J Genet Resour2020;6(1): 41-48 *Homepage: http://sc.journals.umz.ac.ir/*

RESEARCH ARTICLE *DOI: 10.22080/jgr.2020.17859.1171*

J|G|R

In Silico Analysis and Expression of Osmotin-EAAAK-LTP Fused Protein

Minoo Partovi Nasr, Mostafa Motallebi, Mohammad Reza Zamani* and Esmat Jourabchi

National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran

ARTICLE INFO

Article history:
Received 16 December 2019
Accepted 17 January 2020
Available online 29 January 2020

Keywords:
Antifungal activity
In silico
Lipid transfer protein
Osmotin
Pathogenesis-related proteins

Corresponding author:
✉ M. Zamani
zamani@nigeb.ac.ir

p-ISSN 2423-4257
e-ISSN 2588-2589

ABSTRACT

Antifungal agents are causing different problems in the agriculture industry. Plants are using various defense mechanisms for resistance against fungal pathogens. Some examples of these mechanisms are making physical barriers, producing chemical components and pathogenesis-related proteins such as lipid transfer protein (LTP) and Osmotin which can inhibit the growth of fungi at micro-molar concentrations. In this study, *Osmotin* and *LTP* genes were fused by the EAAAK linker to produce a single-fused gene construct. An in silico approach was used to predict and analyze Osmotin-EAAAK-LTP fused protein. Secondary and tertiary structure and mRNA formation of fused protein were predicted using bioinformatics tools. The designed construct was chemically synthesized and cloned in the pUC57 cloning vector. To express the fused protein gene was subcloned in expression vector pET-21b (+) with a hexahistidine tag. This gene was used for prokaryotic expression in *E.* coli BL21 (DE3) host. Different expression conditions were examined for expressing of fused protein. The fused protein was expressed with 1 mM IPTG after 3 hours of incubation at 28°C. The expression of 36.5 kDa protein was confirmed by western blotting. The study of antifungal activity of expressed fused protein was achieved by radial diffusion assay. This protein was able to exhibit antifungal activity towards experimented plant pathogenic fungi under *in vitro* conditions.

Please cite this paper as: Partovi Nasr M, Motallebi M, Zamani MR, Jourabchi E. 2020. *In silico* analysis and expression of osmotin-EAAAK-LTP fused protein. *J Genet Resour* 6(1): 41-48. doi: 10.22080/jgr.2020.17859.1171

Introduction

Plants have different mechanisms for fighting against fungal pathogens. Some examples of these mechanisms are making physical barriers, producing chemical components, hypersensitive response (HR) and production of pathogenesis-related proteins (PRPs) such as Osmotin and LTP (Freeman and Beattie, 2008). These proteins have low molecular weight and they show resistance to low pH rate (pH<3), heat and proteases. Pathogenesis-related proteins are categorized into 14 various groups (Van Loon and Van Strien, 1999). Osmotin and Lipid Transfer Protein (LTP) are in PR-5 and PR-14 families, respectively (Kader, 1996; Mani *et L.*, 2012). An important common feature of most PRs is their antifungal effect; some PRs exhibited also antibacterial, insecticidal, nematicidal and antiviral action. Plasma membrane permeability of PR-5 and PR-14 proteins contribute to plasmolysis and damage of fungal and bacterial pathogens, inhibiting their growth and development (Edreva, 2005). LTPs in plants are small homogeneous high-frequency proteins (9-10 kDa) which often have 8 cysteines with 4 disulfide bonds (de Oliveira Carvalho and Gomes, 2007). These proteins have two subfamilies: LTP1 and LTP2 (Douliez *et al.*, 2001). Studies in LTPs demonstrate high structural similarities between different plant species such as rice, corn and wheat (Sarowar *et al.*, 2009). Osmotins are 26 kDa which classified in different groups based on their basic primary structures and biological and enzymatic activities (Min *et al.*, 2004). The members of the PR-5 family are homologous to Thaumatin sweet proteins (TLPs) (Singh *et al.*, 1987). Researches revealed that Osmotin and LTP proteins have

antifungal effects against wide ranges of fungal pathogens (Abad *et al.*, 1996; Kim *et al.*, 2002).
Fusion proteins have two or more different domains in one molecule which are constructed naturally or synthetically. These proteins provide new functional activities for microorganisms at molecular levels (Aroul-Selvam *et al.*, 2004). End to end genetic fusion of sequences is an easy way to fuse genes and it can be expressed as one peptide chain in an appropriate host organism. Recombination and fusion of two proteins with similar activities can enhance their special characteristics. For instance, the fusion of two antifungal proteins can induce resistance in plants to higher ranges of fungal pathogens (Karri and Bharadwaja, 2013). Tandem fusion of proteins is performed by the connection of two separate proteins using peptide linkers. The existence of linkers is ubiquitously observed in naturally occurring multi-domain proteins with the function of maintaining the necessary distance to reduce steric hindrance and/or permit favorable domain-domain interaction between two protein moieties (Yu *et al.*, 2015). Researchers have employed various types of naturally occurring linkers in their synthetic fusion constructs. For example, the immunoglobulin hinge region functions as a linker in many recombinant therapeutic proteins (Wu *et al.*, 2007).
This study aimed to design fused *Osmotin* and *LTP* genes, investigation of prokaryotic expression and assaying its antifungal activity. This study deals with *in silico* analysis of *Osmotin* and *LTP* genes fusion in different databases using the EAAAK linker. We report the activity of the fused protein *in vitro* against different plant pathogenic fungi.

Material and Methods

In silico analysis

The gene sequences were obtained from the NCBI GenBank (www.ncbi.nlm.nih.gov/genbank/) (*Osmotin* (X95308) and *LTP* (U16721.1)) and saved in FASTA format. The sequences were translated into amino acid sequences using the ExPASy website. Secondary and tertiary structure and mRNA formation of fused protein were predicted using bioinformatics tools. Analysis tools for the design of fused protein were obtained from the mfold RNA Fold web server, PSIPRED website, I-TASSER website, and SynLinker web system.

Expression

The sequence of the fused gene was optimized to match codons of the host. The codons of the fused gene were chosen based on the selected host. Rare codons of *Osmotin* and *LTP* genes were verified and Osmotin-EAAAK-LTP synthesized in pUC57 cloning vector (Shanghai ShineGene Molecular Biotech, P. R. China). The fused gene was isolated from pUC57 by enzymatic double digestion using *Nco*I and *Xho*I and the 927 bp sequence was obtained.
The fused gene was subcloned in pET-21b (+) (Novagen, USA), prokaryotic expression vector with an inbuilt His-tag. Fused protein was expressed in BL21 (DE3) (Novagen, USA). To optimize the fused protein expression, tests were designed to examine the effect of induction time (h), temperature (°C) and IPTG concentration (mM). The experiment was managed using four levels for each factor. Variable factors employed include: a) IPTG concentration of 0.2, 0.5, 0.7 mM and 1, b) Incubation time of 1,3,6 and 24 hour and c) Temperature of 23,28,33 and 37 °C.
The recombinant plasmid containing the fused gene was transformed into *E*. coli BL21 (DE3). An overnight pre-culture of a single colony was used to inoculate 100 ml of Luria-Bertani (LB) media which supplemented with Ampicillin (100 mg/ml). It was grown at 37 °C until an optical density (OD600) of 0.6 was reached. Protein expression was induced by adding 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) to the culture at 28 °C for 3 hours and cells were shaken. Expression was confirmed by western blotting protein assay.

Western blotting analysis

For immunodetection of the expressed fused protein, protein samples were electrophoresed using SDS-PAGE (12%), followed by electrotransfer to a polyvinylidene fluoride (PVDF) membrane. The immunoblots were developed with an antibody against His-tag, according to the manufacturer's instructions (Roche, USA). The anti-His tag antibody against pET-21b(+) expressing vector has been conjugated to horseradish peroxidase (HRP). 4-

Chloro-1-naphthol was used as a substrate for HRP results in a colored precipitate.

Antifungal assays

For detection of antifungal activity of fused protein radial diffusion assay as a fungal growth-inhibitory test was used. The extracted protein of non-induced bacteria and bacteria with an empty vector considered a negative control.

In radial diffusion assay, the area of growth inhibition for antifungal activity based on the modified method of Broglie *et al*. (Brogue *et al.*, 1991) was checked using 100 × 15 mm Petri plates containing 25 mL of potato dextrose agar (PDA). After the mycelia colony had expanded, 5 mm holes were made at a distance of 2-5 mm away from the rim of the mycelial colony. After the addition of protein, the plates were incubated at 28 °C until mycelia growth has enveloped a peripheral hole containing the negative control and had produced crescents of inhibition around the holes containing fused protein. The experimented fungal species were *Botrytis cinerea, Sclerotinia sclerotiorum, Verticillium dahlia and, Rhizoctonia solani*.

Results

The main purpose of this work was to construct a fused protein of Osmotin-EAAAK-LTP to obtain antifungal activity. Bioinformatics observations illustrated that between different possible linkers [AAA, GGGGS, $(EAAAK)_n$, etc.] EAAAK was the best choice. α-helical structure of the EAAAK linker helps Osmotin and LTP proteins keep their intact structures and prevents protein domains interactions.

The secondary structure of mRNA was predicted by the mfold RNA Fold web server. Collected data showed that 5' segment of mRNA (starting of translation) do not have inappropriate structure. Moreover, minimal free energy (MFE) of this structure is acceptable energy for the translation of intended mRNA and it is suitable for the prokaryotic expression system (Fig. 1).

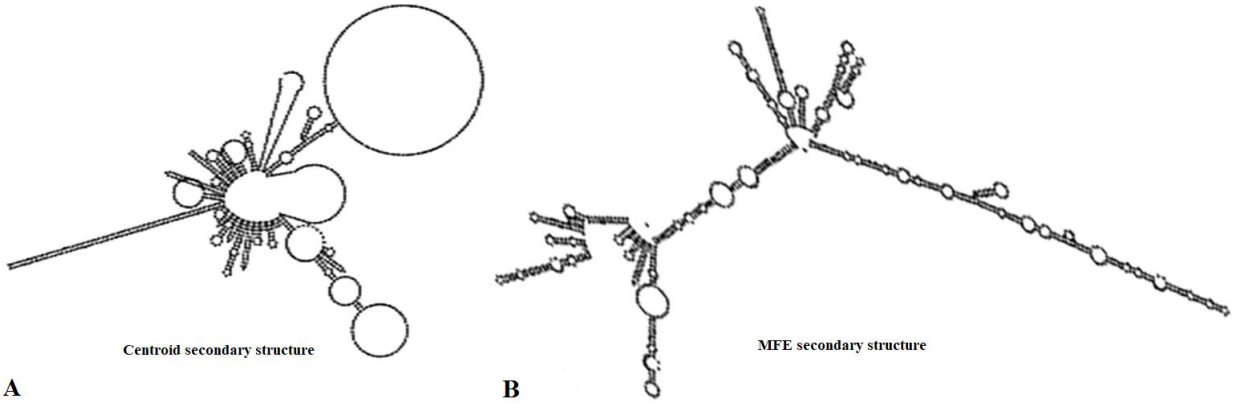

Fig. 1. Prediction of the secondary structure of mRNA of fused protein: A) Secondary structure of mRNA of LTP and Osmotin genes; B) minimal free energy (MFE) of mRNA secondary structure.

Prediction of the secondary structure of the fused protein was obtained by PSIPRED website. PSIPERD servers were employed to calculate the α-helix, β-sheet, and random coil probability at each amino acid position and to make an initial prediction with the highest probabilities. The available results illustrated that the linker has an α-helix structure which facilitates the separation of proteins and it is a barrier for their interactions. Also, Osmotin and LTP proteins maintained their structures and no changes found (Fig. 2). The tertiary structure of the fused protein was predicted by I-TASSER website (Yang and Zhang, 2015). Based on this webserver, the mentioned protein domains were separated and do not connect. Therefore, it is anticipated that they can function independently. Based on the analysis by SynLinker web system N-terminal of Osmotin and C-terminal of LTP proteins used for fusion. The results of bioinformatics prediction demonstrated that the EAAAK linker could detach proteins perfectly (Fig. 3).

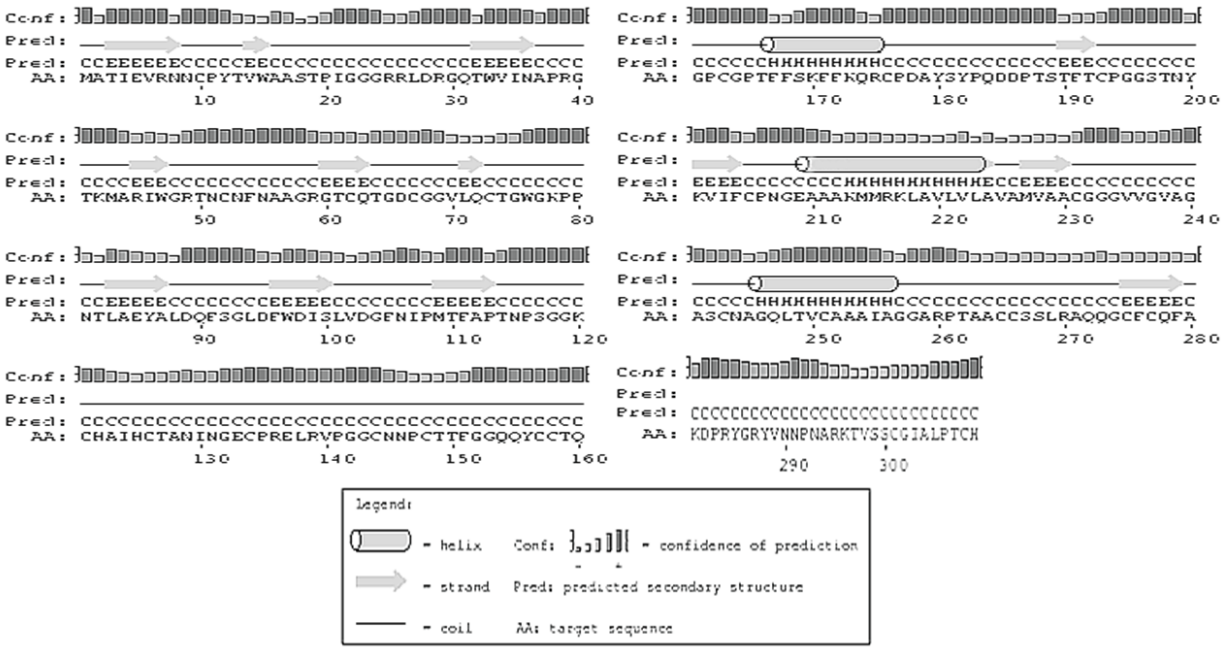

Fig. 2. Prediction of the secondary structure of the fused protein.

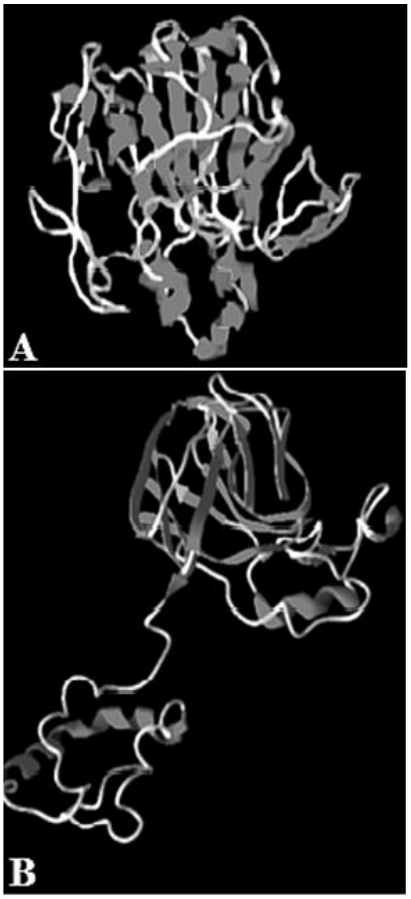

Fig. 3. Prediction of the tertiary structure of fused protein: A) I-TASSER web server; B) SynLinker web server.

The expected size of the His-tagged recombinant fusion protein is 36.5 kDa which calculated from the deduced amino acid sequence (Gasteiger *et al.*, 2005). The 36.5 kDa protein band was confirmed by western blot analysis. Transformed *E.* coli with an empty vector used as a negative control (Fig. 4).

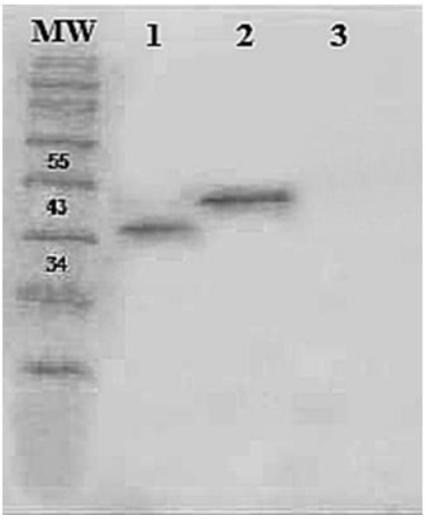

Fig. 4. Western blot showing the comparison of banding patterns of the fused protein and 50 kDa known protein: Line1= fused protein; Line 2= known protein (~50 kDa) as a positive control; Line 3= negative control (transformed *E. coli* with empty vector); MW= Molecular weight marker.

The antifungal activity of the fused protein was investigated using a radial diffusion assay. To study the antifungal activity of the fused protein, the expressed protein was extracted from transformed bacteria. The inhibitory effect was evaluated *in vitro* against four major phytopathogenic fungi. According to radial diffusion assay results, the hyphal growth of *S. sclerotiorum*, *V. dahlia*, and *R. solani* substantially decreased using fused protein. No inhibition was observed in the *B. cinerea* fungus (Fig. 5).

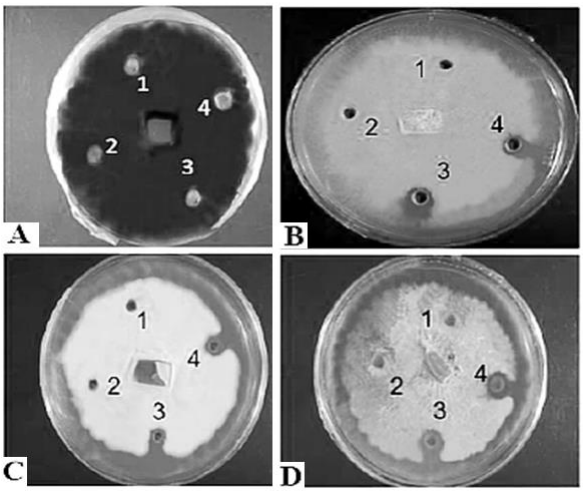

Fig. 5. Radial diffusion assay: A) *B. cinerea*; B) *S. sclerotiorum*; C) *V. dahila* D) *R. solani.* [1= Protein of non-induced bacteria; 2= Protein of bacteria with empty vector (as negative controls); 3 and 4= Extracted fused protein (two replicates)].

Discussion

Plants are exposed to a large number of pathogenic fungi; although they do not have an immune system, they have evolved a variety of potent defense mechanisms, including the synthesis of low-molecular-weight compounds, proteins and peptides that have antifungal activity (Tulasi and Nadimpalli, 1997). Osmotin and LTP are two important members of pathogenesis-related proteins (PRPs), which have a significant role in plant defense mechanisms (Van Loon and Van Strien, 1999). PRPs have destructive effects on the plasma membrane, cell wall, and growth of fungi (Edreva, 2005). Osmotin alters the plasma membrane and the osmotic equilibrium of the cell (Lorito *et al.*, 1996). It has antifungal activity both *in vitro* and *in vivo* (Liu *et al.*, 1996) and it works as penetrating the fungal membrane and finally causes cell death (Narasimhan *et al.*, 2005). The defensive role of plant LTPs is their ability to inhibit fungal pathogens (Blilou *et al.*, 2000). Antifungal tests of LTP protein isolated from sunflower demonstrated that it exerts a fungistatic effect (Regente and De La Canal, 2000).

Gene fusion techniques suggested that by fusion of two or more genes, the resulting fused protein could exhibit multiple functional properties derived from each of its components (Uhlén *et al.*, 1992). It was reported that transgenic plants expressing fused proteins revealed resistance to a wide range of pathogenic fungal and bacterial agents (Osusky *et al.*, 2004). In another study, it was shown that the tandem combination of two plant Defensins by a linker peptide sequence exhibited potent antifungal action towards plant pathogenic fungi (*Botrytis cinerea*, *Fusarium moniliforme*, and *Rhizoctonia solani*) (Karri and Bharadwaja, 2013).

The successful construction of fused proteins requires the selection of desired component proteins and suitable linkers to connect protein domains (Chen *et al.*, 2010). The design and construction of fusion protein have been developed by the end-to-end method (Yu *et al.*, 2015). Linkers in fusion proteins generally consist of stable peptide sequences, including the glycine-serine linker $(GGGGS)_n$ and α-helix-forming peptide linkers, such as $A(EAAAK)_nA$ (n= 2–5), which can provide structure flexibility, improve protein stability, or increase biological activity (Amet *et al*., 2009; Bai and Shen, 2006; Uhlén *et al.*, 1992; Wriggers *et al.*, 2005). In tandem fusion protein use of the linker is critical to maintaining the main features of proteins intact (Zhang *et al.*, 2009). Studies revealed that the EAAAK linker with α-helical structure can keep distance between proteins and minimize the domain interaction (Arai *et al.*, 2004). Comparative study of two fused genes employed helical (EAAAK) and non-helical (GGGGS) linker sequences indicates that helical linker to fused hybrid protein had much higher antimicrobial activity in comparison with non-helical linker (Lee *et al.*, 2013). The results of this study indicate that the EAAAK motif can be used to effectively separate two proteins and produce a fused protein. The first 80 nucleotides

of the *Osmotin* gene which was related to the pro-peptide sequence was omitted from the final sequence. Furthermore, this gene had a vacuolar signal which was not functional in the prokaryotic host and it was deleted. Bioinformatics data indicated that Osmotin protein should be located first in the fused protein to let its three-dimensional steric structures preserved. In this work, we report cloning, expression and antifungal activity of fused protein Osmotin-EAAAK-LTP. The *E. coli* system has been used to produce numerous amounts of recombinant proteins because of ease of handling, inexpensive media and large-scale production (Makrides, 1996).

Many reports are indicating that fusion proteins have antifungal activity for protecting plants (Karri and Bharadwaja, 2013; Li *et al.*, 2008; Patkar and Chattoo, 2006). The antifungal activity of the recombinant fused protein was tested *in vitro* by using plant pathogens. This to our knowledge is a first report of expressing antifungal fused protein made up of Osmotin and LTP in bacteria.

In conclusion, fused of Osmotin and LTP proteins let us produce a fused protein against phytopathogenic fungi. The design of fused protein with the EAAAK linker was assayed by various bioinformatics analyses. Web-based analysis admitted that Osmotin and LTP can be fused with EAAAK linker without spatial avoidance. In this research, the results based on the antifungal activity revealed that Osmotin-EAAAK-LTP has inhibitory effects against some experimented fungi. This fused protein represents an antifungal activity which can be utilized for biotechnological applications to reduce dependence on chemical fungicides.

Acknowledgments

This research was financially supported by the National Institute of Genetic Engineering and Biotechnology (NIGEB) of I. R. Iran. The authors thank the 568 project in the National Institute of Genetic Engineering and Biotechnology (NIGEB) of I. R. Iran.

Conflicts of interest

The authors have no conflict of interest to declare.

References

Abad LR, D'Urzo MP, Liu D, Narasimhan ML, Reuveni M, Zhu JK, Bressan RA. 1996. Antifungal activity of tobacco osmotin has specificity and involves plasma membrane permeabilization. Plant Sci 118(1): 11-23.

Amet N, Lee HF, Shen W. 2009. Insertion of the designed helical linker led to increased expression of tf-based fusion proteins. Pharm Res 26(3): 523-528.

Arai R, Wriggers W, Nishikawa Y, Nagamune T, Fujisawa T. 2004. Conformations of variably linked chimeric proteins evaluated by synchrotron X-ray small-angle scattering. Proteins 57: 829-838.

Aroul-Selvam R, Hubbard T, Sasidharan R. 2004. Domain insertions in protein structures. J Mol Biol 338(4): 633-641.

Bai Y, Shen WC. (2006). Improving the oral efficacy of recombinant granulocyte colony-stimulating factor and transferrin fusion protein by spacer optimization. Pharm Res 23(9): 2116-2121.

Blilou I, Ocampo JA, García-Garrido, JM. 2000. Induction of Ltp (lipid transfer protein) and Pal (phenylalanine ammonia-lyase) gene expression in rice roots colonized by the arbuscular mycorrhizal fungus Glomus mosseae. J Exp Bot 51(353): 1969-1977.

Brogue K, Chet I, Holliday M, Cressman R, Biddle P, Knowlton S, Broglie R. 1991. Transgenic plants with enhanced resistance to the fungal pathogen Rhizoctonia solani. Science 254(5035): 1194-1197.

Chen X, Bai Y, Zaro JL, Shen WC. 2010. Design of an in vivo cleavable disulfide linker in recombinant fusion proteins. Biotechniques 49(1): 513-518.

Carvalho Ade O, Gomes VM. 2007. Role of plant lipid transfer proteins in plant cell physiology-a concise review. Peptides 28(5): 1144-1153.

Douliez JP, Pato C, Rabesona H, Mollé D, Marion D. 2001. Disulfide bond assignment, lipid transfer activity and secondary structure of a 7-kDa plant lipid transfer protein, LTP2. Eur J Biochem 268(5): 1400-1403.

Edreva A. 2005. Pathogenesis-related proteins: research progress in the last 15 years. Gen Appl Plant Physiol 31(1-2): 105-124.

Freeman BC, Beattie GA. 2008. An overview of plant defenses against pathogens and herbivores. The Plant Health Instr 6(11). doi: 10.4172/2157-7471.1000322.

Gasteiger E, Hoogland C, Gattiker A, Wilkins MR, Appel RD, Bairoch A. 2005. Protein identification and analysis tools on the ExPASy server. The proteomics protocols handbook, Springer.

Kader JC. 1996. Lipid-transfer proteins in plants. Annu Rev Plant Biol 47(1): 627-654.

Karri V, Bharadwaja KP. 2013. Tandem combination of Trigonella foenum-graecum defensin (Tfgd2) and Raphanus sativus antifungal protein (RsAFP2) generates a more potent antifungal protein. Funct Integr Genomics 13(4): 435-443.

Kim H, Mun JH, Byun BH, Hwang HJ, Kwon YM, Kim SG. 2002. Molecular cloning and characterization of the gene encoding osmotin protein in Petunia hybrida. Plant Sci 162(5): 745-752.

Lee M, Bang K, Kwon H, Cho S. 2013. Enhanced antibacterial activity of an attacin-coleoptericin hybrid protein fused with a helical linker. Mol Biol Rep 40(6): 3953-3960.

Li HP, Zhang JB, Shi RP, Huang T, Fischer R, Liao YC. 2008. Engineering Fusarium head blight resistance in wheat by expression of a fusion protein containing a Fusarium-specific antibody and an antifungal peptide. Mol Plant Microbe interact 21(9): 1242-1248.

Liu D, Rhodes D, D'Urzo MP, Xu Y, Narasimhan ML, Hasegawa PM, Abad L. 1996. In vivo and in vitro activity of truncated osmotin that is secreted into the extracellular matrix. Plant Sci 121(2): 123-131.

Lorito M, Woo SL, D'ambrosio M, Harman GE, Hayes CK, Kubicek CP, Scala F. 1996. Synergistic interaction between cell wall degrading enzymes and membrane affecting compounds. Mol Plant Microbe Interact 9(3): 206-213.

Makrides SC. 1996. Strategies for achieving high-level expression of genes in Escherichia coli. Microbiol Mol Biol Rev 60(3) 512-538.

Mani T, Sivakumar KC, Manjula S. 2012. Expression and functional analysis of two osmotin (PR5) isoforms with differential antifungal activity from Piper colubrinum: prediction of structure-function relationship by bioinformatics approach. Mol biotechnol 52(3): 251-261.

Min K, Ha SC, Hasegawa PM, Bressan RA, Yun DJ, Kim KK. 2004. Crystal structure of osmotin, a plant antifungal protein. Proteins 54(1): 170-173.

Narasimhan ML, Coca MA, Jin J, Yamauchi T, Ito Y, Kadowaki T, Hasegawa PM. 2005. Osmotin is a homolog of mammalian adiponectin and controls apoptosis in yeast through a homolog of mammalian adiponectin receptor. Mol cell 17(2): 171-180.

Osusky M, Osuska L, Hancock RE, Kay WW, Misra S. 2004. Transgenic potatoes expressing a novel cationic peptide are resistant to late blight and pink rot. Transgenic Res 13(2): 181-190.

Patkar RN, Chattoo BB. 2006. Transgenic indica rice expressing ns-LTP-like protein shows enhanced resistance to both fungal and bacterial pathogens. Mol Breeding 17(2): 159-171.

Regente MC, De La Canal L. 2000. Purification, characterization and antifungal properties of a lipid-transfer protein from sunflower (Helianthus annuus) seeds. Physiol Plant 110(2): 158-163.

Sarowar S, Kim YJ, Kim KD, Hwang BK, Ok SH, Shin JS. 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(3): 419-427.

Singh NK, Bracker CA, Hasegawa PM, Handa AK, Buckel S, Hermodson MA, Bressan RA. 1987. Characterization of osmotin: a thaumatin-like protein associated with osmotic adaptation in plant cells. Plant Physiol 85(2): 529-536.

Tulasi RB, Nadimpalli SK. 1997. Purification of α-mannosidase activity from Indian lablab beans. Biochem Mol Biol Int 41(5): 925-931.

Uhlén M, Forsberg G, Moks T, Hartmanis M, Nilsson B. 1992. Fusion proteins in biotechnology. Curr Opin Biotechnol 3(4): 363-369.

Van Loon LC, Van Strien EA. 1999. The families of pathogenesis-related proteins,

their activities, and comparative analysis of PR-1 type proteins. Physiol Mol Plant P 55(2): 85-97.

Wriggers W, Chakravarty S, Jennings PA. 2005. Control of protein functional dynamics by peptide linkers. Biopolymers 80(6): 736-746.

Wu C, Ying H, Grinnell C, Bryant S, Miller R, Clabbers A, Santora L. 2007. Simultaneous targeting of multiple disease mediators by a dual-variable-domain immunoglobulin. Nat Biotechnol 25(11): 1290-1297.

Yang J, Zhang Y. 2015. I-TASSER server: new development for protein structure and function predictions. Nucleic Acids Research 43(W1): W174-W181.

Yu K, Liu C, Kim BG, Lee DY. 2015. Synthetic fusion protein design and applications. Biotechnol Adv 33(1): 155-164.

Zhang J, Yun J, Shang Z, Zhang X, Pan B. 2009. Design and optimization of a linker for fusion protein construction. Prog Nat Sci 19(10): 1197-1200.