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Improved antifungal activity of barley derived chitinase I gene that overexpress a 32 kDa recombinant chitinase in *Escherichia coli* host



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ABSTRACT

Agricultural crops suffer many diseases, including fungal and bacterial infections, causing significant yield losses. The identification and characterisation of pathogenesis-related protein genes, such as chitinases, can lead to reduction in pathogen growth, thereby increasing tolerance against fungal pathogens. In the present study, the chitinase I gene was isolated from the genomic DNA of Barley (*Hordeum vulgare* L.) cultivar, Haider-93. The isolated DNA was used as template for the amplification of the ~935 bp full-length chitinase I gene. Based on the sequence of the amplified gene fragment, class I barley chitinase shares 93% amino acid sequence homology with class II wheat chitinase. Interestingly, barley class I chitinase and class II chitinase do not share sequence homology. Furthermore, the amplified fragment was expressed in *Escherichia coli* Rosetta strain under the control of T7 promoter in pET 30a vector. Recombinant chitinase protein of 35 kDa exhibited highest expression at 0.5 mM concentration of IPTG. Expressed recombinant protein of 35 kDa was purified to homogeneity with affinity chromatography. Following purification, a Western blot assay for recombinant chitinase protein measuring 35 kDa was developed with His-tag specific antibodies. The purified recombinant chitinase protein was demonstrated to inhibit significantly the important phytopathogenic fungi *Alternaria solani*, *Fusarium spp*, *Rhizoctonia solani* and *Verticillium dahliae* compared to the control at concentrations of 80 µg and 200 µg.

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Introduction

Agricultural crops have great economic importance worldwide, and with the population increase, the crops produced are not sufficient to feed the human population. Additionally,

the agricultural crops are attacked by multiple pathogens including bacterial, viral or fungal causing reduction in both quantity and quality of the overall yield. Almost 26–30% of the yield losses for sugar beet, cotton and wheat are caused by fungal pathogens alone.¹ Moreover, fungi cause 35%, 39% and 40% of the damage in maize, potato and rice, respectively.¹ Fungi, such as *Fusarium solani* and *Fusarium oxysporum*, are the cause of wilting and damping off diseases, whereas *Alternaria alternate* causes leaf spot in various agriculturally important

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crops.² *Rhizoctonia solani* causes many diseases including black scurf in potatoes,³ root rot in sugar beet⁴ and sheath blight in rice.⁵ Similarly, *Verticillium dahliae* is the cause of verticillium wilt in many plants including potato,^{6,7} cotton,⁸ peppermint⁹ and others.

The first structure of the fungus that comes into contact with the host (plants in the case of phytopathogenic fungi) is its cell wall. Chitin, the main constituent of the fungal cell wall, is a homopolymer of N-acetylglucosamine units. In nature, plants possess natural defence systems against fungal attack. Certain plants produce antifungal proteins to defend against fungal growth.¹⁰ Isolating these antifungal proteins and inserting them into different plants increases resistance against fungal pathogens in these new host plants. These proteins include chitinases and glucanases.¹¹ Chitinases are present in almost all organisms including bacteria,¹² fungi,¹³ insects,¹⁴ plants¹⁵ and mammals. Chitinases act on chitin as their substrate and hydrolyse it to mono- and oligomers.¹⁶ A chitinase is classified as an endochitinase if it breaks chitin in internal sites by acting randomly; or an exochitinase, if it breaks the chitin from either nonreducing or reducing ends.¹⁷ They have different roles in different organisms but in plants chitinases are mainly involved in resistance against fungal pathogens.¹⁸ Barley possesses endochitinases (class I and class II) that can be used to control fungal pathogens.¹⁹

Escherichia coli is the most widely used bacterial expression system for producing heterologous proteins.²⁰ To obtain a high yield of recombinant protein, the gene is usually expressed at its highest possible level. The purpose of this study was to isolate chitinase I gene from Barley and express it in an *E. coli* expression system to reveal its antifungal activity against four economically important phytopathogenic fungi; *Alternaria solani*, *Fusarium spp.*, *R. solani* and *V. dahliae*.

Materials and methods

Plant material

Barley (*Hordeum vulgare* L.) variety Haider-93 grown in CEMB field was used as source material for isolation of chitinase I gene. The deduced sequence of chitinase I gene was used to obtain the amino acid sequence of the gene, which was further used for alignment and construction of a phylogenetic tree.

Gene amplification and amino acid sequence homology studies

Genomic DNA was extracted from young Barley leaves using Genomic DNA Purification kit (Thermoscientific, K0512). The 935 bp chitinase I gene was amplified with 5'-AGAGCGTTCGTGTTGTTTCG-3' forward and 5'-CTGTAGCAGTCGAGGTTGTTG-3' reverse primer in a PCR reaction mixture comprised of 2 µL of 10× PCR buffer, 1.5 mM MgCl₂, 0.1 mM dNTPs, 1 pmol each of forward and reverse primers, 50 ng DNA and 2 units of *Taq* polymerase (Thermoscientific). The PCR amplification was carried out under the following conditions: initial denaturation at 95 °C for 5 min, followed by 35 cycles of amplification (94 °C for 45 s, 61 °C for

45 s, and 72 °C for 45 s) with a final extension of 10 min at 72 °C. The amplified fragment was analysed on 0.8% agarose gel by electrophoresis. The amplified fragment of ~935 bp was cloned into the pCR 2.1 vector (Invitrogen, K4500-01) for sequencing. The Barley chitinase clone was sequenced through the facility at Macrogen (Korea), and edited and analysed using the BioEdit tool. The sequences of other plant chitinases used for comparison were downloaded from the National Centre of Biotechnology Information (NCBI). Multiple sequence alignment of amino acid sequences of plant chitinase retrieved from GenBank and our deduced sequence was done using Clustal W (Mega v 6) software. A phylogenetic tree was constructed using Neighbour joining method and the reliability was measured by boot strap analysis with 1500 trials using Mega v 6 software. The protein families of barley chitinase class I and class II were compared using conserved domain database, CDD, using <http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>.

Construction of a prokaryote expression vector and expression of recombinant chitinase

A ~935 bp chitinase I gene was ligated in pET30a (Addgene) expression vector having N/C-terminal His-tag sequence and expressed under T7 promoter to create a pET-chil construct. The ligation was transformed into competent cells of *E. coli* through the heat shock method as described by Froger and Hall.²¹ The pET-chil construct DNA was mixed with 50 µL of chemically competent *E. coli* DH5α cells and after a short incubation on ice, heat shock was given at 42 °C for 45 s and placed back on ice. SOC media was added and the transformed cells were incubated at 37 °C for 30 min with agitation.

Then, the mix was spread onto LB media plates with kanamycin for antibiotic selection and incubated at 37 °C for 16–18 h. Positive clones were confirmed through restriction digestion with *Eco*R1. For expression analyses, the pET-chil construct was transformed in *E. coli* host Rosetta. *E. coli* Rosetta cells were transformed with the prokaryotic expression vector pET-Chil encoding the chitinase I protein and grown in shaking culture (220 rpm, 37 °C) in LB supplemented with 50 µg/mL kanamycin to an OD of 0.6 at 600 nm. Protein expression was induced by the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma) for 3 h (220 rpm 30 °C). Induced cells were harvested by centrifugation at 4000 × *g* for 10 min at 4 °C. The pellet was weighed, resuspended in 1× phosphate saline buffer (1:10 W/V) and centrifuged at 4000 × *g* for 10 min. Lysozyme (4 mg/mL) was added in the pellet and kept at room temperature for 20–30 min for complete cell lysis. The lysate was sonicated (20 min at 16 W) and then cleared by centrifugation at 14,000 × *g* for 15 min at 4 °C.

Purification was performed at 4 °C inside a Kelvinator chromatography refrigerator using a 16/20 XK column with volume of 20 mL and height of 15 mL which was packed with Chelating Fast Flow Sepharose adsorbent (GE Healthcare; catalogue No. 17-0575-01). The column was charged with 0.2 M NiCl₂ solution. Binding buffer (500 mM NaCl, 20 mM sodium phosphate buffer, pH 7.4) was added to balance the Sepharose adsorbent. The supernatant containing the protein was filtered through 0.45 µm membrane to remove cell debris. The filtered

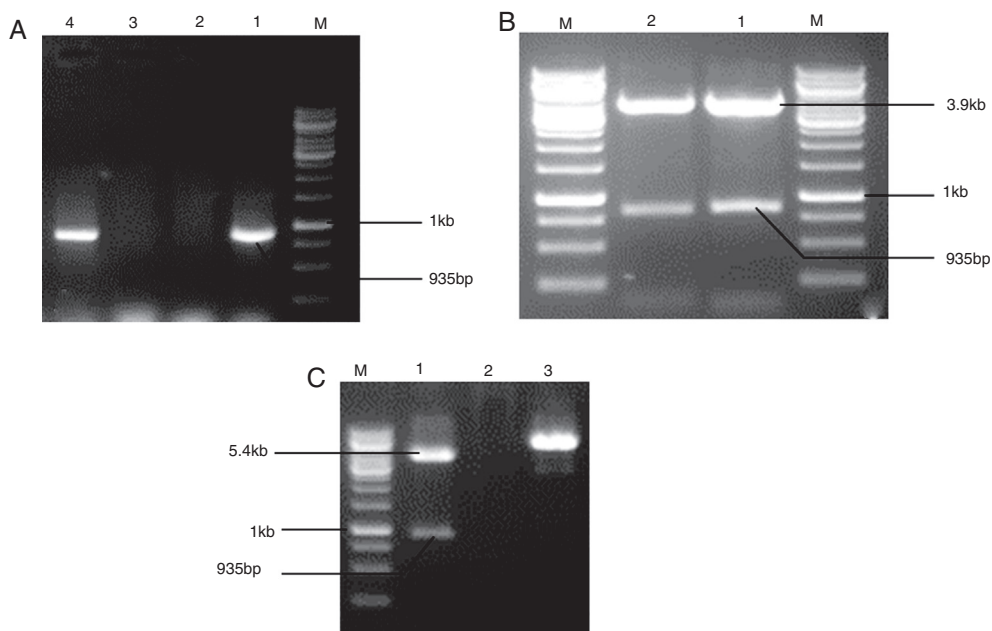


Fig. 1 – Amplification and cloning of chitinase I gene in TA vector and in *E. coli* expression vector pET30a. (A) Lane 1: 1 kb DNA ladder; lanes 2–4: samples showing amplification of ~935 bp of chitinase I gene. **(B)** Restriction digestion of plasmid pTA:chiI harbouring chitinase I gene cloned in pCR2.1 vector with EcoRI enzyme. Lanes 1–2: pTA:chiI DNA samples; M represent 1 kb DNA ladder. The release of ~3.9 kb DNA fragment represent pCR2.1 vector while ~935 bp depict chitinase gene. **(C)** Restriction digestion of pET-chiI recombinant plasmid with EcoRI to confirm ligation of chitinase gene in *E. coli* expression vector. Lane M: 1 kb DNA ladder, lanes 1–3: pET-chiI plasmid DNA. The release of ~5.4 kb DNA fragment represent pET30a vector while ~935 bp depict chitinase gene.

protein supernatant was then loaded onto the column at a linear flow rate of 150 cm/h and the time for complete loading was calculated. Washing was performed first with wash solution 1 (20 mM imidazole + binding buffer, pH 7.4) and then with wash buffer 2 (40 mM imidazole + binding buffer, pH 7.4) such that all of the unbound solutes and other protein contaminants were completely removed. Elution of the His-tag recombinant chitinase I protein was done by providing step-wise increasing concentrations of imidazole (ThermoFisher Scientific; catalogue No. AC30187-0010). Five concentrations of elution buffer were prepared from 70 mM to 150 mM. Five fractions (1 mL each) for each elution were selected and analysed by Bradford assay to check the protein concentration. At each step, fractions were analysed by SDS-PAGE on 12% polyacrylamide gel and stained with Coomassie blue (ThermoScientific). Protein size was determined using Perfect Protein™ Markers (cat #926-98000 WesternSure®). Recombinant protein was fractionated by SDS-PAGE on 12% gel using a Mini Protean II tetra cell (cat # 1658004; BioRad). The resolved purified recombinant protein was transferred to PVDF (polyvinylidenedifluoride) membrane (cat # RPN303E; Amersham) using a Trans-blot Cell (BioRad) blocked with 5% non-fat dry milk with His-tag specific antisera. Recombinant chitinase was detected with rabbit polyclonal His-tag antiserum (1: 5000 dilution; cat # sc-804; Santa Cruz). An appropriate antibody conjugated with peroxidase was used in detection with the ECL system (Amersham Biosciences).

Antifungal activity assay

The phytopathogenic fungi *A. solani*, *Fusarium spp.*, *R. solani* and *V. dahliae* were provided by the Institute of Agricultural Sciences, University of the Punjab Lahore-Pakistan. Antifungal assays of purified recombinant chitinase protein were performed on the four phytopathogenic fungi by employing cylinder plate method.²¹ For this, three wells were made on PDA (potato dextrose agar) medium by the cylinder plate method. The PDA block containing mycelia of the test fungi was inoculated in the centre of the petri plates (23 °C; 48 h). When the colony size increased to 3–4 cm in diameter, the wells were filled with different concentrations of purified chitinase protein; 80 µg, 200 µg, and control. For the control well, recombinant chitinase protein was placed in boiling water for 5 min and poured into the respective well. The plates were then incubated at 23 °C for 48 h. Zone of inhibition created by the recombinant chitinase protein was marked and measured in comparison with negative control.

Results

PCR amplification and amino acid sequence comparison of chitinase genes

A ~935 bp chitinase I gene was successfully amplified from genomic DNA of barley using specific primers (Fig. 1A). A

Table 1 – Amino acid sequence identity of barley chitinase with other plant chitinases.

Plant species	Chi class	GenBank Accession No.	Identity (%)
<i>Secale cereal</i>	I	Q9FRV1	95
<i>Triticum aestivum</i>	II	AAX83262	93
<i>Brachypodium distachyon</i>	I	XP_003563256.2	88
<i>Triticum aestivum</i>	I	AAR11388.1	80
<i>Triticum aestivum</i>	III	BAB82473.1	80
<i>Secale cereal</i>	I	AAG53609.1	80
<i>Festuca arundinacea</i>	I	ACJ23248.1	76
<i>Elaeis guineensis</i>	I	XP_010941404.1	72
<i>Oryza sativa</i>	I	CAA82849.1	72
<i>Oryza sativa</i>	I	BAA03751	72
<i>Gossypium hirsutum</i>	I	CAA92277	72
<i>Acacia koa</i>	I	AFY08283.1	71
<i>Solanum lycopersicum</i>	I	CAA78845.1	70
<i>Nicotiana tabacum</i>	I	AAB23374	69
<i>Pisum sativum</i>	I	CAA45359.1	69
<i>Canavalia ensiformis</i>	II	CAA07413.1	67
<i>Oryza sativa</i>	II	BAA31997	59
<i>Solanum tuberosum</i>	II	Q43835	58
<i>Hordeum vulgare</i>	II	AGS38341.1	58
<i>Solanum lycopersicum</i>	II	CAA78846.1	57
<i>Cucumis melo</i>	II	AAF69836.1	57
<i>Heterodera glycines</i>	I	AAN14979.1	57
<i>Chenopodium amaranticolor</i>	IV	BAA22966	42
<i>Vitis vinifera</i>	IV	AAB65776	42
<i>Oryza sativa</i>	IV	BAA19793	41
<i>Zea mays</i>	IV	P29022	40
<i>Triticum aestivum</i>	II	AAD28730	40
<i>Zea mays</i>	I	ACX37090.1	40
<i>Triticum aestivum</i>	IV	AAD28733	39
<i>Gossypium hirsutum</i>	VII	Q7X7Q1	39
<i>Arabidopsis thaliana</i>	IV	NP_191010	38

sharp and defined fragment can be seen in Fig. 1A. To confirm insertion, plasmid DNA was subjected to restriction digestion by EcoRI, generating two fragments; a ~3.9 kb fragment representing pCR2.1 vector and a ~935 bp fragment representing chitinase I gene (Fig. 1B). The 935 bp of Chi I encoded a polypeptide of 318 amino acids. BLAST and NCBI search using the amino acid sequence indicated that it has high degree of sequence similarity with proteins encoding class I chitinase of *Secale cereal*, chitinases of class I, II & III of wheat (*Triticum aestivum*), and class I chitinase of other plants including *Festuca arundinacea*, *Elaeis guineensis*, *Oryza sativa*, *Gossypium hirsutum*, *Acacia koa*, *Solanum lycopersicum*, but has a low degree of sequence similarity with class IV chitinase of wheat (*T. aestivum*) and *Arabidopsis thaliana*, and class VII chitinase of *G. hirsutum* (Table 1). Moreover, it showed 58% identity with a class II chitinase of *Hordeum vulgare* (Fig. 2). Its highest identity is with class I chitinase of *S. cereal*, at 95% (Fig. 2). Wheat chitinase class II (AAX83262) does not have a chitinase binding

domain and has only a catalytic site but barley class I chitinase has 93% amino acid sequence similarity to it while in comparison, barley class I chitinase has only 58% similarity to barley chitinase class II (AGS38341.1) (Supplementary Figure 1).

Expression of recombinant chitinase protein in prokaryote host and purification

Amplified ~935 bp chitinase I gene was cloned behind T7 promoter in fusion with a His-tag in a prokaryote expression system. Recombinant clone, pET-chiI, upon restriction digestion release two size specific fragments: a ~5.4 kb fragment representing pET30a vector and a ~935 bp fragment representing the chitinase I gene (Fig. 1C). Expression of the ~35 kDa recombinant chitinase protein was optimised using SDS-PAGE and it was found that 0.5 mM IPTG was best for recombinant protein expression (Fig. 3A). The expression was lowest at 0.2 mM concentration of IPTG while highest at 0.5 mM concentration of IPTG compared to the control, non-transformed Rosetta cells where recombinant protein is not expressed. Subsequently, the recombinant chitinase protein was purified through affinity chromatography and a thick band of ~35 kDa was observed in eluted fractions obtained at 125 mM and 150 mM imidazole concentration (Fig. 3B). The purified recombinant protein fractions were electrophoretically transferred onto PVDF (polyvinylidene fluoride) membrane (Millipore, USA) and incubated with polyclonal anti-His-HRP antibody (Santa Cruz, UK). An apparently homogeneous band of approximately 35 kDa was detected using Chemiluminescent HRP Substrate (Millipore) (Fig. 3C).

Antifungal inhibition activity assays

Purified recombinant chitinase protein, measuring ~35 kDa when applied on four important phytopathogenic fungi, exhibited strong inhibitory effect on the mycelial growth of the subjected fungi. Significant growth retardation in the mycelial growth of *A. solani*, *Fusarium spp* and *V. dahliae* was observed at 200 µg concentration of purified chitinase protein while the zone of inhibition was less around the wells where 80 µg of the purified protein was applied. In the control sample, no inhibitory effect was measured (Fig. 4). In *R. solani*, less inhibition was observed at 80 µg concentration of the purified protein compared to other tested fungi while at 200 µg concentration, a zone of inhibition can be seen (Fig. 4). It can be concluded that recombinant chitinase protein, when over-expressed exhibits strong and significant antifungal potential. Hyphal growth of the inoculated fungi was restricted to a particular zone of inhibition.

Discussion

Chitinases belong to group of pathogenesis-related (PR) proteins in plants^{22–25} and several of them have been employed to enhance the tolerance of the plant towards a particular pathogen through genetic transformation technologies.^{26,27} Our aim was to isolate a plant based chitinase I gene, over express it into a prokaryote expression vector under T7 promoter and reveal the antifungal potential of the purified

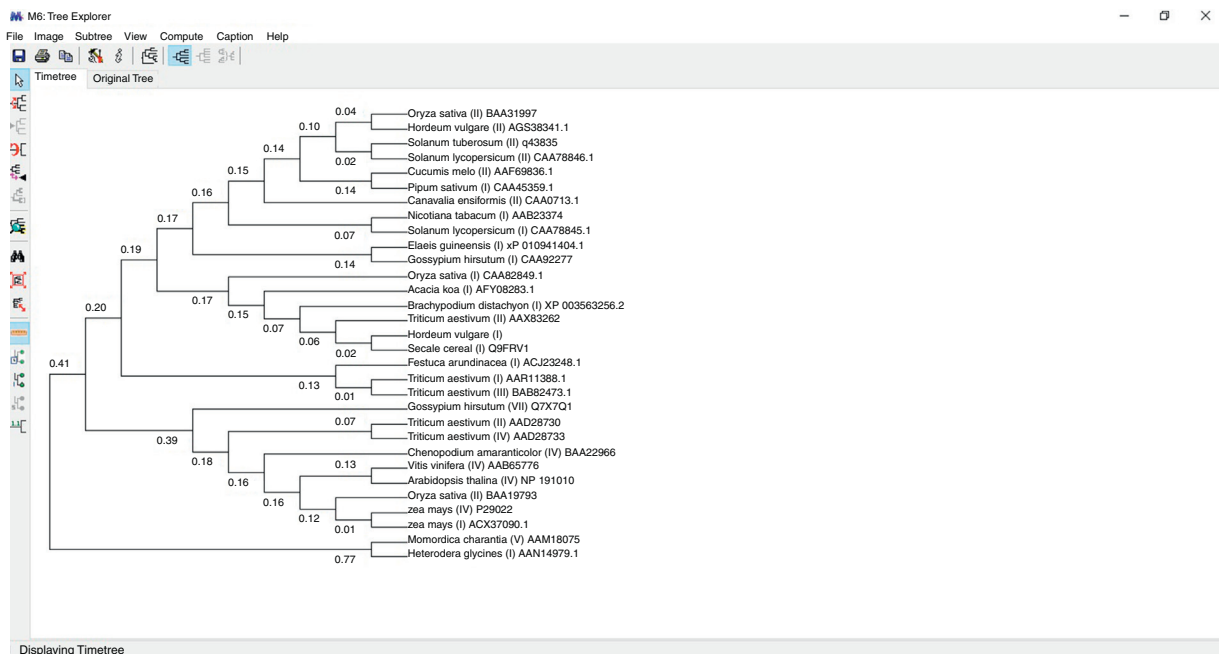


Fig. 2 – Phylogenetic analysis of amino acid sequences of *Hordeum vulgare* derived chitinase I gene in comparison with other plant chitinases. The branch length indicates the percentage of sequence similarity. Chitinase class and accession number is mentioned against particular crop plant.

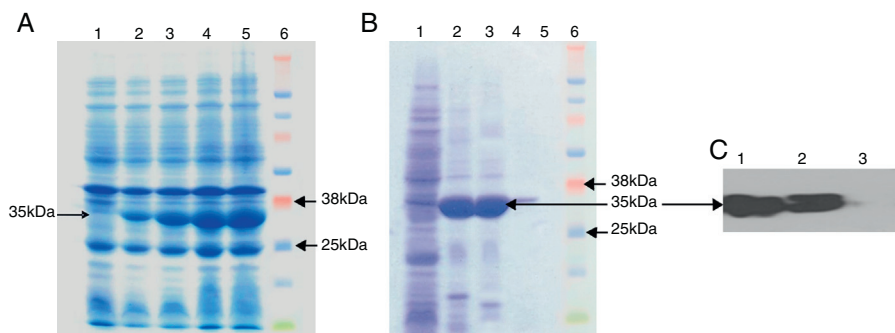


Fig. 3 – Expression analysis of recombinant chitinase protein expressed in *E. coli* host Rosetta. (A) SDS-PAGE (12%) representing expression of a ~35 kDa recombinant chitinase protein optimised at various concentrations of IPTG. Lane 1: cell lysate from un-induced colony of Rosetta, lane 2: cell lysate from transformed Rosetta induced with 0.2 mM IPTG, lane 3: cell lysate from transformed Rosetta induced with 0.3 mM IPTG, lane 4: cell lysate from transformed Rosetta induced with 0.4 mM IPTG and lane 5: cell lysate from transformed Rosetta induced with 0.5 mM IPTG, lane 6: pre-stained protein marker, (B) SDS-PAGE fractionating purified fractions of recombinant chitinase protein. Lane 1: total cell lysate from un-transformed Rosetta, lanes 2–3: purified fraction of recombinant chitinase protein sample, lane 6: pre-stained protein marker, (C) Western blot of purified recombinant chitinase protein. The protein was detected through Histag specific antibodies using ECL system. Lanes 1–2: purified fraction of 35 kDa chitinase protein.

recombinant protein against four important phytopathogenic fungi.

In our study, we selected a plant chitinase as they are usually endo-chitinases and contain lysozyme activity. While, in contrast, the bacterial chitinases are exo-chitinases and hydrolyse the chromogenic trisaccharide analogue pNPP (p-nitrophenyl-P-D-N, N'-diacetylchitobiose) as the substrate. Additionally, plant chitinase I is reported to exhibit high antifungal activity compared to other plant chitinases.²⁸ Barley-derived chitinase genes have been used to enhance fungal resistance in many crops like brassica,¹⁹ wheat,²⁹ rice³⁰

and carrot.³¹ The chitinase I gene is evolutionarily conserved and is present in both monocot and dicot plants such as cocoa,³² potato³³ and rice.³⁴ The chitinase I gene also has a chitin binding domain, which is absent in class II chitinase and hence make it superior in antifungal potential.

We documented an interesting finding that barley-derived chitinase I gene and II gene not only differs in presence of chitin binding domain but also do not share sequence homology. Rather, barley chitinase class I shares 93% amino acid sequence homology with chitinase class II of wheat. Furthermore, we employed pET expression system for expression

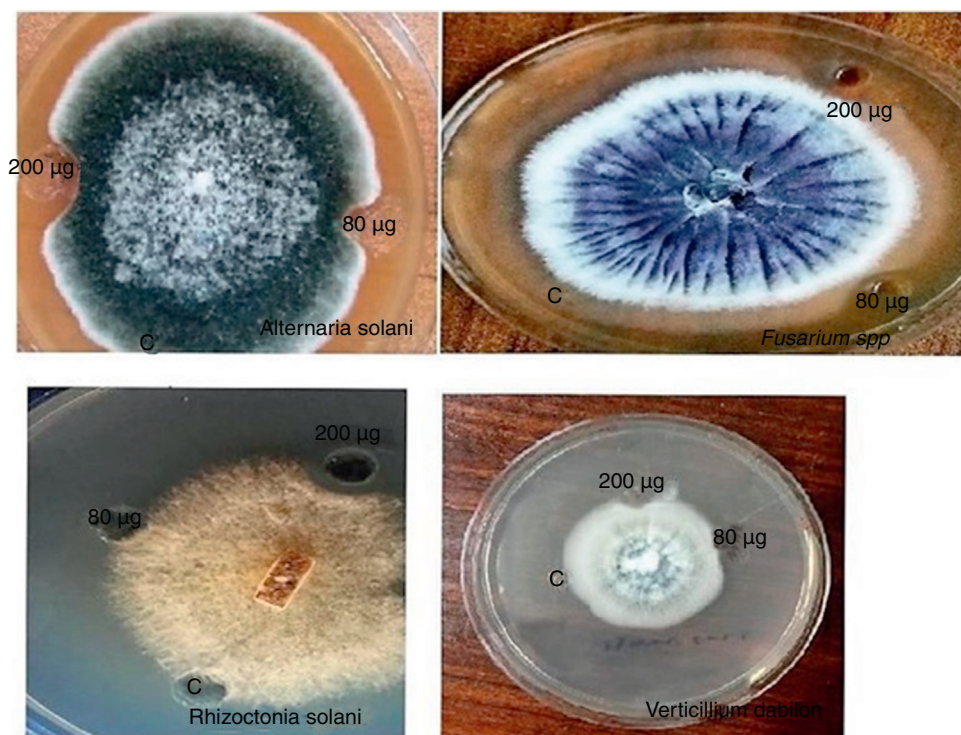


Fig. 4 – Antifungal activity assay of purified recombinant chitinase protein towards (A) *Alternaria solani*, (B) *Fusarium spp.*, (C) *Verticillium dahliae*, (D) *Rhizoctonia solani*. Each selected fungus was subjected to two protein treatments, 80 µg and 200 µg along with control. The letter ‘C’ represent control that contains heat deactivated purified chitinase protein.

studies of chitinase recombinant protein as previously done by Liu and Naismith.³⁵ This is the most widely used system for the cloning and *in vivo* expression of recombinant proteins in *E. coli*. The reason behind this is the high selectivity of the pET system's bacteriophage T7 RNA polymerase for its cognate promoter sequences, the high level of activity of the polymerase and the high translation efficiency mediated by the T7 gene 10 translation initiation signals.³⁶ We employed IPTG as an inducing agent as it is reported to provide better induction.³⁷

Our antifungal result showed that 80 µg of recombinant chitinase protein has a clear zone of inhibition while 200 µg of the protein has an increased zone of inhibition. There are reports³⁸ that rice chitinase had no significant effect on fungi tested at 100 µg and had only subtle effect at a higher dose of 300 µg. Kirubakaran and Sakthivel used *E. coli* strain BL-21 as host and pET 28a+ vector for the overexpression of chitinase I gene and analysed its antifungal activity against different fungal pathogens and reported that 300 µg of enzyme activity has broad spectrum anti-fungal inhibition.²² This finding demonstrates that our chitinase isolate has much better inhibitory effect on test fungi at low doses than isolates used in other studies.

In conclusion, Chitinases that cleave chitin polymers in the fungal cell wall are used to increase resistance to fungal pathogens. And for this purpose, adequate antifungal activity of the chitinase is mandatory. Therefore, screening chitinase with high antifungal activity is important to meet the need to

improve resistance against phytopathogens by gene manipulation. Attempts have been made by several researchers to identify new chitinase genes with enhanced antifungal potential. Like Liu et al. expressed two chitinase genes, LbCHI31 and LbCHI32 from *Limonium bicolor* in *E. coli* BL21 strain and it has significant inhibition against *A. alternata*.³⁹ In a similar study, Itoh et al. overexpressed a cell surface-expressed chitinase from *Paenibacillus* in *E. coli*.⁴⁰ In another study, horse-tail derived chitinase gene was overproduced in *E. coli*.⁴¹ In a similar study, a chitinase gene (pcht28), isolated from *Lycopersicon chilense* was transformed to strawberry and its expression was checked against *V. dahliae*. It showed more resistance against these fungi compared to control, untransformed. This suggests pcht28 plays a role in defence against fungal disease caused by *V. dahliae*.⁴² Mishra et al. transformed the *Trichoderma* endochitinase gene into guava and measured its inhibitory action against *F. oxysporum*, the cause of fusarium wilt.⁴³ In our findings, overexpression of chitinase I gene results in elevated chitinolytic activity, induction of expression of the recombinant chitinase gene and increased inhibition for fungal phytopathogens. All these results collectively indicate that the chitinase I gene derived from barley can be useful in gene engineering against plant diseases.

Conflicts of interest

The authors declare no conflicts of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bjm.2017.05.007.

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