

Molecular Cloning and Characterization of a Chitinase Gene from the Thermophilic Fungus, *Thermomyces lanuginosus*-RMB

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Abstract: A chitinase gene from the thermophilic fungus, *Thermomyces lanuginosus* was amplified from the genomic DNA of the fungus, by PCR technique. The PCR product was cloned into pXcmkn12 vector by TA cloning technique and sequenced. The gene contained five introns and six exons. The deduced protein sequence coded for 390 amino acids and exhibited very high degree of similarity to other endochitinases of mesophilic and thermophilic fungi. The highly conserved catalytic motif, DGLDIDWEYP, suggests that it belongs to family 18 chitinases.

Keywords: Thermophilic fungus, *Thermomyces lanuginosus*, Family 18 Chitinase, Introns

I. Introduction

Chitin is a linear, insoluble, homopolymer made up of β , 1-4 linked N-acetyl glucosamine (GlcNac). It is a major component of cuticles of insects and shells of crustaceans. It also forms an important component of the cell wall of many fungal species. Thus, chitin is the second most abundant biopolymer next to cellulose. Therefore, chitin degradation and chitinases are of biotechnological importance. Chitinases degrade chitin into mainly mono and dimeric units of GlcNac. These enzymes are ubiquitous and are reported from bacteria, yeasts, higher fungi, plants and animals. In plants and animals, their role is mainly defensive.

The chitinases are classified into exo- and endochitinases based on their mode of action. Endochitinases (EC 3.2.1.14) cleave the polymer randomly whereas exochitinases (EC 3.2.1.29) catalyze progressive release of chitobiose residues from the non reducing end of the polymer. N-acetyl- β -D-glucosaminidases (EC 3.2.1.30) release GlcNac residues from chitin oligomers in an exo-fashion. However, based on their sequence and structural similarities they are classified into family 18 and family 19 glycosidases. Family 18 chitinases are structurally different from family 19 chitinases and consist of a highly conserved catalytic motif (DxxDxDWEYP) and are inhibited by allosamidin, a pseudotrisaccharide isolated from *Streptomyces* sp. Bacterial, fungal, animal, and some of the plant chitinases belong to family 18 glycosyl hydrolases whereas most of the plant chitinases and some bacterial chitinases belong to family 19 glycosyl hydrolases [1, 2]. It is interesting to note that the family 19 chitinases have no sequence similarity to family 18 chitinases and are not inhibited by allosamidin.

Recently, chitinases have received considerable attention as they could be used in augmenting plant defense system against chitin containing pathogens such as fungi and nematodes. Fungal chitinases are found to be up to 100 times more active than the corresponding plant enzymes and effective on a much wider range of pathogens and non toxic to plants even at high concentrations [3]. Moreover they may not involve in gene silencing in transgenic plants. Therefore, fungal chitinases are considered as the best choice for production of transgenic plants against fungal pathogens.

A large number of genes encoding chitinases have been isolated and analyzed from a wide variety of fungi including yeast and filamentous fungi such as *Saccharomyces cerevisiae* [4], *Apanocladium album* [5], *Candida albicans* [6], *Rhizopus oligosporus* [7], *Trichoderma harzianum* [8, 9], *Trichoderma reesei* [10], *Trichoderma atroviride* [11], *Thermomyces lanuginosus* [12], *Aspergillus nidulans* [13] and *Gliogladium roseum* [14].

Genes encoding for chitinases have been successfully used for the production of transgenic plants to effectively control a large number of plant pathogenic fungi, which are major pathogens leading to crop loss [3, 15–18]. In the light of stringent IPRs, the gene/plasmid constructs harbouring commercially important genes such as chitinase gene are not freely available and therefore, attempts were made to clone the gene from a thermophilic fungus, which grows optimally at 50° C. Here we report the molecular cloning and characterization of a chitinase gene from an Indian isolate of the thermophilic fungus, *Thermomyces lanuginosus*-RMB. Sequence data shows that the chitinase gene isolated in the present study belongs to family 18 chitinase, similar to the chitinases found in the mycoparasitic fungi (*Trichoderma* sp.), that have been very widely used for

production of transgenic plants. Therefore, the thermophilic fungal chitinase with broad temperature optimum and better thermostability could also be exploited to generate transgenic plants against fungal pathogens.

II. Materials and Methods

T. lanuginosus-RMB was obtained from Dr. Ramesh Maheshwari, Indian Institute of Science, Bangalore-560012. Restriction enzymes, dNTPs, ligase and *Taq* polymerase were from Bangalore Genie Pvt. Ltd., Bangalore, India. The restriction enzyme *XcmI* was obtained from New England Biolabs, USA. The plasmid pXcmkn12 was kindly provided by Dr. Seiichi Yasuda, Department of Microbial genetics, National Institute of Genetics, Mishima, Japan. All other chemicals used were of analytical grade obtained from Indian manufacturers.

2.1 Growth conditions

The fungus was grown in a modified Vogel's medium containing 2% glucose and 0.01% Vogel's trace elements stock solution [19]. The pH of the medium was adjusted to 6.5 with KOH and the medium was sterilized by autoclaving at 15 psi for 20 min. The mycelial inoculum was prepared by incubating the spores in 50 ml of mannitol-asparagine medium according to Palanivelu *et al.* [20], containing 0.2% yeast extract in a 250 ml Erlenmeyer flask, at 50° C for 12-16 hr on a gyratory shaker at 150 rpm. Fungal culture was raised in 100 ml medium in 500 ml Erlenmeyer flasks using a 3% mycelial inoculum (Cell density ~10 mg dry wt/ml) at 50° C for 12-15 hr on a gyratory shaker. The mycelium was harvested by suction filtration and washed twice with distilled water. Moisture was removed by pressing the mycelium several times between layers of blotting paper and then weighed and stored at -20° C.

2.2 Isolation of genomic and plasmid DNAs

High molecular weight genomic DNA was isolated according to the method of Basha and Palanivelu [21]. Plasmid DNAs were isolated by a modified alkaline lysis procedure published by Basha and Palanivelu [22]. The genomic and plasmid DNAs and PCR products were routinely analyzed by 0.8% agarose gel electrophoresis.

2.3 PCR conditions for amplification of genomic DNA

The genomic DNA was amplified by PCR technique using the forward and reverse primers ATTGGGCCATTTATGGT and TTTCTCGATGACATTGTG, respectively. The PCR reaction mixture in a total volume of 25 µl contained 2.5 µl 10X buffer, 1.0 µl dNTP mix (10 mM), 0.5 µl primers (10 pmoles/µl), 0.5 µl template DNA (0.1 µg), 0.5 µl *Taq* polymerase (3 units/µl). After 4 min of initial denaturation at 94° C, the genomic DNA was amplified with the following conditions, 94° C/30 sec; 55° C/60 sec; 72° C/2 min for 30 cycles and the final extension was done at 72° C for 10 min.

2.4 Extraction of DNA from agarose gels, restriction digestion and ligation

Extraction of DNA from agarose gels, restriction digestion and ligation were done according to the recommendations of the manufacturers. The ligation reaction in a total volume of 20 µl, contained 7 µl eluted PCR product, 1 µl pXcmkn12 backbone, 10 µl of 10X ligase buffer and 2 µl of ligase. The ligation mixture was incubated at 16° C for 24 hr and used to transform freshly prepared *E. coli* (DH5α) competent cells. The recombinant plasmids from the transformants were digested with *Bam*HI to release the fragment. The restriction digestion reaction in a total volume of 20 µl, contained 10 µl recombinant plasmid, 2 µl 10X BSA, 2 µl of 10X *Bam*HI buffer, 1 µl of *Bam*HI enzyme and 5 µl of Milli Q water. The reaction was incubated at 37° C for 2 hr and analyzed in 1% agarose gels.

2.5 Sequencing of the recombinant DNA plasmid

The plasmid from recombinant clones containing the insert was purified by polyethylene glycol (PEG) procedure and sequenced.

2.6 Bioinformatics analyses of chitinases sequences

Multiple sequence alignment was done using the software TCOFFEE (advance version) available in the website www.tcoffee.org. BLAST analyses were done from the PubMed site.

III. Results and Discussion

3.1 Isolation of genomic DNA from *Thermomyces lanuginosus*-RMB

Fig. 1 shows the agarose gel electrophoresis of genomic DNA isolated from the fungus. It showed a single band of high molecular weight. The DNA concentrations were quantified spectrophotometrically. The final genomic DNA concentration was adjusted to $1\mu\text{g}/\mu\text{l}$ with 1X TE buffer (pH 8.0).

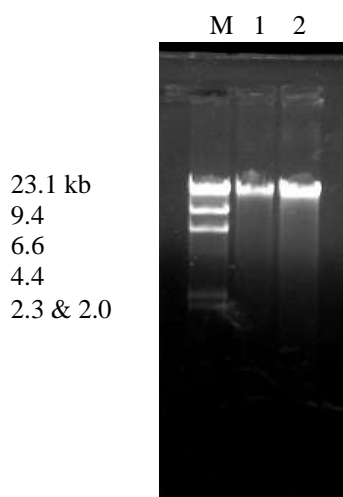


Fig. 1. Agarose gel electrophoresis of genomic DNA from *T. lanuginosus*

Lane M, DNA Marker (λ DNA digested with *Hind*III)

Lane 1, 5 μl genomic DNA

Lane 2, 10 μl genomic DNA

3.2 Amplification of the genomic DNA

The genomic DNA was amplified according to the conditions described in the Materials and Methods. The amplified PCR product was analyzed by agarose gel electrophoresis (Fig. 2).

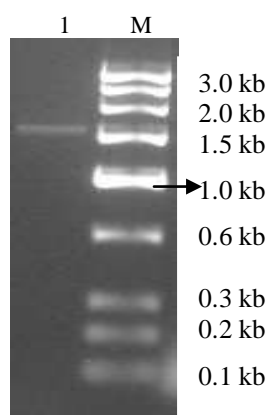


Fig. 2. Agarose gel electrophoresis of PCR amplified product

Lane 1, PCR amplified product

Lane M, Low range DNA ruler

It showed a single band suggesting that the PCR conditions were optimal and no non-specific amplifications were seen. The amplicon size was about 1.7 kb in length.

3.3 Molecular cloning of the PCR product in pXcmkn12 vector

The plasmid pXcmkn12 was used to transform *E. coli* DH5 α and the plasmid was isolated from a transformed colony according to the method published by Basha and Palanivelu [22] and purified by using Bangalore Genie gel extraction kit. The eluted plasmid was cleaved with the restriction enzyme *Xcm*I and the backbone was again purified by using Bangalore Genie gel extraction kit. The amplified product was also purified from the gel by using the above kit. The eluted fragments were found to be homogenous (Fig. 3).

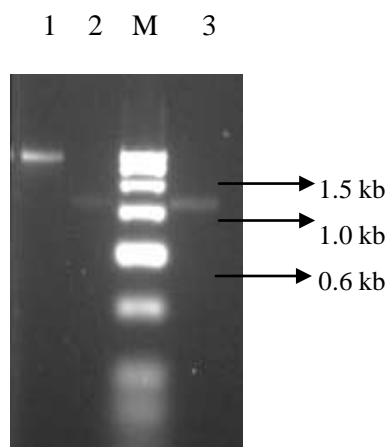


Fig. 3. Agarose gel electrophoresis of eluted pXcmkn12 backbone and the PCR amplified product

Lane 1, pXcmkn12

Lanes 2 & 3, PCR amplified product

Lane M, Low range DNA ruler

The pXcmkn12 plasmid backbone and the PCR product were subjected to ligation according to manufacturer's recommendation, for 16 hr at 16° C. The ligated mixture was used to transform *E. coli* DH5 α competent cells. The ampicillin resistant colonies were patched again in LB plates containing 100 μ g/ml ampicillin and then selected for further analysis. The plasmids were isolated from the recombinant clones as reported earlier [22] and were analyzed by agarose gel electrophoresis. A mobility shift was observed from the plasmids isolated from the recombinant clones. The transformants were further confirmed by digestion with *Bam*HI. (The *Bam*HI digestion releases any insert cloned into pXcmkn12). The recombinant plasmids released an expected 1.7 kb insert which was named pXchi-PPLV2 (Fig. 4).

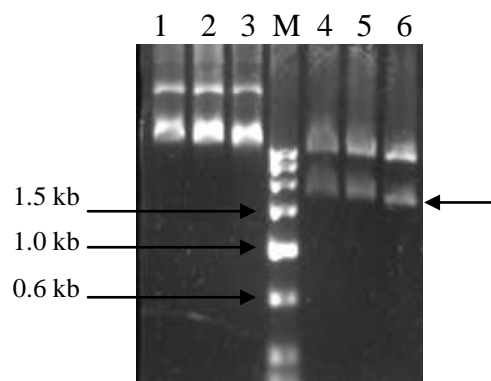


Fig. 4. Agarose gel electrophoresis of recombinant plasmids before and after *Bam*HI digestion

Lanes 1, 2 & 3, Plasmids from recombinant clones

Lane M, Low range DNA ruler

Lanes 4, 5 & 6, *Bam*HI digested recombinant plasmids
(The insert released, is indicated by the arrow mark).

3.4 Sequencing and sequence analysis

The PEG purified recombinant plasmid DNA from pXchi-PPLV2 was sequenced. The sequence using the forward primer (807 nucleotides) and the reverse primer sequence (823 nucleotides) were assembled to obtain the complete sequence (1630 nucleotides). The complete genomic sequence is shown in Fig. 5. (The complete genomic DNA sequence is deposited in the Genbank; Genbank accession No. JQ801444).

*ATTGGGCCATTATGGT*CGCAACCAUAAUCCCCAAGACCUGCCUGCCGAGAAACUAACGCAUAUUCUAUACGCCUUUGCCAAUGUGAGGCCGGAUAGUGGUGAAGUGUACCUUACCGACACCUGGUCGACACGGACAAGCAUUAACCGAGUGACUCCUGGAAUGACACCGGGACCAACGUCUACGGCUGCAUCAAAACAACUCUUUCUCCUCAAGAAGCGGCAUCGUAAACUCAAGGUUCUGCUGUCUAUUGGCGGGUGGACGUAUUCUAUCAAACCUUGCCCAGCCAGCCAGCACCCGAGGCCGGACGCGAGACCUUUGCCGAACCGCCACGAGAUUGGUGCUAGAUCUAGGGCUGGACGGUCUCGAUAUCGACUGGGAAUACCCACAAGACGAUAACAGGCGCGUGACUUUGUGGCUCUGCUGCGCAAUUGCCGGGAGCAUUUGGAUUACCCCGCGGGCCAAAUCGGAGGUUCCUCCUGACCAUUGCCUGCCCAGCUGGUCCUAAACUUUACCAAGCUUCGCCUGCCAAAAAUGACACCGUACUUGGACUUUACAAUCUAAUGGGCUACGACUAUGCUGGCUCUUGGGACCAACUCGCCGGCCACCAGGCCAACAUUUUCCCCUCUUCACCAACCCUGCCUCUACGCAUUCUCAACCGAUGCAGCCUACGACACUACAUCAGUCAGCGCGUGCCAUGGUAUUGGGCAUGCCGUCUACGGGCGCGCAUUCGAAACACCAUUGGCCCGGAACGCCGUUCAGUGGUGGUGAGGGAAGCUGGGAGCAAGGCGUGUGGGACUAAAGCUUUGCCCCGGCCUGGUGCUACGGAGCACGUUGAUCCUAAUCAUCGGGGCGUCGUGGUCGUACGAUCCCCAGACCAGAACUAUGGUCACCUAUGAUAAACGUUGCCGUUGCCGAGAUCAAGGCCAACUUCGUCCGCGGAGCCGGCCUUGGUGGAGGUAUGUGGUGGGAGAGUAGUGCUGACCGAGGCGGGAAGACGGCCAAUAAAGCUGAUGGCAGUCUCAUCGGAACCUUUGUGGACGGAUUGGGUGCGUUUUCGCAUUGGAUCAGUCGCCUAAACAACCUUGAUUAUCCGGAGAGCAAGUAUGACAAUCUCCGGGCGUCUCCCGGGAGAGUGAAUCUGAACUCGACGGUGGCACAUGGGUAUAUGCACCGUUCGCACACACGUACGAGUACACAAUUUACGACUACAGCAUUAAGGAUAGAGAAUCACAAUGTCATCGAGAAAA

Fig. 6. mRNA sequence of the chitinase after deletion of the introns

The stop codon and poly (A) signal sequence are underlined.

The forward and reverse primer sequences are indicated in italics.

The first amino acid W, starts from UGG of the forward primer (underlined)

The mRNA sequence was translated using ExpASy tool and found it was almost identical (98%) to the original sequence published by Guo and Li [12] by cDNA cloning strategy. However, the chitinase from *Thermomyces lanuginosus*-RMB showed 5 amino acid changes from the Chinese isolate (indicated in brackets) (Fig. 7).

WAIYGRNHNPDLPKAEKLTILYAFANVRPDSGEVYLTDTSWSDTDKHYPSDSWNDTGTNV
YGCIKQLFLLKRRHRKLVLLSIGGWTYSSNL*(F)AQPASTEAGRETFARTATRLVLDLGLDG
LDIDWEYPQDDNQARDFVALLRKREHLDP*(A)AGPNRRFLLTIACPAGPNNFTKRLRPK*(E)
MTPYLDFYNLMG*(A)YDY*(N)AGSWDQLAGHQANIFPSSSTNPASTPFSTDAALRHYISVSGVPS
SKMVLGMPLYGRAFQNTNGPGTSPFSGVGEQVWDYKALPRPGATEHVDPNIGASWS
YDQTRTMVYDQVAVAEIKANFVRGAGLGGGMWESSADRGGKTANKADGSLIGTFVDG
LGVFALDQSPNNLDYPESKYDNLRAFGPE

Fig. 7. Deduced protein sequence of the chitinase gene from *T. lanuginosus*-RMB

*Amino acids which are different from the Chinese isolate are given in brackets

These changes may be due to variation in the strains. As the chitinase gene from the Chinese isolate of *Thermomyces lanuginosus* was cloned and sequenced by cDNA cloning method [12], no intron and exon data are available for comparison.

3.5 Bioinformatics analyses of the chitinase sequences

The nucleotide sequence and the translated protein sequence were analyzed by BLAST. The Blast(n) results showed maximum score (699) with chitinase sequence of the *Thermomyces lanuginosus* as expected. Interestingly, an endochitinase from *Thermoascus auranticus* and Class V chitinases from *Taloromyces stipitatus* and *Penicillium marneffeii* had high scores of 588, 570 and 563, respectively. The Blast(p) analysis gave maximum identity (100%) with chitinase mRNA from *Thermomyces lanuginosus* (Chinese isolate), followed by endochitinases of *Chaetomium globosum* (90% identity) and *Penicillium chrysogenum* (87% identity) (data not shown).

Trichoderma preparations from *T. harzianum* or *T. atroviride* are commonly used as bio-control agents against many plant pathogens. Moreover, chitinase gene of *T. harzianum* has been found to be very effective in transgenic plants against many fungal pathogens. Therefore, the chitinases of these two organisms were analyzed for similarity/identity by ClustalW with the chitinase sequences from *T. lanuginosus*. Multiple sequence analysis of the thermophilic fungal chitinases with *T. harzianum* and *T. atroviride* chitinases showed the conserved catalytic blocks typical of family 18 chitinases (Marked) (Fig. 8). *T. lanuginosus*-RMB sequence

showed very high homology with the *T. harzianum* and *T. atroviride* chitinases. The truncated *T. lanuginosus*-RMB sequence covers from 56th amino acid and up to the last C- terminal amino acid of the original sequence [12]. Interestingly the catalytic region is completely conserved confirming the chitinase from *T. lanuginosus*-RMB belongs to family 18 endochitinase type and hence may be effectively used for production of transgenic plants to protect crop plants from fungal pathogens. Further experiments are in progress.

sp P48827 CHI4_TRIHA	MLS-----FL--GKSVALLAALQATLSSPKPGH--RRASVEKRANGYAN	40
tr Chit-TL-RMB	-----	0
tr Q4FCX2 Q4FCX2_THELA	MLVKYRVFAPFLWSGLYRRVFCSLHLHTIHAGRVLSPPI-QEKAQGYLS	49
tr Q9UV47 Q9UV47_TRIAT	MLG-----FL--GKSVALLAALQATLISASPVTANDVSVEKRASGYAN	41
sp P48827 CHI4_TRIHA	SVYFTNWGIYDRNFQPADLVASDVTHVIYSFMNLQAD-GTVISGDTYADY	89
tr Chit	-----WAIYGRNHNPDLPKAEKLTHILYAFANVRPDSGEVYLTDTWSDT	44
tr Q4FCX2 Q4FCX2_THELA	VQYFVNWAIYGRNHNPDLPKAEKLTHILYAFANVRPDSGEVYLTDTWSDT	99
tr Q9UV47 Q9UV47_TRIAT	AVYFTNWGIYGRNFQPNLVASDITHVIYSFMNFQAD-GTVVSGDAYADY	90
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sp P48827 CHI4_TRIHA	EKHYADDSWNDVGTNAYGCVKQLFKVKKANRGLKVLLSIGGWTWSTN-FP	138
tr Chit-TL-RMB	DKHYPSDSWNDTGTNVYGCIKQLFLLKRRHRKLVLSIGGWTYSSNLF	94
tr Q4FCX2 Q4FCX2_THELA	DKHYPSDSWNDTGTNVYGCIKQLFLLKRRHRKLVLSIGGWTYSSN-FA	148
tr Q9UV47 Q9UV47_TRIAT	QKHYDDDSWNDVGNAYGCVKQLFKLKKANRNLKVLMSIGGWTWSTN-FP	139
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sp P48827 CHI4_TRIHA	SAASTDANRKNFAKTAITFMKDWGFDGIDVWEYEDATQASNMILLKE	188
tr Chit-TL-RMB	QPASTEAGRETFAATATRLVLDLGLDGLDIDWEYEQDDNQARDFVALLRK	144
tr Q4FCX2 Q4FCX2_THELA	QPASTEAGRETFAATATRLVLDLGLDGLDIDWEYEQDDNQARDFVALLRK	198
tr Q9UV47 Q9UV47_TRIAT	SAASTDANRKNFAKTAITFMKDWGFDGIDVWEYEDADTQATNMVLLLKE	189
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sp P48827 CHI4_TRIHA	VRSQRDAYAAQYAPGYHFLLTIAAPAGKDNYSKLRILA-DLGQVLDYINLM	237
tr Chit-TL-RMB	CREHLDYPAA--GPNRRFLLTIAACPAGPNNFTKLRLPKEMTPYLDFYNLM	192
tr Q4FCX2 Q4FCX2_THELA	CREHLDY-AA--GPNRRFLLTIAACPAGPNNFTKLRLP-EMTPYLDFYNLM	244
tr Q9UV47 Q9UV47_TRIAT	IRSQLDAYAAQYAPGYHFLLSIAAPAGPEHYSFLHMS-DLGQVLDYVNL	238
	* . : * * * . * . : * * * . * . * . * . * . * . * . * . * . * . *	
sp P48827 CHI4_TRIHA	-AYDY-AGSFSPLTGHANLFFNPNPNATPFNTDSAVKDYIN-GGVPAN	284
tr Chit-TL-RMB	GAYDYNAGSWDQLAGHQANIFPSSTNPASTPFSTDAALRHYISVSGVPSS	242
tr Q4FCX2 Q4FCX2_THELA	-AYDN-AGSWDQLAGHQANIFPSSTNPASTPFSTDAALRHYISVSGVPSS	292
tr Q9UV47 Q9UV47_TRIAT	-AYDY-AGSWSSYSGHDANLFANPNPNSSPYNTDQAIKDYIK-GGVPAS	285
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sp P48827 CHI4_TRIHA	KIVLGMPIYGRSFQNTAGIGQTYNGVSGSWEAGIWDYKALPKAGATVQY	334
tr Chit-TL-RMB	KMVLGMPYGRAFGNTNGPPTPFSVGVGEGSWEQGVWDYKALPRPGATEHV	292
tr Q4FCX2 Q4FCX2_THELA	KMVLGMPYGRAFGNTNGPPTPFSVGVGEGSWEQGVWDYKALPRPGATEHV	342
tr Q9UV47 Q9UV47_TRIAT	KIVLGMPIYGRAFEFSTGGIGQTYSGIGSGSWENGIWDYKALPKAGATVQY	335
	* . * . * . * . * . * . * . * . * . * . * . * . * . * . * . * . *	
sp P48827 CHI4_TRIHA	DSVAKGYYSYNATKELISFDTMINTKVAYLKSGLGGSWFWEASADK	384
tr Chit-TL-RMB	DPNIGASWSYD-QTRTMVTYDNVAVAEIKANFVRGAGLGGGMWESSADR	341
tr Q4FCX2 Q4FCX2_THELA	DPNIGASWSYDQTRTMVTYDNVAVAEIKANFVRGAGLGGGMWESSADR	392
tr Q9UV47 Q9UV47_TRIAT	DSVAQAYYSYDPSKELISFDTMINTKVSYLKNLGGSWFWEASADK	385
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sp P48827 CHI4_TRIHA	KGA-----DSVIGTSHRALG---GLDTPQNLLSYPNISKYDNIKNGL--N	423
tr Chit-TL-RMB	GGKTANKADGSLIGTFVDGLGV-FALDQSPNNLDYPESKYDNLNLAGFPGE	390
tr Q4FCX2 Q4FCX2_THELA	GGKTANKADGSLIGTFVDGLGGVFALDQSPNNLDYPESKYDNLNLAGFPGE	442
tr Q9UV47 Q9UV47_TRIAT	TGS-----DSLIGTSHRALG---SLDSTQNLLSYPN-----Q	415
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Fig: 8. ClustalW analysis of thermophilic fungal chitinases with *Trichoderma* chitinases
 The highly conserved catalytic block in family 18 chitinases is highlighted.
 No change was observed in the conserved blocks between the original sequence (Chinese isolate) and from *T. lanuginosus*-RMB (Indian isolate, present communication).

IV. Conclusion

A chitinase gene is cloned and sequenced along with its introns and exons for the first time from the genomic DNA of the thermophilic fungus, *Thermomyces lanuginosus* RMB. The deduced protein sequence is found to be very similar to other mycoparasitic fungal chitinase sequences. The catalytic motif DGLDIDWEYP is highly conserved in the thermophilic fungal chitinase. Therefore, this gene could be used for the production of transgenic plants against fungal pathogens.

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