[26]. The ability to produce lytic enzymes has been shown to be a crucial property of these and other mycoparasitic fungi. Most of the studies on the expression and regulation of these lytic enzymes have been performed in liquid cultures supplemented with different C-sources like chitin, glucose, β-1,4-linked N-acetylglucosamine, fungal cell walls and their antifungal effects determined *in vitro*. A set of chitinolytic enzymes secreted by various strains of *T. harzianum*, when grown on chitin as the sole C-source consists N-acetylglucosamine, endochitinase and exochitinases. Combining a 42 kDa endochitinase and a 40 kDa chitobiosidase from *T. harzianum* strain P1 resulted in a synergistic increase in antifungal activity[4]. A variety of synergistic interactions have been found when different enzymes were combined or associated with biotic or abiotic antifungal agents[26]. Present studies confirm that many chitinolytic enzymes secreted by the antagonistic fungus are believed to be involved in suppressing *C. falcatum* growth synergistically.

Analyses of the isolated RNA in non-denaturing agarose gel revealed the presence of 28S rRNA, 18S rRNA and 5.8S rRNA as discrete bands with mRNA appearing faintly as a smear between the top two rRNA bands. The purified mRNA from total RNA, as a template for RT–PCR synthesized an expected 246 bp partial cDNA product (Figure 3). Re-amplification with same primers under the same conditions yielded the same product, thereby confirming their specificity. Further PCR amplification of recombinants with the primers yielded the 260 bp amplicon. Sequencing of the partial cDNA endochitinase showed the sequence length as 246 bp (Figure 4). Homology analysis of the 246 bp sequence (Genbank accession number AY762230) with existing sequences in gene databank revealed high level of identity at the nucleotide and protein

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Figure 5. Comparison of deduced amino acid sequence of partial endochitinase from *T. harzianum* T5 with endochitinases of other related species. Differences between the amino acid sequences are indicated with an asterisk. Dashes indicate gaps introduced into the alignment.
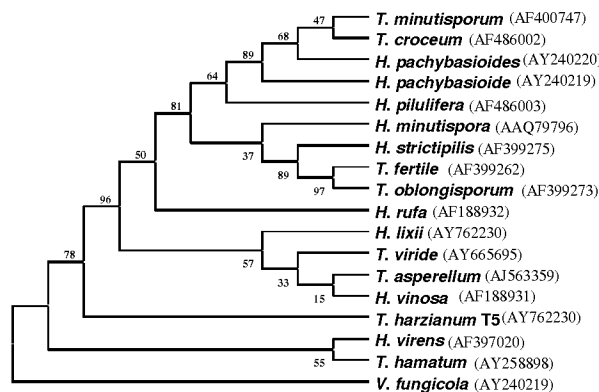

Figure 6. Inferred phylogenetic relationship of *T. harzianum* T5 and related species. Numbers shown represent bootstrap support values (%).

levels (Figure 5). The high level of bootstrap values indicated the reliability of the clustering (Figure 6).

The deduced partial cDNA sequence of chitinase will be used as a probe to pick the full-length chitinase gene from the cDNA library to be constructed. Cloning of genes encoding for lytic enzymes, characterizing their products, and elucidating their individual roles in the mycoparasitic activity of *T. harzianum* and other species of *Trichoderma* or *Gliocladium* has opened ways to improve the biocontrol capacity of these fungi. Also, the genes coding for these lytic enzymes were expressed in plants to resist invading pathogens. Cloning of genes encoding for endochitinase from *Trichoderma* spp. has already been reported[3,12,27–30]. A chitinase gene *ech42* of 1447 bp was obtained from *T. atroviride*, which had three introns in the sequence[31]. Lorito *et al.*[32] successfully improved disease resistance by inserting the *ech42* gene into tobacco and potato plants. The transgenic lines were highly tolerant or completely resistant to foliar pathogens *Alternaria alternata*, *A. solani* and *Botrytis cinerea* and the soil-borne pathogen *Rhizoctonia solani*. To engineer broad, durable resistance to diseases, dual transfer of the exochitinase,

N-acetyl glucosaminidase and endochitinase which are synergistic is essential. Such co-expression of an N-acetyl glucosaminidase and an endochitinase in apple acted synergistically to reduce apple scab caused by *Venturia inaequalis*[33]. Attempts are being made to isolate the coding sequences for N-acetyl glucosaminidase and endochitinase from *T. harzianum* strain T5 based on the present studies to pyramid the genes in sugarcane for high level of resistance to the red rot pathogen.

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Oxygen isotope enrichment (Δ^{18}O) is a potential screening approach for higher leaf yield in tea (*Camillia sinesis*) accessions

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Natural variation in Δ^{18}O$_{lb}$ in existing tea accessions is significantly high (18.36–25.31‰) and also showed a strong positive relationship with the harvested leaf yield ($r = 0.484, P < 0.05, n = 20$). Interestingly, the relative values of Δ^{18}O of the crosses (genetic cross) were higher than either of the individual parent. This trend was also reflected in the leaf yield. This study highlights the relevance of ^{18}O enrichment approach for screening tea accessions for both higher transpiration rate and leaf yields.

Keywords: Leaf biomass/yield, oxygen isotope enrichment, tea, transpiration rate.

AMONG several physiological traits, total transpiration (*T*) strongly determines the biomass production of plants[1,2].