**Progress in Biological Sciences**
Vol. 5, Number 1, Winter / Spring 2015/133-142

# Antifungal activity of recombinant rice LTP2 on some phytopathogenic fungi

Received: 15 October, 2014; Accepted: 11 March, 2015

**Fatemeh Zebardast, Mohammadreza Zamani*, Mostafa Motallebi and Zahra Moghaddassi-Jahromi**

National Institute of Genetic Engineering and Biotechnology, Tehran, Iran

## ABSTRACT

Plant lipid transfer proteins (LTPs) are members of the pathogenesis-related proteins (PR-14) and some of them exhibit activity against phytopathogenic fungi. To investigate whether rice LTP2 plays a role in antifungal activity, the coding region of an Iranian rice *Ltp2* gene was cloned into expression vector pET24-d(+) and then expressed in *Escherichia coli* Rosetta strain (DE3). The potential antifungal activity of the purified LTP2 was tested on mycelial growth of some important phytopathogenic fungi. The results showed that the rice LTP2 inhibit the growth of *Rhizoctonia solani*, *Botrytis cinerea*, *Sclerotinia sclerotiorum*, *Fusarium sporotrichioides* and *F. oxysporum*. Also, the purified LTP2 protein was shown to strongly inhibit spore germination and consequential mycelia of *Alternaria brassicola*.

**Keywords:** antifungal activity, lipid transfer protein 2, phytopathogenic fungi, prokaryotic expression

* **Corresponding author:** zamani@nigeb.ac.ir

## Introduction

Plant lipid transfer proteins (LTPs) are small molecules (7-10 kDa), which are classified as pathogenesis-related proteins (PR14) (1, 2). They have eight strictly conserved cystein residues that are engaged in forming four disulfide bonds (3). LTPs are able to bind to a variety of lipid molecules and hydrophobic compounds and catalyze their transfer across biomembrane *in vitro* (4, 5). It has also been shown that LTPs are highly resistant to protease, heat and denaturants (6).

Conversely, LTPs are encoded by members of multigenic families (1, 23) which are isolated from various tissues and organs of higher plants such as wheat (7), rice (8), barley (9), maize (10) and *Arabidopsis thalia* (11). It has been shown that LTPs are possibly involved in a range of biological functions including membrane biogenesis (12), biosynthesis of cutin (13), somatic embryogenesis (14), plant defense against pathogen infection (1, 15) and plant response to abiotic stress (16). *In vitro* antimicrobial activities of LTPs and inducibility of the *Ltp* genes in response to pathogen infection, strongly suggest that plant LTPs participate in defense mechanisms against bacterial and fungal pathogens. However, the exact mechanism of their antimicrobial activity is still unclear (1, 17). Recent studies have been reported on the use of LTPs in transgenic plants. When the *Ltp* genes were over-expressed in rice (18), wheat (19) and tobacco (7, 20), they showed enhanced resistance to many fungal diseases. It was observed that LTPs synergistically enhance the antimicrobial activity of other antimicrobial peptides such as defensins and thionins (21).

In this study, to investigate the antifungal activity of LTP2, results on molecular cloning and over expression of this protein in *E. coli* and its fungal inhibition against some phytopathogenic fungi were reported.

## Materials and Methods

### Plant material and fungal strains

The Iranian rice seeds, *Oriza sativa* (TN-03-2219) were obtained from the Seed and Plant Improvement Institute, Karaj, Iran. The phytopathogenic fungal species used in this study include *Rhizoctonia solani* (ABRIICC RS46), *Botrytis cinerea* (ARBIICC Bc2), *Sclerotinia sclerotiorum* (ABRIICc Ss8), *Fusarium oxysporum* (ABRIICC Fo11), *F. sporotrichioides* (IRAN 994c) and *Alternaria brassicola* (ABRIICC Ab3) were obtained from Iranian Research Institute of Plant Protection, Tehran, Iran.

### Construction of recombinant plasmid

Genomic DNA was extracted from 180 mg of rice leaves using the plant DNA extraction MBST kit (Molecular Biological System Transfer, Iran). DNA quality was checked using gel electrophoresis.

The *Ltp*2 gene located on rice chromosome 3 has no intron (23). The PCR primers were designed based on the published sequence of rice *Ltp*2 gene (291 bp). The forward primer (Oltp2B) with *Bpi*I site 5'-GGAAGACAACATGATGAGGAAGTTGGCGGTG-3' and reverse primer (Oltp2X) with *Xho*I site 5'-CCGCTCGAGGTGGCAGGTGGGGAGGGC-3' were used in PCR solution. The amplified fragment was purified with PCR Fragment Recovery Kit (iNtRON Biotechnology). The purified fragment was double digested with *Bpi*I and *Xho*I, and thereafter, subcloned into *Nco*I/*Xho*I digested pET-24d(+) expression vector (Novagen) in frame to His-tag and under the control of T7 promoter, thereby forming the recombinant plasmid pETFZ. The sequence encoding LTP2 was confirmed by sequencing.

**Expression of rice LTP2 in *E. coli***

Recombinant plasmid pETFZ was transformed into *E. coli* Rosetta (DE3) (Novagen) and selected by 34 µg/ml chloramphenicol and 50 µg/ml kanamycine on LB media. An overnight pre-culture of a single colony was used to inoculate 200 ml of LB containing appropriate antibiotics, and the culture was allowed to grow at 37°C until it reached an $OD_{600}$ of 1. Protein expression was induced by adding 0.7 mM IPTG to the cell culture, and the cells were shacked at 37°C for 4 h. The cells were collected by centrifugation (4200 rpm, 20 min, 4°C) and stored at -20°C until use. The empty vector was also introduced into *E. coli* Rosetta (DE3) as a control.

**Purification of recombinant LTP2**

The His-tagged LTP2 was purified under native conditions using Nickel-nitrilotriacetic acid (Ni-NTA) resin (Qiagen). To achieve this, cell pellet was resuspended in 4 ml of the lysis buffer (50 mM $NaH_2PO_4$, 300 mM NaCl, 5 mM imidazole, pH 8.0). The resuspension was then sonicated (15 times for 30 s at amplifier 70 with 45 s of cooling between cycles). The supernatant was collected by centrifugation (13000 rpm, 20 min, 4°C). The supernatant was separated and loaded on the Ni-NTA His-bind resin column (3 ml). Unbounded proteins were washed from the column with the lysis buffer containing imidazole (15 mM). Finally, the bounded protein was eluted with elution buffer (50 mM $NaH_2PO_4$, 300 mM NaCl, 300 mM imidazole, pH 8.0). The purification procedure was carried out at 4°C and the purified LTP2 was checked on a 15% SDS-PAGE. Protein concentration was determined by the Bradford assay (22) using bovine serum albumin as a standard.

**Western blot**

For immunodetection of the expressed LTP2, total protein was extracted from induced *E. coli* Rosetta (DE3). The protein samples were electrophoresed on SDS-PAGE and thereafter, were electrotransferred to PVDF (polyvinylidene fluoride) membrane. The immunoblots were developed with antibody against His-tag, according to the manufacturer's instruction (Roche, USA)

***In vitro* antifungal activity assay**

For detection of antifungal activity of the purified LTP2, the fungal growth inhibition method was used. The zone of inhibition assay for antifungal activity was executed using petri plates containing potato dextrose agar (PDA) medium. After development of the mycelial colony, the appropriate holes were made on agar surface at a distance of 0.5 cm away from the rim of the mycelia.

Aliquots of the purified protein (containing 34 or 17 µg) in elution buffer were applied to the holes. The elution buffer and extracted protein from uninduced *E. coli* served as control. The plates were incubated at 25°C until the mycelia growth had enveloped peripheral holes containing the negative control and had produced crescent of inhibition around holes containing samples with antifungal activity.

**Spore germination inhibition assay and microscopic analysis**

Spore germination inhibition assay was performed using spore suspensions of phytopathogenic fungi. Fungus was grown on PDA media until spores were produced. The spores were collected by adding sterile water to the plate and counted by a homocytometer under microscope and adjusted to concentration of $10^6$ spores/ml with sterile water. Sterile microcentrifuge tubes were used to determine the ability of the purified LTP2 to inhibit germination of spores. Each tube contained 50 µl spore suspension, 100 µl YM liquid media (0.2% yeast extract, malt 2%)

and appropriate concentration of the purified LTP2. The elution buffer served as negative control. The treated and untreated spores were incubated at room temperature. Microscopic observation was carried out after 24 and 48 h by staining with fuchsin. To determine whether the LTP2 protein has fungistatic or fungicidal activity, 48 h treated and untreated spores were plated on PDA and incubated at room temperature for 3 days. Delayed germination of spores indicates fungistatic effect, whereas no germination indicates fungicidal activity of the LTP2 protein

## Results

### Molecular cloning and sequencing

In this research, in order to test the role of *Ltp2* in antifungal activity, the coding region of this gene was obtained by PCR using specific primers on genomic DNA extracted from the Iranian rice leaves and cloned into a cloning vector. The sequencing result showed that the coding region of *Ltp*2 is 291 bp long, which encodes a deduced protein of 96 amino acids (Fig. 1). The sequence of *Ltp*2 is deposited in GenBank (KF511587).

### Heterologous expression and purification of LTP2

The analysis of soluble proteins by SDS-PAGE revealed the presence of the His-tagged protein of expected size (10.5 kDa) in the induced clones (Fig. 2A). The expressed LTP2 protein was then purified by affinity chromatography using Ni-NTA spin column. Soluble proteins were added to the column and the His-tagged protein was eluted with 300 mM imidazole (Fig. 2A). Western blot analysis using anti His-tag confirmed the expressed protein band with expected molecular weight for LTP2 in crude and purified forms (Fig. 2B).

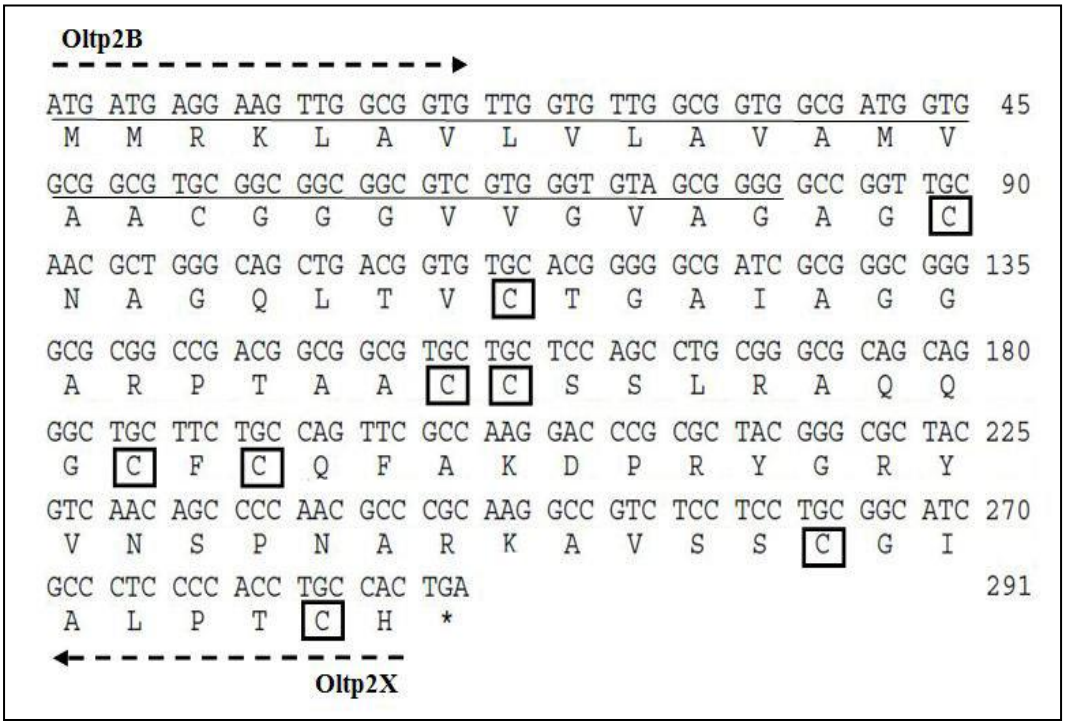


**Figure 1. Nucleotide sequence of rice *Ltp2* gene and its deduced amino acid sequence. Forward (Oltp2B) and reverse (Oltp2x) primers are indicated by arrows. Eight highly conserved cystein residues are boxed. The signal peptide is underlined.**

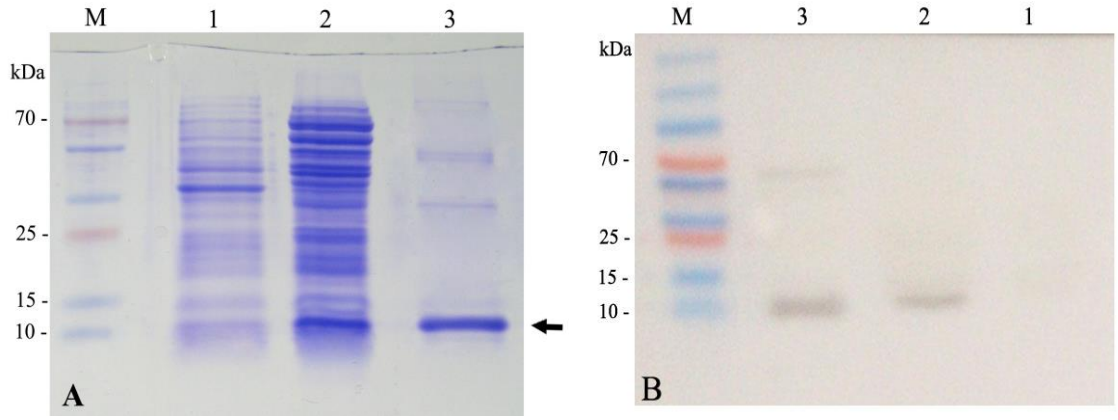


**Figure 2. Analysis of the protein expression, purification and Western blot of the LTP2. (a) SDS-PAGE analysis, (b) Western blot analysis using anti His-tag: 1) Total cell proteins from uninduced *E. coli*; 2) Soluble proteins from induced cells; 3) The partial purification profile of LTP2 using Ni-NTA His Bind Resin; M) Molecular weight marker. The arrow indicates the LTP2 (10.5 kDa).**

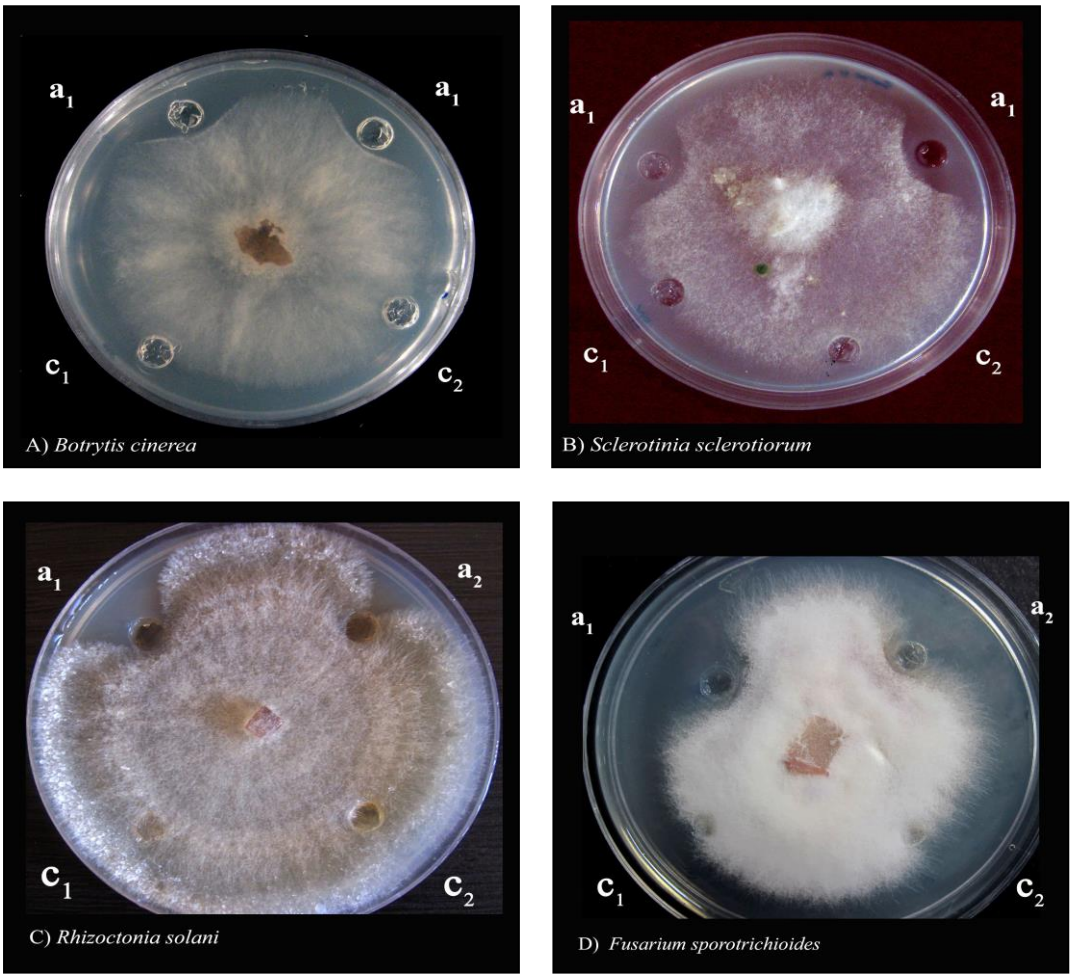


**Fig. 3. Continue**

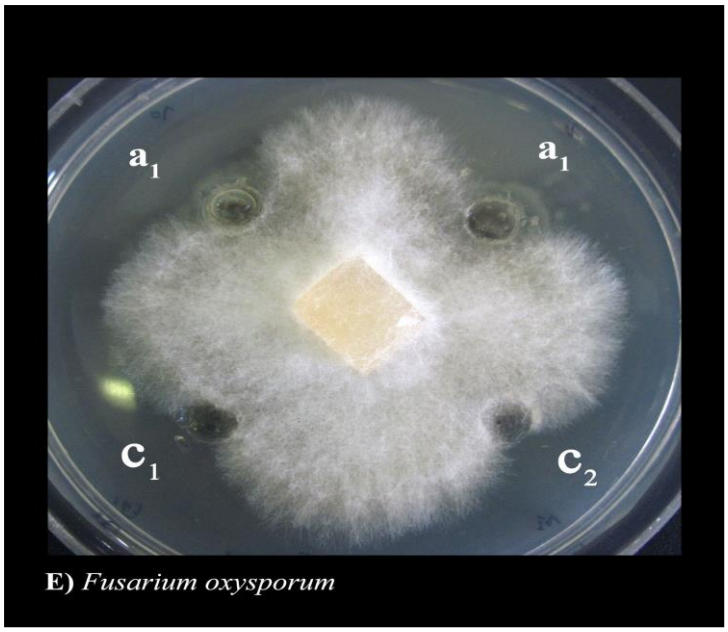


**Figure 3. Antifungal activity assay of the purified rice LTP2: (A) *B. cinerea* (B) *S. sclerotiorum* (C) *R. solani* (D) *F. sporotrichioides* (E) *F. oxysporum*. $a_1$ and $a_2$) 34 and 17 µg of the purified rice LTP2, respectively; $c_1$) Control containing elution buffer, pH 8.0; $c_2$) Control containing extracted protein from uninduced *E. coli.***

### *In vitro* antifungal activity assay

In order to test the antifungal activity of the prokaryotic expressed LTP2 on actively growing culture of a broad-spectrum of important phytopathogenic fungi: *R. solani, B. cinerea, S. sclerotiorum, F. sporotrichioides* and *F. oxysporum*, the purified protein was used in radial diffusion assay. It has been demonstrated that an aliquot of purified LTP2 (34 µg) showed antifungal activity towards these fungal pathogens and formed a crescent of growth inhibition around the purified protein (Fig. 3), whereas the elution buffer, as well as the proteins obtained in non-induced conditions (as negative controls) had no detectable effect on the fungi. The antifungal activity of the purified LTP2 was less effective against *F. oxysporum* mycelial growth (Fig. 3E).

### Spore germination inhibition assay

The purified expressed LTP2 was further tested for possible toxicity against spore germination of *A. brassicola*. Microscopic observations showed that 17 µg of the purified protein strongly inhibited spore germination and consequential mycelial growth even after 48 h as compared to the control (Fig. 4A). When LTP2 concentration decreased to 8.5 µg, the results showed that spore germination and mycelial growth were mainly inhibited (Fig. 4A).

This assay on *F. oxysporum* demonstrated that 17 µg of the purified LTP2 reduced the hyphal elongation, resulting in poor developed mycelia, while the control spores showed well developed growth of mycelium after 24 and 48 h of incubation (Fig. 4B).

Furthermore, when the treated spores of *A. brassicola* with 17 µg of LTP2 were plated on PDA medium, they exhibited germination and mycelial growth, suggesting the fungistatic effect of the protein (data not shown).

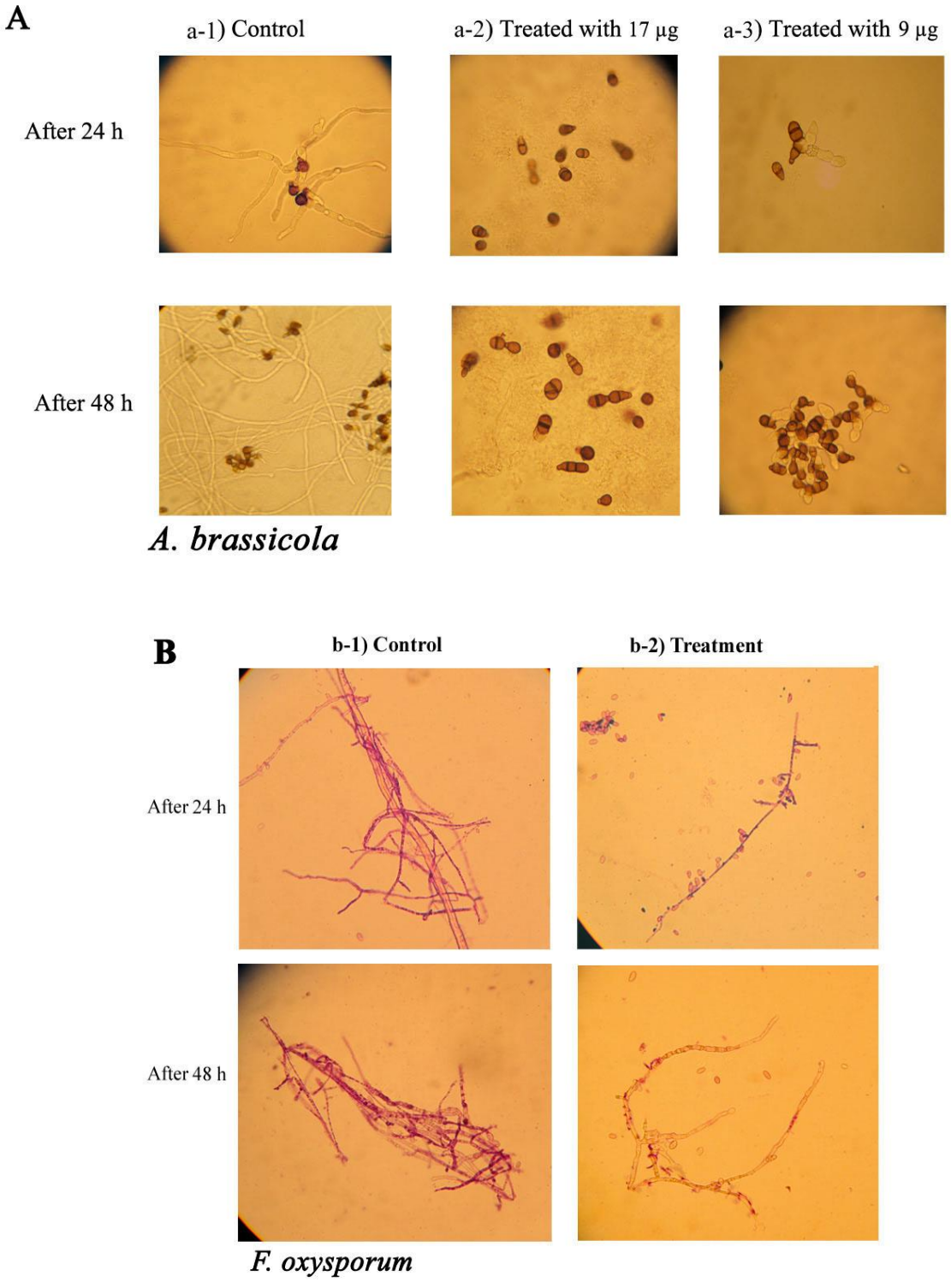


**Figure 4. Light microscopic observation of the treated and untreated spores. (a) *A. brassicola*: a-1) Untreated spores showed the growth of hypha after 24 h and profuse growth of hypha after 48 h; a-2) Spores treated with 17 µg of LTP2 showed no germination after 24 h and even after 48 h; a-3) Spores treated with 9 µg of LTP2 mainly showed inhibition of germination after 24 and 48 h. (b) *F.* oxysporum: b-1) Untreated spores showed normal well developed mycelia after 24 and 48 h; b-2) Spores treated with the purified LTP2 (17 µg) showed poor developed mycelia after 24 and 48 h of incubation.**

## Discussion

Fungal diseases are rated either the most important or the second most important factor contributing to yield losses in many important crops. Fungal diseases contribute about 30% of the total yield loss in some important crops (24). In the past two decades, extensive studies on PR proteins have been achieved by a large researchers (25-26). This high level of interest in PR proteins is mostly due to the antifungal property of these proteins (27). An important common feature of PR proteins, including some LTPs, is their antifungal effect (25,28).

It is reported that not all LTPs are able to inhibit the mycelial growth of fungi (1,17). The results of this study shows that rice LTP2 exhibits inhibitory activity against growth of some important phytopathogenic fungi: *R. solani, B. cinerea, S. sclerotiorum, F. sporotrichioides* and *F. oxysporum* and also strongly inhibited spore germination of *A. brassicola.*

In this research, the pET24-d(+) expression vector was used, because the use of this expression vector yielded appropriate amounts of a related protein in *E. coli* (data not shown). Also, the presence of the histidine-tag did not impair the inhibitory function of the recombinant protein. The same finding has also been reported by Gianotti *et al*. as they expressed two His-tagged cystatins (29).

Expression in *E. coli* as a host is an economical and fast way to produce proteins in an appropriate amount. Generally, protein expression is highly affected by different codon usage (30). *E. coli* Rosetta (DE3) strain compensates tRNA for the rare codons that are commonly used by eukaryotes but rarely used in *E. coli.* Since, there are some rare codons, especially AGG, among rice *Ltp*2 nucleotide sequence, to solve this inappropriate codon usage, the LTP2 in Rosetta (DE3) strain of *E. coli* was expressed in this study. As a result, this study successfully expressed the rice *Ltp*2 gene in prokaryotic expression system. The ability to inhibit the fungal growth of rice LTP2 confirms that the protein has been produced in biological active form.

Based on the results of this study, rice LTP2 exhibits antifungal activity against a range of phytopathogenic fungi at concentration of 34 µg. In a similar study, Ge *et al*. (31) reported that a rice LTP1 showed antifungal effect at concentration of 27 µg. Kirubakaran *et al*. demonstrated that 100 µg of LTP 3F1 protein from wheat inhibit the mycelial growth of a fungal broad-spectrum (7). Also in this research, 17 µg of rice LTP2 was shown to be able to inhibit completely spore germination of *A. brassicola,* while Kirubakaran *et al*. (2008) used 300 µg of purified wheat LTP 3F1 for the inhibition of spore germination of this fungus. Furthermore, this study's observation indicated that rice LTP2 slowed down the hyphal elongation of *F. oxysporum* after spore germination, but did not induce morphological distortions, and the new growing mycelia showed stunned mycelial elongation.

In conclusion, it was shown that LTP2 from rice has potential antifungal activity against some phytopathogenic fungi. These results suggest that the rice *Ltp*2 gene has the potential to be efficiently used for improving plant resistance against fungal pathogens.

## Acknowledgement

This project was supported by National Institute of Genetic Engineering and Biotechnology (NIGEB).

# REFERENCES


1. Kader, J.C. (1996) Lipid-transfer proteins in plants. *Annu. Rev. Plant Physiol Plant Mol. Biol*., **47**, 627–654.

2. Van Loon, L.C., Van Strien, E.A. (1999) The families of pathogenesis-related proteins, their activities and comparative analysis of PR-1 type proteins. *Physiol. Mol. Plant Pathol*., **55**, 85-97.

3. José-Estanyol, M., Gomis-Rüth, F.X., Puigdomènech, P. (2004) The eight-cysteine motif, a versatile structure in plant proteins. *Plant Physiol. Biochem.*, **42(5)**,355-365.

4. Samuel, D., Liu, Y.J., Cheng, C.S., Lyu, P.C. (2002) Purification and characterization of a novel 7-kDa non-specific lipid transfer protein-2 from rice (Oryza sativa). *J. Biol. Chem.*, **277**, 35267–35273.

5. Lee, J.Y., Min, K., Cha, H., Hwang, D.H.S.K.Y., Suh, S.W. (1998) Rice nonspecific lipid transfer protein: the 1.6A° crystal structure in the unliganded state reveals a small hydrophobic cavity. *J. Mol. Biol*., **276**, 437–48.

6. Lindorff-Larsen, K., Winther, J.R. (2001) Surprisingly high stability of barley lipid transfer protein, LTP1, towards denaturant, heat and proteases. *FEBS Lett*., **488**, 145–148.

7. Kirubakaran, S.I., Mubarak Begum, S.M., Ulaganathan, K., Sakthivel, N. (2008) Characterization of a new antifungal lipid transfer protein from wheat. *Plant Physiol. Bioch.*, **46**, 918-927.

8. Liu, Y.J., Samuel, D., Lin, C.H., Lyu, P.C. (2002) Purification and characterization of a novel 7-kDa non-specific lipid transfer protein-2 from rice (Oryza sativa). *Biochem. Biophys. Res. Commun*., **294**, 535–540.

9. Gorjanovic, S., Spillner, E., Beljanski, M., Gorjanovic, R., Pavlovic, M., Gojgic-Cvijovic, G. (2005) Malting barley grain non/specific lipid-transfer protein (ns-LTP): importance for grain protectio. *J. Inst. Brew*., **111(2)**, 99–104

10. Castro, M.S., Gerhardt, I.R., Orru, S., Pucci, P., Bloch, C.Jr. (2003) P urification and characterization of a small (7.3 kDa) putative lipid transfer protein from maize seeds. *J. Chromatogr. B.,* **794**, 109-114.

11. Segura, A., Moreno, M., Garcia-Olmedo, F. (1993) Purification and antipathogenic activity of lipid transfer proteins (LTPs) from the leaves of *Arabidopsis* and spinach. *FEBS Lett*., **332(3)**, 243-246.

12. Arondel, V., Vergnolle, C., Tchang, F., Kader, J.C. (1990) Biofunctional lipid-transfer: Fatty acid-binding proteins in plants. *Mol. cell. Biochim*., **98**, 49-56.

13. Sterk, P., Booij, H., Scheleekens, G.A., Van Kammen, A., de Vries, S.C. (1991) Cell specific expression of the carrot EP2 lipid transfer protein gene. *Plant Cell*., **3**, 907-921.

14. Coutos-Thevenot, P., Jouenne, T., Maes, O., Guerbette, F., Grosbois, M., Le Caer, J.P., Boulay, M., Deloire, A., Kader, J.C., Guerin, J. (1993) Four 9-kDa proteins excreted by somatic embryos of grapevine are isoforms of lipid transfer proteins. *Eur. J Biochem*., **217**, 885-889.

15. Buhot, N., Douliez, J.P., Jacquemard, A., Marion, D., Tran, V., Maume, BF., *et al*. (2001) A lipid transfer protein binds to a receptor involved in the control of plant defence responses. *FEBS Lett.*, **509**, 27–30.

16. Torres-Schumann, S., Godoy, J.A., Pintor-Toro, J.A. (1992) A probable lipid transfer protein gene is induced by NaCl in stems of tomato plants. *Plant Mol. Biol*., **18**,749-757.

17. Carvalho, A.O., Gomes, V.M. (2007) Role of plant lipid transfer proteins in plant cell physiology: A concise review. *Peptides*., **28**, 1144-1153.

18. Patkar, R. N., Chattoo, B. B. (2006) Transgenic indica rice expressing ns-LTP-like protein shows enhanced resistance to both fungal and bacterial pathogens. *Mol. Breeding*., **17**, 159-171.

19. Roy-Barman, S., Sautter, C., Chattoo, B.B. (2006) Expression of the lipid transfer protein Ace-AMP1 in transgenic wheat enhances antifungal activity and defense responses. *Transgenic Res*., **15(4)**, 435-446.

20. Sarowar, S., Kim, Y.J., Kim, K.D., Hwang, B.K., Ok, S.H., Shin, J.S. (2009) Overexpression of lipid transfer protein (LTP) genes enhances resistance to plant pathogens and LTP functions in long-distance systemic signaling in tobacco. *Plant Cell Rep*., **28**, 419-427

21. Marion, D., Douliez, J.P., Gautier, M.F., Elmorjani, K. (2004) Plant Food Allergens. In Mills, E.N.C., Shewry, P.R. (eds.), Plant lipid transfer proteins: relationships between allergenicity and structural, biological and technological properties. Blackwell Science, Oxford, UK, pp. 57-69.

22. Bradford, M.M. (1976) A rapid and sensitive method for the quantiWcation of microgram quantities of protein utilizing the principle of protein–dye binding, Anal. *Biochem*., **72**, 248-254.

23. Boutrot, F., Chantret, N., Gautier, M.F. (2008) Genome-wide analysis of the rice and arabidopsis non-specific lipid transfer protein (nsLtp) gene families and identification of wheat *nsLtp* genes by EST data mining. *BMC Genomics*., **9**, 86.

24. Adams, D.J. (2004) Fungal cell wall chitinase and glucanases. *Microbiol.*, **50**, 2029-2035.

25. Selitrennikoff, CP. (2001) Antifungal Proteins. *Appl. Env. Microbiol*., **67**, 2883-2894.

26. Velazhahan, R., Jayaraj, J., Jeoung, J.M., Liang, G.H., Muthukrishnan, S. (2002) Purification and characterization of an antifungal thaumatin-like protein from sorghum (Sorghum bicolor (L) Moench) leaves. *J. Plant Dis. Protect*., **109**, 452-461.

27. Edreva, A. (2005) Pathogenesis-related proteins: research progress in the last 15 years. *Appl. Plant Physiology*., **31**, 105-124.

28. Van Loon, L.C. (2001) Book of abstracts. Spa, Belgium, pp. 9.

29. Gianotti, A., Rios, W.M., Soarescosta, A., Nogaroto, V., Carmona, A.K., Oliva, M.L.V., Andrade, S.S., Henrique-Silva, F. (2006) Recombinant expression, puriWcation, and functional analysis of two novel cystatins from sugarcane (*Saccharumo Ycinarum*). *Protein Expres. Purif*., **47**, 483-489.

30. Gustafsson, C., Govindarajan, S., Minshull, J. (2004) Codon bias and heterologous protein expression. *Trends Biotechnol*., **22**, 346-353.