

Research article

Characterization of a new antifungal lipid transfer protein from wheat

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Abstract

Lipid transfer proteins (LTPs) are members of the family of pathogenesis-related proteins (PR-14) that are believed to be involved in plant defense responses. In this study, a novel gene *Ltp* 3F1 encoding an antifungal protein from wheat (Sumai 3) was subcloned, overexpressed in *Escherichia coli* BL-21 (DE3) and enriched using ammonium sulfate fractionation followed by gel permeation chromatography. Molecular phylogeny analyses of wheat *Ltp* 3F1 gene showed a strong identity to other plant LTPs. Predicted three-dimensional structural model showed the presence of 6 α -helices and 9 loop turns. The active site catalytic residues Gly30, Pro50, Ala52 and Cys55 may be suggested for catalyzing the reaction involved in lipid binding. SDS–PAGE analysis confirmed the production of recombinant fusion protein. The LTP fusion protein exhibited a broad-spectrum antifungal activity against *Alternaria* sp., *Rhizoctonia solani*, *Curvularia lunata*, *Bipolaris oryzae*, *Cylindrocladium scoparium*, *Botrytis cinerea* and *Sarocladium oryzae*. Gene cassette with cyanamide hydratase (*cah*) marker and *Ltp* 3F1 gene was constructed for genetic transformation in tobacco. Efficient regeneration was achieved in selective media amended with cyanamide. Transgenic plants with normal phenotype were obtained. Results of PCR and Southern, Northern and Western hybridization analyses confirmed the integration and expression of genes in transgenic plants. Experiments with detached leaves from transgenic tobacco expressing *Ltp* 3F1 gene showed fungal resistance. Due to the innate potential of broad-spectrum antifungal activity, wheat *Ltp* 3F1 gene can be used to enhance resistance against fungi in crop plants.

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Keywords: Heterologous expression; Lipid transfer protein; Antifungal activity; Transgenic tobacco

1. Introduction

Plant lipid transfer proteins (LTPs) are small basic proteins of about 9–10 kDa in size and may represent as much as 4% of the total soluble proteins. LTPs participate in the *in vitro* transfer of phospholipids between membranes and can bind acyl chains [1]. Based on these properties, LTPs are thought to be involved in membrane biogenesis and regulation of intracellular fatty acid pools. LTPs are secreted proteins and are located in

the cell wall. Novel roles suggested for plant LTPs include: involvement in cutin formation, embryogenesis, symbiosis and adaptation of plants to various environmental conditions. Defensive role of LTPs has been proposed considering the ability of some members of this family, but not all, to inhibit the growth of plant pathogens [2]. Their extracellular distribution in the exposed surfaces in vascular tissue systems, high abundance and expression in response to infection by pathogens suggest that they are active plant-defense proteins [3]. LTPs can inhibit the growth of fungal pathogens *in vitro*. Interestingly, they are capable of synergistically enhancing the antimicrobial properties of other antimicrobial peptides such as defensins and thionins [4]. LTPs have been classified as members of pathogenesis-related (PR) proteins belonging to the group, PR-14 [5]. The relative activities of different plant LTPs against pathogens vary, suggesting that they have varying degree of selectivity. Velazhahan et al. [6] demonstrated the antifungal activity against *Trichoderma viridae* and *Rhizoctonia solani* using purified protein from pearl

Abbreviations: LTPs, lipid transfer proteins; PR, pathogenesis-related; cDNA, complementary DNA; 8 CM, eight-cysteine motif; IPTG, isopropyl β -thiogalactoside; PBS, phosphate-buffered saline; RMSD, root mean square deviation; MOE, molecular operating environment; NMR, nuclear magnetic resonance.

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millet seeds which had sequence homology with LTPs of cotton, wheat and barley. An antifungal protein purified to homogeneity from sunflower (*Helianthus annuus* L.) seeds (Ha-AP10) exerted a fungistatic effect that inhibits germination of *Fusarium solani* f. sp. *eumartii* spores [7]. Recent studies have demonstrated the antifungal potential of LTPs. Transgenic rice containing the homologous ns-LTP gene (*Ace-AMP1*) of *Allium cepa* showed antimicrobial activity towards *Magnaporthe grisea*, *Rhizoctonia solani* and *Xanthomonas oryzae* pv. *oryzae* [8]. Transgenic wheat showed enhanced antifungal activity against *Blumeria graminis* f. sp. *tritici* [9]. It is reported that not all the PR-proteins are antimicrobial and therefore, in vitro screening of proteins for their antimicrobial potential is important to use them for developing transgenic resistance against pathogens in crop plants. Wheat LTPs with antifungal properties have not been reported so far. In this study, we report our results on molecular cloning and overexpression of wheat LTP (*Ltp* 3F1) gene in *Escherichia coli*, evaluation of its antifungal activity against major phytopathogenic fungi that attack various crop plants and construction of plant transformation cassette with *cah* marker and *Ltp* 3F1 gene for genetic transformation in tobacco.

2. Methods

2.1. Bacterial strains, plasmids and fungal cultures

Escherichia coli strains BL 21 (DE3) and JM109 were used as host. The vector pMAL-p2x (New England Biolabs Inc., Beverly, MA, USA) was used for the overexpression of wheat *Ltp* 3F1 gene (GenBank accession number EF432573). Plant transformation vector pCambia 1300 (Centre for the Application of Molecular Biology to International Agriculture, Canberra, Australia) was used for construction of gene cassette for genetic transformation in tobacco. PCR reagents, T4 DNA ligase and restriction endonuclease were purchased from Promega (Madison, WI, USA). *E. coli* cells with plasmids were grown aerobically in LB (Luria Bertani) medium (Hi-Media, India) or on LB agar plates at 37 °C, supplemented with appropriate concentration of antibiotics (ampicillin 100 µg/ml or kanamycin 50 µg/ml) for selection of transformants. Phytopathogenic fungal species used in this study include *Alternaria* sp., *Bipolaris oryzae*, *Magnaporthe grisea*, *Sarocladium oryzae*, *Rhizoctonia solani*, *Macrophomina phaseolina*, *Botrytis cinerea*, *Pythium* sp., *Fusarium* sp., *Cylindrocladium scoparium*, *Cy. floricolum*, *Pestalotia theae*, *Curvularia lunata*, *Colletotrichum falcatum* and *C. gleosporoides*. All isolates are maintained in the Microbial Culture Collection (MCC), Department of Biotechnology, Pondicherry University, India. The tobacco seeds (*Nicotiana tabacum*, cv. *Sam-sun*) were obtained from Central Tobacco Research Station (CTRI), Rajahmundry, India.

2.2. Nucleic acid techniques

Plasmid isolation, transformation, and standard DNA protocols were performed as described in Sambrook and Russel [10].

2.3. Isolation, characterization and molecular phylogeny of *Ltp* cDNA clone

F. graminearum (GZ3639), a highly virulent isolate, was used at a final concentration of 1.8×10^5 spores/ml to inoculate Sumai 3 (c'Funo' × Taiwan Wheat), a scab resistant cultivar and its mutant susceptible variety. The cDNA library construction and screening was described by Li et al. [11]. Base maps were constructed for placement of defense genes [12]. The positive clone, *Ltp* 3F1 was cloned in the T-vector and sequenced. Open reading frames (ORF's) and amino acid sequences were deduced using ORF finder program and homology search were conducted using BLAST 2.0 program of the National Center of Biotechnology Information (NCBI). The software ClustalW (1.82) from Europe Biotechnology Information was employed for multiple sequence alignment of amino acid sequences of wheat LTP with other monocot LTPs retrieved from GenBank and subsequently a phylogenetic tree was constructed by the neighbor-joining (NJ) method [13] with ClustalX 1.81. The reliability of the tree was measured by bootstrap analysis with 1500 trials and the phylogenetic tree was edited using molecular evolutionary genetics analysis and sequence alignment tool MEGA v 3.1 [14]. The theoretical calculation of isoelectric point and molecular weight were analyzed with 'Expasy' using Compute pI/Mw tool (http://www.expasy.ch/tools/pi_tool.html).

2.4. Molecular modeling

Maize ns-LTP (PDB code 1AFH) was used as the template. Modeling was done using molecular operating environment (MOE version 2001.07, Chemical Computing Group Inc., Montreal, QC, Canada) [15]. Ten intermediate homology models were built as a result of the permutational selection of different loop candidates and side chain rotamers. The intermediate models were averaged to produce the final model by Cartesian average. The validity of the model was tested using WHATCHECK and PROCHECK [16].

2.5. Molecular cloning, overexpression and purification of recombinant protein

The *Ltp* 3F1 gene was amplified using the forward primer (LTP3F1-F) 5'-AGG ATC CTT GAT CGA GAT GGC CCG TT-3' and reverse primer (LTP3F1-R) 5'-CAA GCT TGG AGT GGA AGA ACA ACC-3'. In the forward and reverse primers, the underlined sequences represent the sites of BamHI and HindIII site respectively. The wheat cDNA clone, 3F1 encoding *Ltp* was used as template. The PCR amplified fragments were purified, double digested with BamHI and HindIII, ligated into BamHI–HindIII digested pMAL-p2x, and transformed into *E. coli* BL-21 (DE3) competent cells to construct the recombinant expression plasmid, pMAL-*Ltp*. The clone carrying pMAL-*Ltp* was overexpressed using isopropyl β-D-thiogalactoside (IPTG; 0.4 mM) induction. After 5 h of induction at 37 °C, cells were harvested by centrifugation at $3000 \times g$ for 10 min at 4 °C and were frozen until use.

[17]. The crude lysate was first fractionated by ammonium sulfate precipitation, in which the crude protein was brought to 20% saturation by addition of solid ammonium sulfate. After removal of the pellet by centrifugation, additional ammonium sulfate was added to bring the resultant supernatant to 50% saturation. After centrifugation at 12,000 rpm for 20 min, the supernatant was discarded. The precipitate was collected and dissolved in phosphate-buffered saline (PBS) pH 7.2 and dialyzed against PBS (pH 7.2). The dialyzed protein sample was subjected to gel filtration chromatography on a Sephadex G-75 column equilibrated with the same buffer. Protein elution was carried out with PBS (pH 7.2) at a flow rate was 0.3 ml/min and the eluate was monitored by measuring the absorbance at 280 nm. Protein fractions were analyzed by SDS–PAGE and protein quantification was done by Lowry method [18] using bovine serum albumin as the standard.

2.6. Determination of protein solubility and Factor Xa cleavage assay

In order to determine the solubility percentage of protein when overexpressed in *E. coli*, the Wilkinson and Harrison model was used [19]. Factor Xa (New England Biolabs Inc.) cleavage was carried out using 1 µg of Factor Xa and 50 µg of LTP fusion protein at 4 °C as per the recommendation of manufacturers.

2.7. In vitro antifungal activity, spore germination inhibition assays and microscopic analyses

Antifungal activity assays using various concentrations of LTP (80 µl of a fusion protein solution containing either 100 µg or 300 µg of protein in PBS buffer, pH 7.2) were performed on potato dextrose agar (PDA) as described [20]. The PBS buffer containing 300 µg of maltose binding protein served as control. The fungal hyphae from the periphery of the zone of inhibition produced by the LTP fusion protein was scraped, stained with lactophenol cotton blue and examined under a light microscope (Novex, the Netherlands) (×400) for any morphological changes. A similar observation was also made with fungal hyphae obtained from a normal fungal culture [17]. Spore germination inhibition assays were performed using spore suspensions of phytopathogenic fungi. Fungi were grown on PDA for 10–15 days. Conidial suspensions were collected by adding sterile water to the surface of the mycelium. Suspensions were counted in a Neubauer counting chamber and adjusted to the appropriate concentration (1×10^6 spores/ml). The ability of LTP fusion protein to inhibit conidial germination of fungi was determined by placing conidial suspensions (50 µl) in sterile microcentrifuge tubes containing potato dextrose broth (150 µl) and 300 µg LTP fusion protein or maltose binding protein was added. Conidial germination was examined after 12 h of incubation at 28 °C. To determine whether the LTP fusion protein has fungistatic or fungicidal activity, 12 h treated and control spores were plated on PDA and incubated at 28 °C for 4 days. Delayed germination of spores when plated in PDA indicates

fungistatic effect, whereas no germination indicates fungicidal activity of the LTP fusion protein. Similarly, microscopic observation of the treated and untreated control mycelia or spores was carried out after 12 h by staining with lactophenol cottonblue and examined under a light microscope (Novex, Holland) (×400).

2.8. Construction of gene cassettes with antifungal *Ltp* gene

To construct plant expression vector, the marker gene cyanamide hydratase (*cah*) along with Ubiquitin (*Ubi1*) promoter (EcoRI–HindIII fragment, 3 kb) was first cloned into pCAMBIA 1300 vector. The vector harboring *cah* gene was digested with HindIII, dephosphorylated and the *Ubi1* promoter (HindIII–BamHI fragment) and antifungal *Ltp* 3F1 gene (BamHI–HindIII fragment) were ligated by three-fragment ligation, transformed and maintained in *E. coli* JM109.

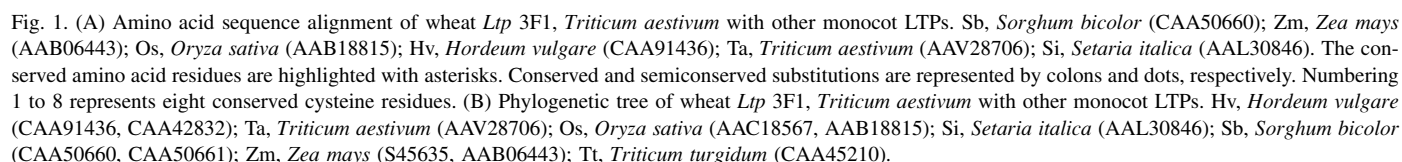
2.9. Genetic transformation of tobacco and molecular analyses of the transformants

The recombinant gene cassette, pCAMBIA-*cah-Ltp* 3F1 was transformed into *Agrobacterium* strain LBA 4404 by the freeze–thaw method. Transformation of tobacco plants was carried out as described [21]. PCR analysis was carried out using the genomic DNA and gene-specific primers for *cah* and *Ltp* genes. For conducting Southern hybridization, analysis total genomic DNA of tobacco leaves was digested with BamHI, electrophoresed and hybridized with probes of *cah* and *Ltp* 3F1 radiolabeled with [α - ^{32} P]dCTP (RadPrime DNA Labeling System, Invitrogen Life Technologies, USA). To perform Western hybridization analysis the total protein was isolated from tobacco leaves and fractionated by 12% SDS–PAGE gel. Hybridization was done using 1:1000 dilution of rabbit antiserum as described by Towbin et al. [22]. To perform Northern hybridization analysis total RNA was isolated from the transgenic and control plants [10]. RNA (12 µg) was run on a 1.2% denaturing agarose gel containing formaldehyde and transferred onto positively charged nylon membrane. The ^{32} P radiolabeled coding sequence of *Ltp* 3F1 gene was used as probe.

2.10. Resistance in transgenic tobacco

To test the antifungal resistance of transgenic tobacco, fungi were grown on PDA. Mycelial agar plug (6 mm) was inoculated in the center of the detached tobacco leaves, incubated at room temperature for 10 days and the observation was made after 10 days. The inoculated leaves of plants expressing *cah* gene alone served as control. The extent of lesion was recorded and the difference in the control and *Ltp*-expressing transgenic plants was observed as described [23]. Experiments were setup in a completely randomized design with three replications. Mean and standard error (S.E.) were calculated and difference between means were tested using Duncan's multiple range test at the level of $p = 0.05$.

sativa (AAB18815), 50.4% identity and 64% similarity with LTP of *Setaria italica* (AAL30846), 62.7% identity and 79.7% similarity with LTP of *Sorghum bicolor* (CAA50660), 71.3% identity and 86.1% similarity with LTP of *Triticum aestivum* (AAV28706) and 60.2% identity and 78.9% similarity with LTP of *Zea mays* (AAB06443). The eight-cysteine motif (8 CM) appears to be a structural scaffold of conserved helical regions connected by variable loops. The position of the eight cysteine residues are conserved where the third and fourth cysteines are consecutive in the polypeptide chain and the fifth and sixth cysteines are separated by only one residue. Phylogenetic tree was constituted on the basis of amino acid sequence alignment of wheat *Ltp* 3F1 with other monocot LTPs (Fig. 1A and B). Wheat *Ltp* 3F1 exhibited high identity



as well as similarity with LTP of *H. vulgare* (CAA91436) and *T. aestivum* (AAV28706) and low identity and similarity with LTPs of other monocot species.

3.2. Molecular modeling

Sequence comparison showed that the wheat *Ltp* 3F1 is homologous to another maize ns-LTP (Protein Data Bank code 1AFH) whose three-dimensional structure has been determined by ^1H NMR spectroscopy data [24]. Three-dimensional structure of wheat *Ltp* 3F1 reported in this study was successfully simulated by homology modeling tool, molecular operating environment (MOE, version 2001.07) [15] using maize ns-LTP as template. The structure of wheat *Ltp* 3F1 shared 53.4% sequence identity with maize ns-LTP (1AFH). The validity of the model was tested using WHATCHECK and PROCHECK [16]. The predicted three-dimensional structural model of wheat *Ltp* 3F1 is presented in Fig. 2. The structure of wheat *Ltp* 3F1 showed the presence of 6 α -helices, absence of β -strand, 9 loop turns and also the presence of 8 conserved cysteine residues that are responsible for the formation of 4 disulfide bridges (Cys29–77, Cys39–54, Cys55–97, Cys75–111). The active site residues Gly30, Pro50, Ala52 and Cys55 present in the hydrophobic core may be suggested for catalyzing the reaction in lipid binding [24]. Root mean square deviation (RMSD) is the measure of the average distance between the backbones of superimposed proteins. The accepted range of RMSD has been reported in between 0–2 [25]. RMSD of query sequence structure and template (1AFH) was found to be 0.932601 Å.

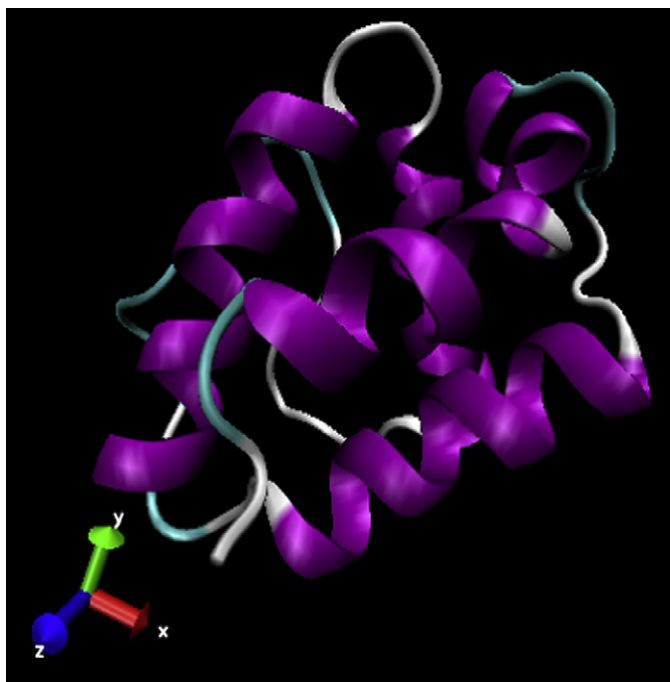


Fig. 2. Predicted three-dimensional structural model of wheat lipid transfer protein 3F1 showed the presence of 6 α -helices and 9 loop turns and other active residues Gly30 Pro50, Ala52 and Cys55 present in the hydrophobic core which may be suggested for lipid binding properties.

3.3. Molecular cloning, overexpression and purification of recombinant protein

The wheat *Ltp* 3F1 gene was cloned in the *E. coli* expression vector, pMAL-p2x and overexpressed in the host *E. coli* BL-21 (DE3). The 40 kDa *E. coli* maltose binding protein (MBP) enhanced the solubility of recombinant protein as reported [26]. SDS–PAGE analysis revealed the accumulation of a 51 kDa MBP–LTP fusion protein only after addition of IPTG to the culture. The uninduced control did not produce LTP fusion protein. When the Wilkinson and Harrison model [19] for theoretical calculation was employed, wheat *Ltp* 3F1 showed 82.7% chance of insolubility when overexpressed in *E. coli*. It is known that recombinant proteins overexpressed in bacteria often form insoluble inclusion bodies, which are aggregates of insoluble proteins that contain most of the expressed protein [27]. To test this possibility, cells were lysed and soluble cytoplasmic proteins were separated from insoluble proteins by sonication followed by centrifugation. The resulting pellets, supernatants and the LTP fusion protein were analyzed by SDS–PAGE. The recombinant 51 kDa fusion protein was found mainly in the supernatant, indicating that the major part of the protein is soluble (Fig. 3). Although the fusion protein was enriched its purity was not insured.

3.4. Determination of protein solubility and Factor Xa cleavage assay

The fusion protein was subjected to Factor Xa cleavage and the cleavage products were analyzed by SDS–PAGE.

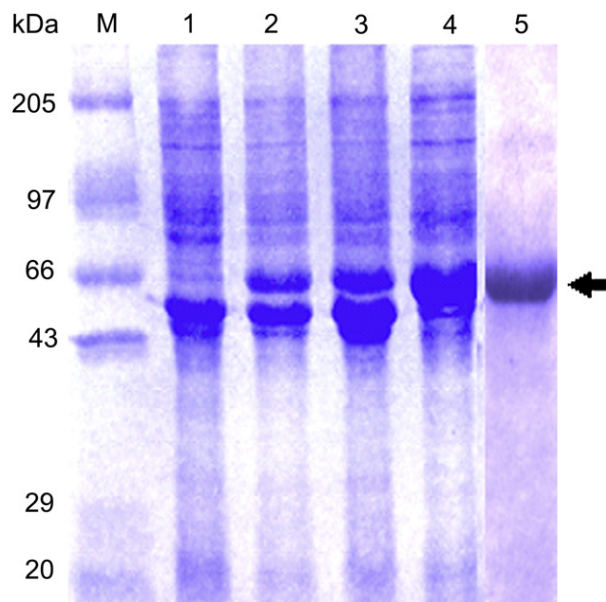


Fig. 3. Gel electrophoresis and Coomassie staining of recombinant LTP fusion protein. Lane M, Protein molecular weight marker (Genei, Bangalore, India); lane 1, total cell protein from uninduced *E. coli* BL-21 (DE3) containing pMAL-LTP; lane 2, total cell protein from induced *E. coli* BL-21 (DE3) containing pMAL-LTP; lane 3, pellet from centrifugation of induced sonicated cells; lane 4, supernatant from centrifugation of induced sonicated cells; lane 5, LTP–MBP fusion protein. The arrow indicates the MBP–LTP fusion protein (51 kDa).

Complete cleavage of the fusion protein was observed after 6 h digestion at 4 °C, with Factor Xa. However, after complete digestion, no protein appeared with an electrophoretic mobility corresponding to the 11 kDa LTP 3F1. Results suggested that the release of the 11 kDa LTP 3F1 from the fusion protein is coupled with its proteolysis. Therefore, the observed degradation of the LTP released from the fusion protein could be due to a bacterial protease that was co-purified with the fusion protein as observed with proteins produced with this expression system [28]. The degradation of the heterologously expressed protein after cleavage with Factor Xa can also be explained by a low stability of the 11 kDa LTP.

3.5. *In vitro* antifungal activity, spore germination inhibition assays and microscopic analyses

The purified fusion protein showed a broad-spectrum antifungal activity at a concentration of 100 and 300 µg against *Alternaria* sp., *R. solani* (Fig. 4A), *C. lunata*, *B. oryzae*, *Cy. scoparium*, *B. cinerea* and *S. oryzae* (data not shown). When the mycelium of *Alternaria* sp. from the periphery of the zone of inhibition produced by purified LTP fusion protein was examined under light microscope, the hyphae appeared to have clear cut mycelial deformations such as swollen mycelium and hyphal tip, poorly developed mycelium with swollen

margin, whereas the mycelium from the control plate was normal, branched, well developed, intact and without any distortion (Fig. 4B). Spore germination inhibition assays of purified fusion protein revealed a fungistatic effect and delayed germination of treated spores when plated in PDA, while no fungicidal effect was observed at the concentration tested against phytopathogenic fungi such as *M. grisea*, *M. phaseolina*, *Fusarium* sp., *B. cinerea*, *S. oryzae* and *B. oryzae* (data not shown). Fungicidal activity was clearly observed against *Alternaria* sp. Microscopic observation of the control spores showed germination but the treated spores did not show germination (Fig. 4C), which was confirmed by plating the control and treated spores in PDA.

3.6. Construction of gene cassettes with antifungal *Ltp* gene

cah marker gene was cloned into pCAMBIA 1300 vector and the resulting vector was designated as pCAMBIA-*cah*. The plant expression cassette pCAMBIA-*cah*-*Ltp* 3F1 was successfully constructed by three-fragment ligation of the vector (pCAMBIA-*cah*) digested with HindIII, *Ubi1* promoter (HindIII–BamHI) fragment and *Ltp* 3F1 gene (BamHI–HindIII) fragment.

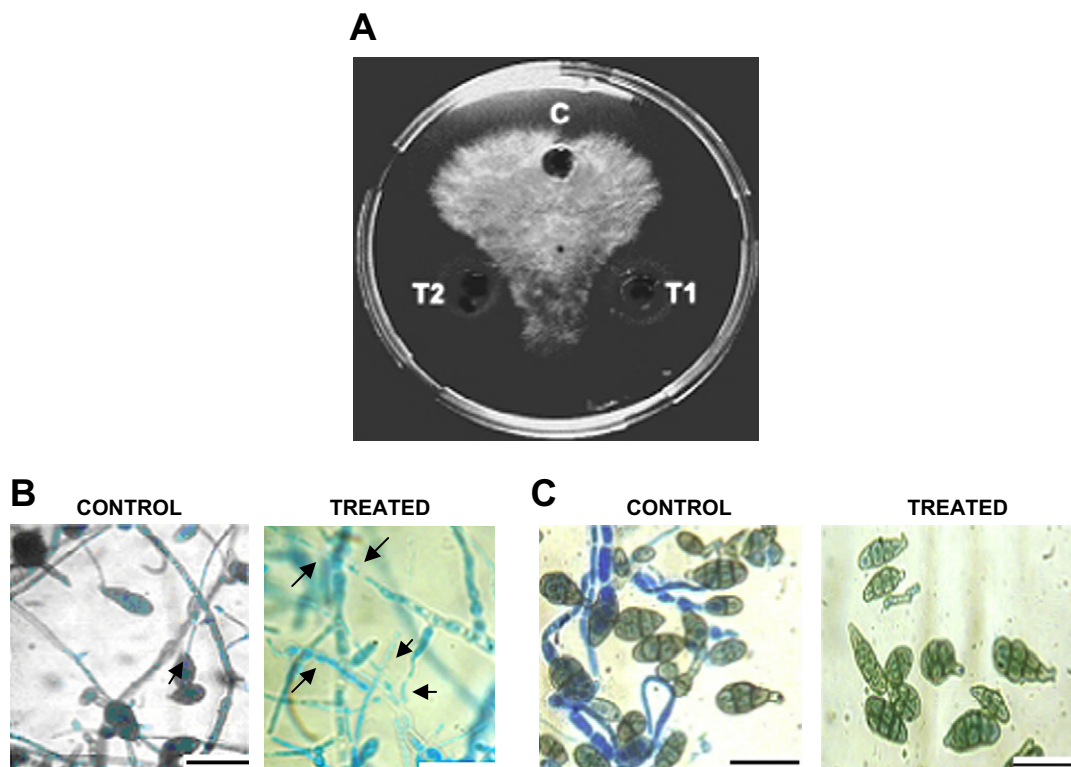


Fig. 4. (A) Inhibitory activity of LTP fusion protein towards *R. solani*. (C) PBS buffer pH 7.2 containing 300 µg maltose binding protein; (T1) 300 µg of LTP fusion protein and (T2) 100 µg of LTP fusion protein. (B) Light microscopic observation of control and treated fungal mycelium of *Alternaria* sp. Control showed normal well developed mycelia with sporulation and treated mycelia showed the lysis of mycelium (arrows) with swollen margin without sporulation. (C) Light microscopic observation of control and treated spores of *Alternaria* sp. Control spores treated with PBS buffer containing MBP showed germination and spores treated with LTP fusion protein did not show germination. Micrographs were taken after 12 h of incubation of the spores with purified LTP fusion protein (300 µg). Bars = 40 µm.

3.7. Genetic transformation of tobacco and molecular analyses of the transformants

The freeze–thaw method facilitated the transformation of pCAMBIA-*cah-Ltp* 3F1 into *Agrobacterium* LBA4404 and the tobacco leaf-disc method was found to be efficient for gene transfer. Efficient regeneration was achieved for tobacco after transformation on the MS medium [29] amended with 5 mM cyanamide. Putative transgenic shoots were randomly selected and rooted in the rooting medium containing 10 mM cyanamide (data not shown). All plants expressing the gene cassettes pCAMBIA-*cah* and pCAMBIA-*cah-Ltp* 3F1 showed normal phenotypes with no indication of cytotoxicity due to the expression of *cah* and *Ltp* in T₀ and T₁ generation (data not shown). T₀ plants were analyzed using PCR and selected lines were transferred to a greenhouse. Eight independent lines were selected and T₁ transgenic plants for each line were analyzed by PCR, Southern hybridization analysis for gene integration, and Western and Northern hybridization analyses for gene expression. When the genomic DNA of cyanamide tolerant transgenic plants was used as template, gene-specific primers amplified 732 bp of *cah* gene and 348 bp *Ltp* 3F1 gene in all plants and in positive control (pCAMBIA-*cah-Ltp* 3F1). Conversely, no such band was observed in the untransformed control under identical conditions (data not shown). Southern hybridization analysis with *cah* (data not shown) and *Ltp* 3F1 gene probes confirmed the single copy in transgenic tobacco lines 2 and 4 and double copy integration in other transgenic lines 1, 3, 5–8 (Fig. 5A). Northern hybridization analysis carried out using the total RNA

isolated from transgenic plants and untransformed control revealed a distinct hybridizable band when probed with ³²P-labeled *Ltp* 3F1 gene and no such band was observed in the untransformed control (Fig. 5B). Western hybridization showed the expression of *cah* (27.7 kDa) in all transgenic plants when hybridized with Cah antibody. In contrast, no corresponding protein band was detected in the untransformed control plant (Fig. 5C).

3.8. Resistance in transgenic tobacco

In order to study the fungal resistance response of LTP-expressing transgenic tobacco, detached leaf fungal assays using phytopathogenic fungi were performed. The results of the fungal infection assay using leaves from the tobacco plants expressing *cah* alone (control) and transgenic plants expressing *Ltp* with single copy of the gene revealed the expression of significant resistance in *Ltp*-expressing transgenic tobacco plants against phytopathogenic fungi. Observations were made 5–10 days after inoculation of fungi, *Bipolaris oryzae*, *Cylindrocladium scoparium* and *Alternaria* sp. The leaves from the control plants showed spreading lesions (diameter 2–3 cm) around the site of contact with the fungus containing the plug. However, the leaves from *Ltp*-expressing transgenic plants showed smaller and restricted lesion (diameter of lesion 0.6–1.7 cm) around the site of contact with the fungus containing plug which is the indication of transgenic resistance. Transgenic plants showed 48–60% reduction in lesion size over control (Table 1 and Fig. 6).

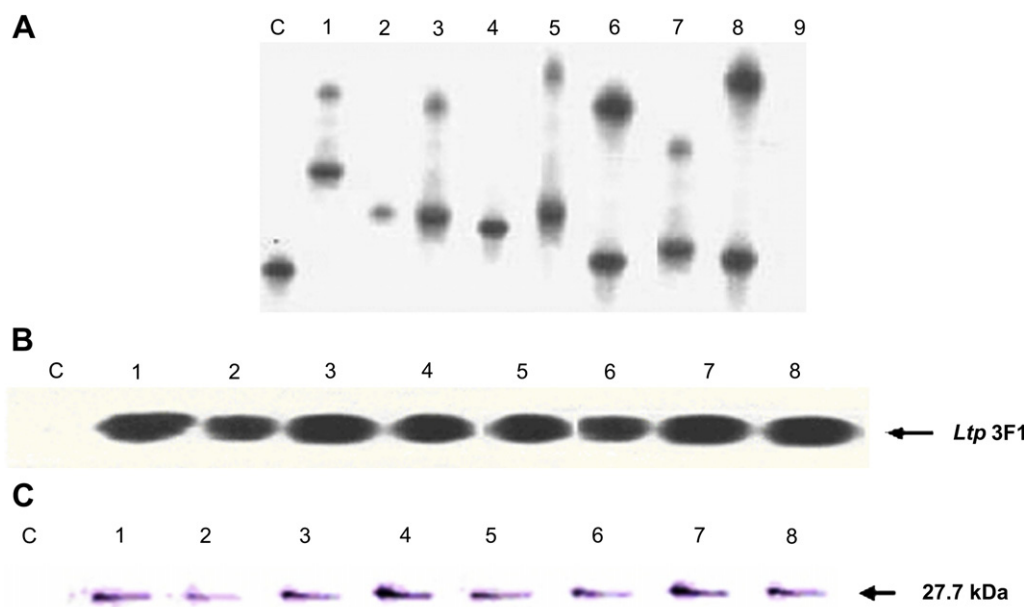


Fig. 5. Molecular analyses of transgenic of transgenic tobacco plants containing pCAMBIA-*cah-Ltp* 3F1. (A) Southern hybridization analysis. Genomic DNA of tobacco was digested with BamHI, electrophoresed and hybridized with ³²P-labeled *Ltp* 3F1 probe. Lane C1, positive control, pCAMBIA-*cah-Ltp* 3F1; lanes 1–8, transgenic lines, and lane 9, untransformed control plant. The number of bands reflects the number of transgenic insertions. (B) Northern hybridization analysis of RNA isolated from transgenic tobacco plants (lines 1–8) hybridized with ³²P-labeled *Ltp* 3F1 probe. Lane C1, untransformed control and lanes 1–8, total RNA from transgenic plants. (C) Western hybridization analysis of total protein extracted from transgenic tobacco plants (lines 1–8) hybridized with *cah* antibody. Lane C1, untransformed control and lanes 1–8, transgenic plants. Arrow indicates the Cah (27.7 kDa).

Table 1
Resistance in transgenic tobacco expressing LTP 3F1

Treatment	Fungus and diameter of lesion (cm) ($\pm$ S.E.)		
	<i>Bipolaris oryzae</i>	<i>Cy. scoparium</i>	<i>Alternaria</i> sp.
Control	3.0 $\pm$ 0.2 ^a	2.5 $\pm$ 0.3 ^a	2.0 $\pm$ 0.2 ^a
Transgenic lines			
1	1.2 $\pm$ 0.2 ^{cd}	1.3 $\pm$ 0.3 ^{bcd}	0.8 $\pm$ 0.2 ^c
2	1.4 $\pm$ 0.1 ^{bc}	1.2 $\pm$ 0.1 ^c	0.9 $\pm$ 0.1 ^c
3	1.1 $\pm$ 0.3 ^{cd}	1.4 $\pm$ 0.2 ^{bc}	1.0 $\pm$ 0.3 ^{bc}
4	1.3 $\pm$ 0.1 ^c	1.2 $\pm$ 0.1 ^c	1.0 $\pm$ 0.2 ^{bc}
5	1.2 $\pm$ 0.2 ^{cd}	1.3 $\pm$ 0.4 ^{bcd}	1.1 $\pm$ 0.2 ^{bc}
6	1.4 $\pm$ 0.1 ^{bc}	1.1 $\pm$ 0.2 ^{cd}	0.9 $\pm$ 0.4 ^{bc}
7	1.2 $\pm$ 0.4 ^{bcd}	1.4 $\pm$ 0.1 ^{bc}	1.0 $\pm$ 0.3 ^{bc}
8	1.3 $\pm$ 0.2 ^{bcd}	1.2 $\pm$ 0.2 ^{cd}	1.1 $\pm$ 0.2 ^{bc}

Means within the column followed by different letters are significantly different according to Duncan's multiple range test ($p = 0.05$). Data represent the mean of three replications with five leaves per replication.

4. Discussion

Plant diseases are a major concern to agriculture production. It has been reported that total loss as a consequence of plant diseases reaches 25% of the yield in Western countries and almost 50% in developing countries. Of this, one third yield loss is due to fungal diseases [30]. It is generally assumed that a wide variety of plant proteins play important roles in the defense of plants against bacterial and fungal pathogens. LTPs are members of PR-proteins and are ubiquitous in the plant kingdom [5]. Among LTPs, those proteins that can enhance inter-membrane transfer without lipid specificity are termed as non-specific lipid transfer proteins (ns-LTP). They form a multigenic family and more than 50 amino acid sequences of plant ns-LTPs are registered in the databanks. Until now, two main families with different molecular masses have been isolated, one composed by proteins of molecular mass of

about 9 kDa and the other, by proteins of molecular mass of 7 kDa, referred to as ns-LTP1 and ns-LTP2, respectively [31]. Plant ns-LTPs appear ideally designed to bind and carry not only lipids but also a variety of organic molecules, thus they are suitable for biotechnological applications [32]. Wang et al. [33] reported ns-LTP from mung bean with antifungal and antibacterial activities against *F. solani*, *F. oxysporum*, *Pythium aphanidermatum*, *Sclerotium rolfsii* and *Staphylococcus aureus*. Antimicrobial potential of LTPs against phytopathogens has been well documented [2]. Velazhahan et al. [6] reported the isolation of an antifungal protein purified from pearl millet seeds with extensive N-terminal sequence similarity to lipid transfer proteins of cotton, wheat and barley. An antifungal LTP protein from sunflower (*Helianthus annuus* L.) seeds showed inhibition of spore germination against *F. solani* f. sp. *eumartii* at a concentration of 40 μ g/ml and 50% mycelial growth inhibition at 6.5 μ g/ml (0.65 μ M) [7].

Phylogenetic analysis of amino acid sequences indicated that wheat LTP had higher sequence identity and similarity to LTPs of barley (CAA91436) and wheat (AAV28706). Amino acid sequence analysis of LTP3F1 suggested its typical features of plant LTPs, viz., absence of tryptophan and phenylalanine, and conservation of eight cysteine residues that could form a network of disulfide bridges necessary for the maintenance of the tertiary structure of the molecule together with the central helical core, while the variable loops would provide the sequences required for the specific functions of the proteins [34]. Structural biology is a complementary approach to search the biological function of proteins. Three-dimensional structure of wheat LTP 3F1 was simulated by the homology modeling tool MOE using the NMR structure of maize ns-LTP (1AFH) as template. Accordingly, the global fold involving four helical fragments connected by three loops

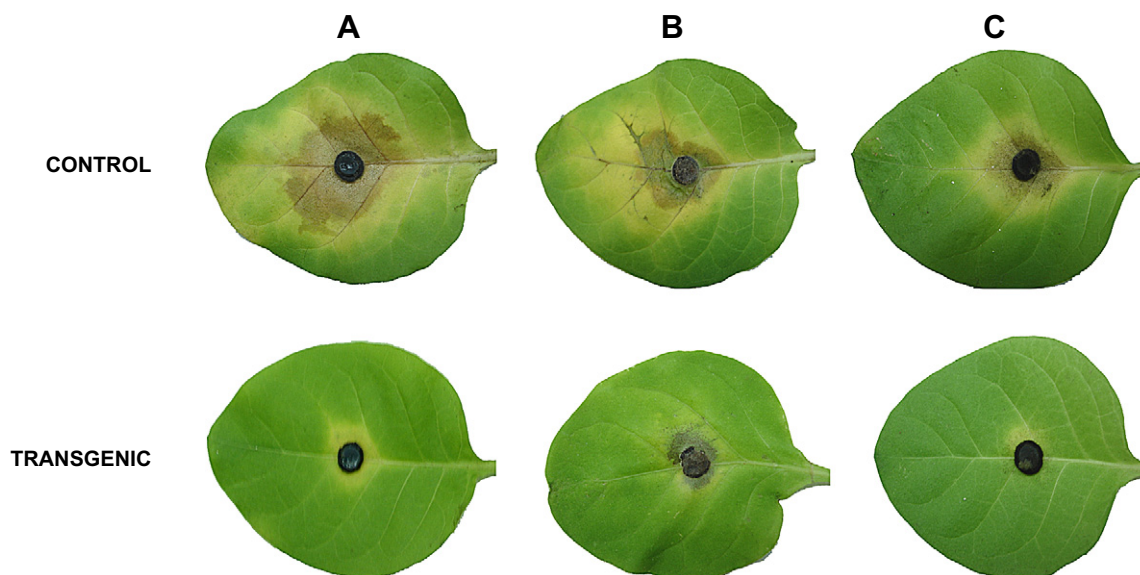


Fig. 6. Transgenic resistance of tobacco leaves expressing lipid transfer protein (*Ltp* 3F1). Detached leaf from control tobacco plant expressing *cah* alone showed spreading lesions due to (A) *Bipolaris oryzae*, (B) *Cylindrocladium scoparium* and (C) *Alternaria* sp. infection. Detached leaf from *Ltp*-expressing tobacco plant (line 2) showed smaller and restricted lesion. Observations were made 10 days after inoculation.

and a C-terminal tail without a regular secondary structure stabilized by four disulfide bridges presumably formed due to the presence of eight cysteine residues were predicted in wheat LTP 3F1. The striking feature of this structure is the existence of an internal hydrophobic cavity running through the whole molecule. As a whole, the global fold of this protein is similar to that of previously described LTP extracted from wheat seeds [35]. Based on the current knowledge of plant LTPs, the present study was aimed at characterization and overexpression of a wheat *Ltp* 3F1 gene and to evaluate its antifungal potential against devastating phytopathogenic fungi. We have successfully expressed the wheat *Ltp* 3F1 gene in the pMAL-p2x *E. coli* expression system and the protein was enriched using ammonium sulfate fractionation and gel permeation chromatography. However, we could not recover the purified LTP domain from the fusion protein after Factor Xa digestion. Because a wide range of proteins can be expressed and purified by fusion protein technology, in this study, expression vector (pMAL-p2x) with MBP as fusion partner was selected to construct the recombinant vector. The antifungal potential of recombinant fusion protein was evaluated using *in vitro* bioassays before being designated as antifungal.

Transgenic rice containing the homologous ns-LTP gene (*Ace*-AMP1) of *Allium cepa* showed antimicrobial activity towards *M. grisea*, *R. solani* and *X. oryzae* pv. *oryzae* [8]. Transgenic geranium carrying the same gene exhibited enhanced resistance against *Botrytis cinerea* [36]. In the present investigation we have constructed *Ltp* 3F1 harboring plant transformation gene cassette using pCambia 1300 as base vector and *cah* gene as marker. Gene cassette pCambia-*cah*-*Ltp* 3F1 was transformed into tobacco by the *Agrobacterium* method. Efficient regeneration of tobacco was achieved in selective medium and the transgenic plants showed normal phenotype with no indication of cytotoxicity due to expression of *cah* and *Ltp* 3F1. Molecular analyses of transgenic tobacco plants by PCR and Southern hybridization analyses confirmed *cah* and *Ltp* 3F1 gene integrations. The influence of transgene copy number on level of gene expression is known to be complex. Although it was anticipated that increasing transgene copy number would increase expression level, it is now known that multiple gene copies frequently lead to co-suppression and gene silencing [37]. Transgene copy number can be positively or negatively associated with transgene expression. However, it is generally considered that selecting plants with low gene copy number decreases the possibility of transgene co-suppression. Single or low gene copy (2 copies) integrations through *Agrobacterium*-mediated transformation have been well documented in this study as reported earlier [38]. Western and Northern hybridization analyses of transgenic tobacco confirmed the expression of *cah* and *Ltp* 3F1 respectively in transgenic lines. Results from detached leaf fungal assays showed the antifungal resistance of transgenic plants expressing LTP.

The present investigation reported an antifungal LTP and its heterologous expression. This is the first report to demonstrate that LTP from wheat has antifungal activity against major phytopathogenic fungi that attack economically important crops

such as rice, clover and banana. Due to the innate potential of broad-spectrum antimicrobial activity the wheat *Ltp* 3F1 gene can be efficiently employed for the genetic engineering of crops for disease resistance.

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