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SYNERGISTIC ACTION OF TLP AND PR1 PROTEINS REVEALED THEIR ENHANCED INTERACTION WITH FUNGAL CELL-WALL COMPONENTS: EVIDENCE FROM *IN SILICO* STUDIES

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ABSTRACT

Various pathogenesis-related (PR) proteins have been used in combination for effective disease management in plants. However, the mechanism underlying their synergistic effect has not been elucidated at the molecular level. Here, we explore the fundamental molecular mechanism of synergism between thaumatin-like protein (TLP) and PR1 proteins using an *in-silico* approach. This approach revealed physicochemical properties, predicted subcellular localization, performed topological and domain analyses, analyzed conserved motifs, performed phylogenetic analysis, predicted secondary and tertiary structures, and conducted molecular docking studies of TLP and PR1 proteins alone and in combination with significant components of the fungal cell wall. Physicochemical analyses revealed that TLP is a neutral protein, whereas PR1 is a negatively charged, acidic protein. Both proteins were found to be thermostable based on their aliphatic index values. Similarly, GRAVY values indicated that TLP was hydrophobic, while PR1 was hydrophilic. Furthermore, subcellular localization predicted that both proteins were extracellular. Through topological analysis, TLP was found to be a transmembrane protein, whereas PR1 protein was found on the outer side of the membrane. Lastly, protein-ligand interaction studies via docking between TLP and PR1 alone and in combination with fungal cell wall components such as chitin, chitosan, glucan, and mannan showed a stronger interaction for the TLP-PR1 combination than for TLP and PR1 alone. It can be concluded that these proteins may have more vigorous anti-pathogenic activity co-expressed in plants, hence resulting in broad-spectrum resistance against fungal pathogens.

***Keywords*:** Thaumatin-like protein (TLP), Pathogenesis-related protein 1 (PR1), Plant innate immunity, Fungal cell wall components, Molecular docking, Broad-spectrum disease resistance, Plant–fungal interactions.

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1. INTRODUCTION

Each ecological system has ~250,000 fungi, some of which cause severe damage to crop growth and production (Yasmin et al., 2017). Though plants evolved specific adaptations to cope with these drastic agents, they don't have an immune system like animals. These adaptations include lignification of the cell wall, hypersensitive response (HR) and production of antioxidants as well as PR proteins e.g. reactive oxygen species (ROS), jasmonic acid (JA), late embryogenesis-abundant (LEA) proteins, different phytohormones like salicylic acid (SA), methyl jasmonate (MeJA), ethylene (ET), proline, sugar, anti-microbial compounds (like phytoalexins), abscisic acid (ABA). Pathogenesis-related (PR) proteins were first observed to accumulate in tobacco in response to infection with fungal pathogens (Cao et al., 2016). The widespread distribution of these PR proteins led to their classification into 17 groups based on sequence homology and mode of action. PR1 and PR5 are more common pathogenesis-related proteins and are constitutively expressed in almost all of the plant's parts, including roots and pollen. Furthermore, the production of these PR proteins is induced by biotic and abiotic stresses, indicating their role in the plant defense system (van Loon et al., 2006). PR1 family proteins are paradoxical in the arena of plant–microbe interactions. During pathogen interaction, they have been found to accumulate in abundance in the apoplast, yet their exact role has remained unclear for more than five decades. Some preliminary studies explored their role as growth-limiting agents in zoospores of *Phytophthora infestans* (Niderman et al., 1995; Woloshuk et al., 1991). They have also been reported to have antimicrobial activity owing to their ability to confiscate sterols from the pathogen

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membrane (Gamir et al., 2017). Pathogen infection and salicylic acid (a plant hormone) have been found to upregulate expression of these PR1 proteins; hence they their expression is induced by certain external stimuli. As a result, systemic acquired resistance (SAR) and pattern-associated triggered immunity (PTI) are induced. So, PR1 protein expression can be used as a marker to assess for resistance response (van Loon et al., 2006). Though literature reports that PR1 proteins have crucial role in defense but its exact function and mechanisms of action protein remains vague (Sung et al., 2020).

TLP belong to PR5 family and have ability to promptly accumulate to extraordinary level in response to stresses (biotic/abiotic) and demonstrate anti-fungal activity in numerous plant species (Petre et al., 2011). They have found to be effective mycoparastic agents against filamentous fungi including *Botrytis cinerea, Mycosphaerella arachidicola, Fusarium oxysporum and Trichoderma viride* (Garcia-Casado et al., 2000; Chu & Ng, 2003; Ho et al., 2007). Moreover, they can also retard the fungal growth with xylanase inhibitor and β-glucanase activities by hydrolyzing cell wall components (β-1,3-glucans). Enhanced resistance against various fungal pathogens was observed in transgenic plants overexpressing thaumatin like proteins. Some studies also showed their involvement in plant developmental processes i.e. fruit ripening and anti-freeze activity (Singh et al., 2013). Up till now, many valuable plants species have been engineered to produce various versions of TLPs resulting in enhanced resistance against pathogenic diseases. Since overexpression of these proteins enable crop plants to withstand fungal pathogen infection (Kalpana et al., 2006). For instance, overexpression of rice thaumatin-like-protein in tobacco showed increased resistance against fungal pathogen (Velazhahan & Muthukrishnan, 2003). In addition, PR1 protein has been explored to interact with other proteins and depict synergistic effect for the control of fungal pathogens. These interactions have been validated through various molecular approaches i.e. BiFC (bimolecular florescence complementation) Y2H (Yeast two hybrid system), pull-down assay, co-immunoprecipitation (CoIP) and Glutathione S-transferase pull-down (Wang et al., 2020). Moreover, co-expression of TLP (TaTLP1) and PR1 (TaPR1) enhanced antifungal activity in wheat against wheat leaf rust, exhibiting interaction between members of PR1 and PR5 (TLP) families (Wang et al., 2020). These engineered plants may be helpful to reduce the use of chemical pesticides which are hazardous and cause various health and environmental issues.

This study was designed to explore the binding affinity of a combination of PR proteins with fungal cell wall components. The focus was *in-silico* analysis of TLP (thaumatin-like protein) and PR1 proteins to explore physico-chemical properties, prediction of subcellular localization, topological analysis, domain analysis, conserved motif analysis, phylogenetic analysis, secondary and tertiary structure prediction and docking analysis with fungal cell wall components. Hence, a possible outcome of the co-expression of two antifungal proteins was predicted.

2. MATERIALS AND METHODS

2.1. Sequence Characterization

Nucleotide and protein sequences of Thaumatin-like protein and PR1 protein were retrieved from NCBI with accession number XM_026027151 and AJR16763. ExPASy translate tool (https://web.expasy.org/translate/) was used to translate nucleotide sequence into respective amino acid sequence whereas ExPASy server (https://web.expasy.org/protparam/) was used to determine physico-chemical properties of the two proteins by protparam tool (Gasteiger et al., 2003).

Cellular localization of TLP and PR1 proteins was predicted by using web servers like: CELLO v.2.5 (http://cello.life.nctu.edu.tw/) (Yu et al., 2006); WoLF PSORT (http://wolfpsort.seq.cbrc.jp/) (Horton et al., 2007); and EuLoc (http://euloc.mbc.nctu.edu.tw/) (Chang et al., 2013). Signal peptides were detected by PrediSi (http://www.predisi.de/) (Hiller et al., 2004) and SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/) (Petersen et al., 2011).

Topological analysis of TLP and PR1 proteins was executed using online tools including: TMpred (http://www.ch.embnet.org/software/TMPRED_form.html) (Hofman, 1993); TMAP (http://www.bioinformatics.nl/cgi-bin/emboss/tmap) (Persson & Argos, 1994); PHDhtm (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_htm.html) (Rost et al., 1996); HMMTOP (http://www.enzim.hu/hmmtop/) (Tusnady & Simon, 2001); TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) (Krogh et al., 2001) and Phobius (http://phobius.sbc.su.se/) (Käll et al., 2004); Motifs were analyzed using MEME Suit (http://meme-suite.org/tools/meme/) (Bailey et al., 2009). The MEME Suite used to predict the sequence motifs in DNA, RNA and proteins is very integrated and powerful set of web-based tools. Such motifs have pivotal role in study of regulation of gene expression and molecular interactions in the cell by encoding their detection, characterization and biological functions. Almost 100 sequences of both TLP and PR1 were used for this analysis.

2.2. Structural Characterization

Secondary structure analysis was performed by using SOPMA (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopm.html) (Geourjon & Deleage, 1995) and PSIPRED Server

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(McGuffin et al., 2000). Conserved domains were analyzed using Pfam (https://pfam.xfam.org/search/sequence) (Finn et al., 2016).

Homology modeling was performed to predict 3D structure of TLP and PR1 proteins using Phyre[2], SWISS-MODEL, Robetta, ORION, PS2 and I-Tasser. The template structure must have at least 60% query coverage and 25% sequence identity as per selection criteria reported by Fischer et al. (2001) (Fischer et al., 2001). Numerous models were predicted for each protein and best was selected based on quality and stereochemical properties through VERIFY 3D (http://services.mbi. ucla.edu/Verify_3D/) (Lüthy et al., 1992), Ramachandran plot (services.mbi.ucla.edu/PROCHECK/) (Laskowski et al., 1996), ERRAT (services.mbi.ucla.edu/ERRAT/) (Colovos & Yeates, 1993). Ramachandran plot was calculated by ProCheck server which is based on the distribution of backbone dihedral angles. Moreover, structural evaluation and superimposition of the model protein with template protein was calculated by FATCAT (https://fatcat.godziklab.org/) server and UCSF chimera. Further refinement of the selected structures was accomplished by ModRefiner (https://zhanglab.ccmb.med.umich.edu/ModRefiner/) (Xu & Zhang, 2011).

2.3. Molecular Docking Studies

The proteins under study are hypothesized to interact with major fungal cell wall components—namely chitin, β-glucan, chitosan, and mannan—to exert their antifungal activity. Accordingly, chitin, glucan, chitosan, and mannan were selected as ligands for molecular docking analyses. The 3D structures of ligands (Fungal cell wall components i.e. chitin, glucan, chitosan and mannan) were retrieved from a database PubChem (pubchem.ncbi.nlm.nih.gov). These ligands were screened against targeted proteins by executing computational molecular docking (Parvaiz et al., 2019). AutoDockVina was used for flexible ligand-protein docking. All steps for docking were performed iteratively for each protein and complex of both proteins. TLP and PR1 were docked by using Zserver (Venkatraman et al., 2009). Different grid boxes were drawn for different proteins. For studying molecular interaction of TLP with all ligands grid box having coordinates; center-x =8.322, center-y = -6.459 center-z = 12.358, size_x = 86 size_y = 92 and size_z =40 was set. To find molecular interaction of PR1 with all ligands grid box having coordinates; center-x = -1.497, center-y = 4.415, center-z = -3.335, size_x = 110, size_y = 110 and size_z = 110 was set. To studying molecular interaction of TLP-PR1 complex with all ligands grid box having coordinates; center-x = -0.22, center-y =14.62, center-z = 0.256, size_x = 66 size_y =108 and size_z =46 was set. Polar hydrogen molecules were added to all protein molecules and then docking analysis was carried out. The most stable confirmation of complex was selected based on energy score and RMSD value. Further, the binding was evaluated by PyMol and Chimera.

3. RESULTS

3.1. Sequence Characterization

Amino acid sequences of TLP and PR1 were characterized for physico-chemical properties i.e. molecular weight, amino acid and atomic compositions, instability index, isoelectric point and GRAVY (Grand Average of Hydropathicity) using ProtParam tool of EXPASY (Table 1). Thaumatin-like protein and PR1 consist of 276 and 149 amino acids with molecular weight of 28 and 15 k Dalton, respectively. The calculated isoelectric point (pI= 6.79) showed that TLP is neutral protein with zero net charge as it has same number of negatively charged and positively charged residues, while isoelectric point (pI= 4.23) of PR1 indicated it to be an acidic protein with negative charge as it has 17 negatively charged residues and 5 positively charged residues. The instability indices of TLP (43.51) and PR1 (45.76) proposed them as unstable proteins as any of the protein with instability index greater than 40 is considered to be unstable (Guruprasad et al., 1990). The aliphatic index considered as a positive factor for the upsurge of thermostability of globular proteins (Ikai, 1980) so according to protparam TLP and PR1 are thermostable as their aliphatic indices are 66.70 and 56.44 respectively. Similarly, GRAVY values of TLP i.e. 0.132 suggested it as hydrophobic protein and GRAVY value of PR1 i.e. -0.343 suggested it as hydrophilic. The GRAVY value for a protein or peptide is calculated as the summation of hydropathy values of all the amino acid residues, divided by the number of residues in the sequence. Positive GRAVY value showed that protein is hydrophobic in nature and vice versa (Kyte & Doolittle, 1982).

Three different tools were used to predict the subcellular localization of both proteins. According to Cello and Euloc prediction TLP and PR1 both are extracellular proteins. While Wolfpsort found that nearest members of TLP are localized in mitochondria, vacuole, E.R, chloroplast, cytoplasm and extracellular while nearest member of PR1 are localized in chloroplast and cytoplasm. The tools, SignalP 4.1 and PrediSi predicted the absence and presence of signal peptide in PR1 and TLP, respectively.

Topological analysis of both proteins using several online tools predicted the existence of transmembrane helices in TLP and lack of transmembrane domain in PR1. According to these tools TLP has almost 20-30 residues transmembrane domain while PR1 is outer membrane protein.

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Table 1: Physico-chemical properties of TLP and PR1 calculated by Protparam

Parameters	TLP			PR1		
Mol. Weight	28 k Dal			15 k Dal		
No. of amino acids	276			149		
Theoretical *pI*	6.79			4.23		
Instability index (II)	43.51 (unstable)			45.76 (unstable)		
No. of Negatively Charged Residues (Asp + Glu)	21			17		
No. of Positively Charged Residues (Arg + Lys)	21			5		
Atomic Composition	Carbon	1218		Carbon	674	
	Hydrogen	1883		Hydrogen	985	
	Nitrogen	359		Nitrogen	189	
	Oxygen	374		Oxygen	232	
	Sulfur	16		Sulfur	7	
Amino Acid Composition	Amino acid	No.	%age	Amino acid	No.	%age
	Ala (A)	52	18.8	Ala (A)	23	15.4
	Arg (R)	19	6.9	Arg (R)	3	2.0
	Asn (N)	10	3.6	Asn (N)	9	6.0
	Asp (D)	10	3.6	Asp (D)	12	8.1
	Cys (C)	14	5.1	Cys (C)	6	4.0
	Gln (Q)	5	1.8	Gln (Q)	6	4.0
	Glu (E)	11	4.0	Glu (E)	5	3.4
	Gly (G)	34	12.3	Gly (G)	13	8.7
	His (H)	3	1.1	His (H)	4	2.7
	Ile (I)	2	0.7	Ile (I)	3	2.0
	Leu (L)	17	6.2	Leu (L)	3	2.0
	Lys (K)	2	0.7	Lys (K)	2	1.3
	Met (M)	2	0.7	Met (M)	1	0.7
	Phe (F)	17	6.2	Phe (F)	2	1.3
	Pro (P)	15	5.4	Pro (P)	5	3.4
	Ser (S)	17	6.2	Ser (S)	19	12.8
	Thr (T)	19	6.9	Thr (T)	6	4.0
	Trp (W)	3	1.1	Trp (W)	6	4.0
	Tyr (Y)	4	1.4	Tyr (Y)	8	5.4
	Val (V)	20	7.2	Val (V)	13	8.7
	Pyl (O)	0	0.0	Pyl (O)	0	0.0
	Sec (U)	0	0.	Sec (U)	0	0.0
Aliphatic Index	66.70			56.44		
Grand average of Hydropathicity (GRAVY)	0.132			-0.343		

Conserved motifs were predicted by MEME suit. Almost 100 sequences of TLP retrieved from NCBI-Blast were scanned for motif prediction. 7 conserved motifs were found. Same was done for PR1 and 5 conserved motifs were found (Fig. 1; Table 2).

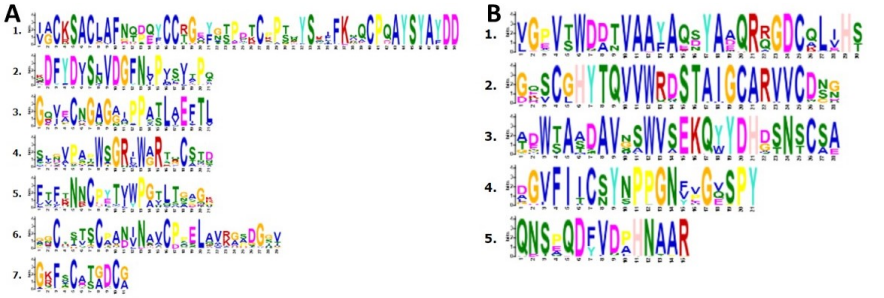

Fig. 1: Conserved motifs of (A) TLP and (B) PR1 predicted by MEME suit.

Table 2: Conserved motif of TLP and PR1 predicted by MEME suit

TLP	PR1
IACKSACLAFNQDZYCCRGEYGTPDTCPPTQYSKIFKNQCPQAYSYAYDD	VGPVTWDDTVAAYAQSYAAQRRGDCQLIHS
KDFYDVSLVDGFNLPVSVTPQ	GQSCGHYTQVVWRDSTAIGCARVVCDNG
GQVECNGAGAIPPATLAEFTL	ADWTAADAVNSWVSEKQYYDHDSNSCSA
SJGVPAPWSGRIWARTQCSTD	DGVFIICSYNPPGNFVGVSPY
FTFTNNCPYTVWPGTLTGAGK	QNSPQDFVDPHNAAR
GGCSSTSCPABINAVCPPELAVRGSDGGV	
GKFSCATGDCG	

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3.2. Structural Characterization

Only 1% proteins have been characterized experimentally to determine the 3D structure and function of proteins despite achievements in molecular biology and next-generation sequencing (NGS). Nowadays it's very easy to determine the structure and function of unknown proteins by using bioinformatics tools. Secondary structure of both proteins was predicated by SOPMA and PSIPRED. SOPMA predicted that TLP has 27.17% alpha-helix, 23.55% extended strands, 10.87% beta turns and 38.41% random coils. According to PSIPRED prediction TLP has 19.5% strands, 15.7% alpha helix and 64.8% coils. While according to SOPMA analysis PR1 shows 32.89 % alpha-helix, 18.12% extended strands, 8.77% beta turns and 40.27% random coils and according to PSIPRED prediction PR1 has 19.5% strands, 33.5% alpha helix and 47% coils. Secondary structure of TLP and PR1 is shown in Fig. 2.

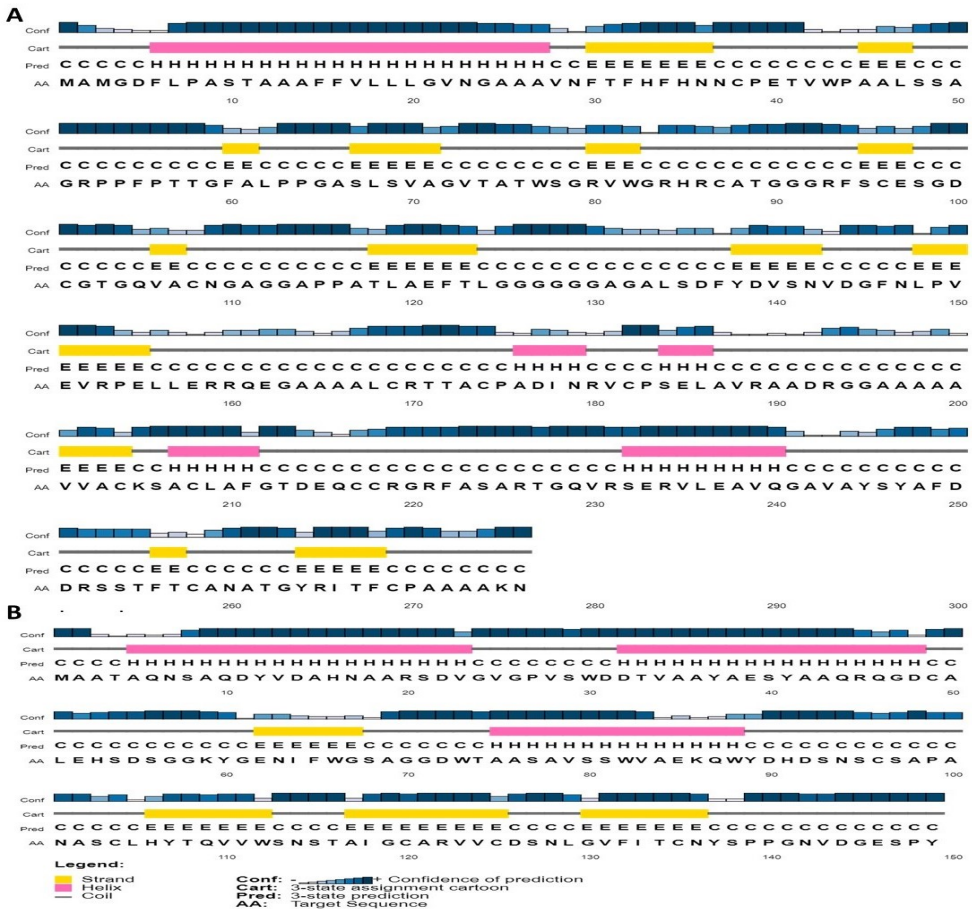

Fig. 2: Secondary structure of (A) Thaumatin-like protein (B) PR1 predicted by PSIPRED.

Pfam tool was used for prediction of domain analysis which showed that TLP has single domain belongs to thaumatin family and PR1 also have single domain belongs to Cysteine-rich secretory protein family. Conservation of these domains in numerous monocot and dicot species was confirmed by multiple sequence alignment. 3D structure of proteins was predicted by homology modeling using Phyre[2], SWISS-MODEL, ORION, (PS)[2], Robetta and I-Tasser. Predicted models for each protein were evaluated for their quality and stereochemical properties through Ramachandran plot, VERIFY 3D and ERRAT (Table 3). Parameters of protein geometry including C_β deviations, ramachandran outliers, poor and favored rotamers, bad bonds and bad angels of selected models were determined through MolProbity (Table 4). The best model was selected and was further refined by ModRefiner. Backbone topology, hydrogen bonds and side chain positioning of predicted model are drawn closer to their native structure and it also helps to improve the physical quality of structure. Structures were evaluated and their superimposition with template protein was determined by UCSF chimera and FATCAT server. The evaluation of TLP and PR1 with their respective templates showed Q-score of 0.865 and 0.880, P value of 0.00e+00 and RMSD of 0.641Å and 0.77Å, respectively.

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Table 3: NIH Server (SAVES) evaluation for selection of best models by using tools like ERRAT, Verify_3D and PROCHECK.

Protein	Models	Verify3D (%)	ERRAT Quality Factor (%)	PROCHECK Ramachandran (%)
	Phyre[2]	80.08	59.05	87.7
	Swiss-Model	83.33	82.27	84.5
Thaumatin-like Protein	ORION	80.91	51.08	85.1
(TLP)	(PS)[2]	68.84	45.88	85.8
	Robetta	77.68	82.81	87.8
	Phyre[2]	77.08	71.32	83.1
	Swiss-Model	97.92	84.55	80.6
Pathogenesis-related 1	ORION	97.22	50.73	82.3
(PR1)	(PS)[2]	92.62	52.48	84.5
	Robetta	99.11	85.22	100

Table 4: MolProbity analyses of selected models of TLP and PR

Models	Poor Rotamers (<0.3%)	Favored Rotamers (>98%)	Ramachandran Outliers (<0.05%)	Ramachandran Favored (>98%)	Cβ deviations >0.25Å (0%)	Residues with bad bonds (0%)	Residues with bad angles (<0.1%)
TLP (%)	0.61	98.77	0.00	96.73	0.00	0.00	0.36
PR1 (%)	0.00	100	0.00	100	0.00	0.13	0.19

The predicted models were submitted to Protein Model DataBase (PMDB) with PMDB ID: PM0083502 and PMDB ID: PM0083503 for TLP and PR1, respectively. The 3D models and their superimposition are shown in Fig. 3 and 4.

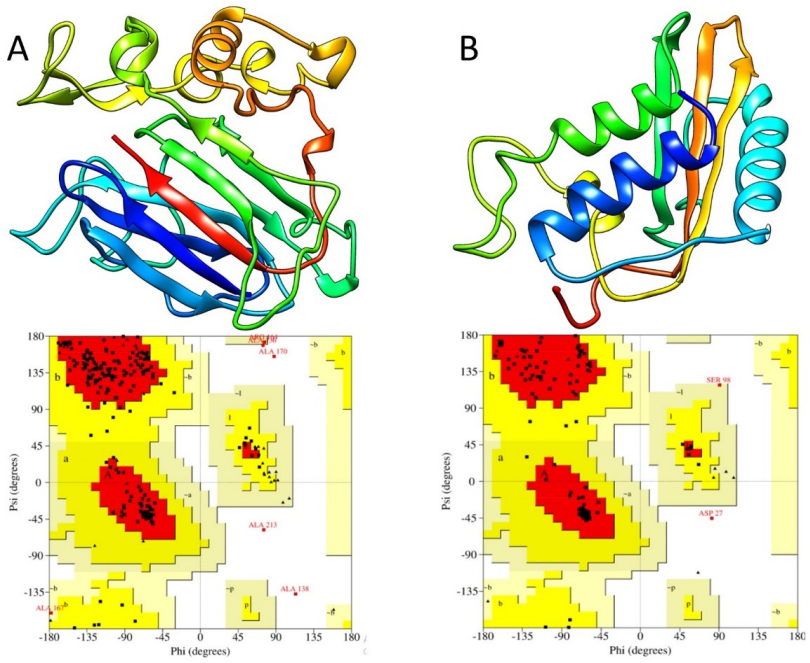

Fig. 3: (A) 3D structure of TLP (PMDB ID: PM0083502) with Ramachandran plot. Ramachandran plot indicated that 85% residues are in favored region. (B) 3D structure of PR1 (PMDB ID: PM0083503) with Ramachandran plot. Ramachandran plot showed that 90% residues are in favored region.

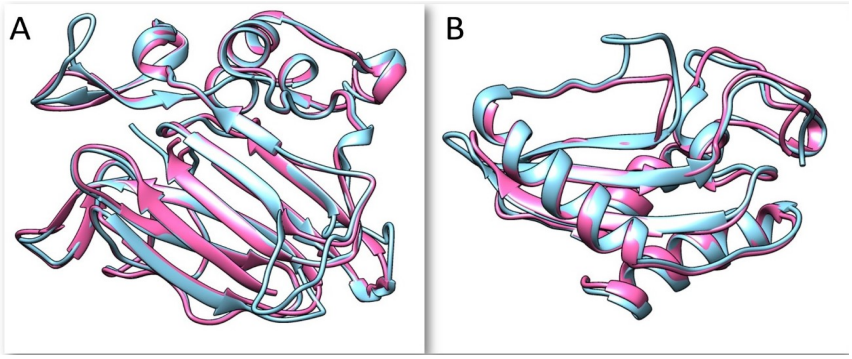

Fig. 4: Superimposition of (A) Thaumatin-like Protein having 0.641Å RMSD (B) PR1 having 0.77Å RMSD with their respective templates.

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3.3. Molecular Docking Studies

Interaction of TLP and PR1 with chitin, glucan, chitosan and mannans by molecular docking apprehended antifungal activity of these proteins. Interaction of TLP and PR1 proteins with fungal cell wall residues exposed their chitinolytic activity, hence these proteins may be involved in degradation of fungal cell wall by hydrolyzing β-1,4-linkage of N-acetylglucosamine and 1,3-β-D-glucosidic linkages in β-1,3-glucans (Leubner-Metzger & Meins Jr, 1999). In current study, chitin, chitosan, mannan and glucan were used as ligands. The 3D structure of all ligand molecules was downloaded from PubChem database whereas autodock vina was used for molecular docking. The interaction of most ligands with their binding sites on receptors can be characterized in terms of energy score. Energy score with the most negative values shows the most favorable binding. Chimera and pymol were used to find and visualize active sites and interacting residues of protein.

Both of the proteins TLP and PR1 showed interaction with the key components of fungal cell wall separately and showed stronger interaction in form of complex. In case of TLP, Asp148, Asn150, Asp163 and Val172showed hydrogen bonding with chitin. For PR1, Tyr132 and Ser133 showed hydrogen bonding with chitin. While in complex of TLP and PR1 Arg124, Ala138, Leu139, Cys140 and Thr142 showed hydrogen bonding with chitin. Chitosan interacts with Asn118 and Vl173 of TLP, Tyr84 and Asp85 of PR1 and Glu11, Asp148, Asn150, Arg164 and Val172 of TLP-PR1 complex by hydrogen bonding. Glucan also showed hydrogen bonding with Gly30, Gly63, Gly75, Gln76 and Val77 of TLP, Ser76 and Asn96 of PR1 and Cys58, Ser66, Cys67, Glu68 and Arg160 of TLP-PR1 complex. Similarly, Thr2 and Leu128 of TLP, Tyr36 and Ser48 of PR1, Glu11, His56, Asn150, Gly166 and Val172 of TLP-PR1 complex appeared to have hydrogen bonding with mannan. Active sites and interaction of TLP, PR1 and TLP-PR1 complex with chitin, chitosan, glucan and mannan is shown in Fig. 5 and 6, respectively. The predicted information is crucial to explore interaction of TLP and PR1 proteins with the major components of fungal cell wall which exposed their antifungal activity. Moreover, these results will also help to develop plant varieties having resistance against fungal diseases by working on their active sites explored by docking.

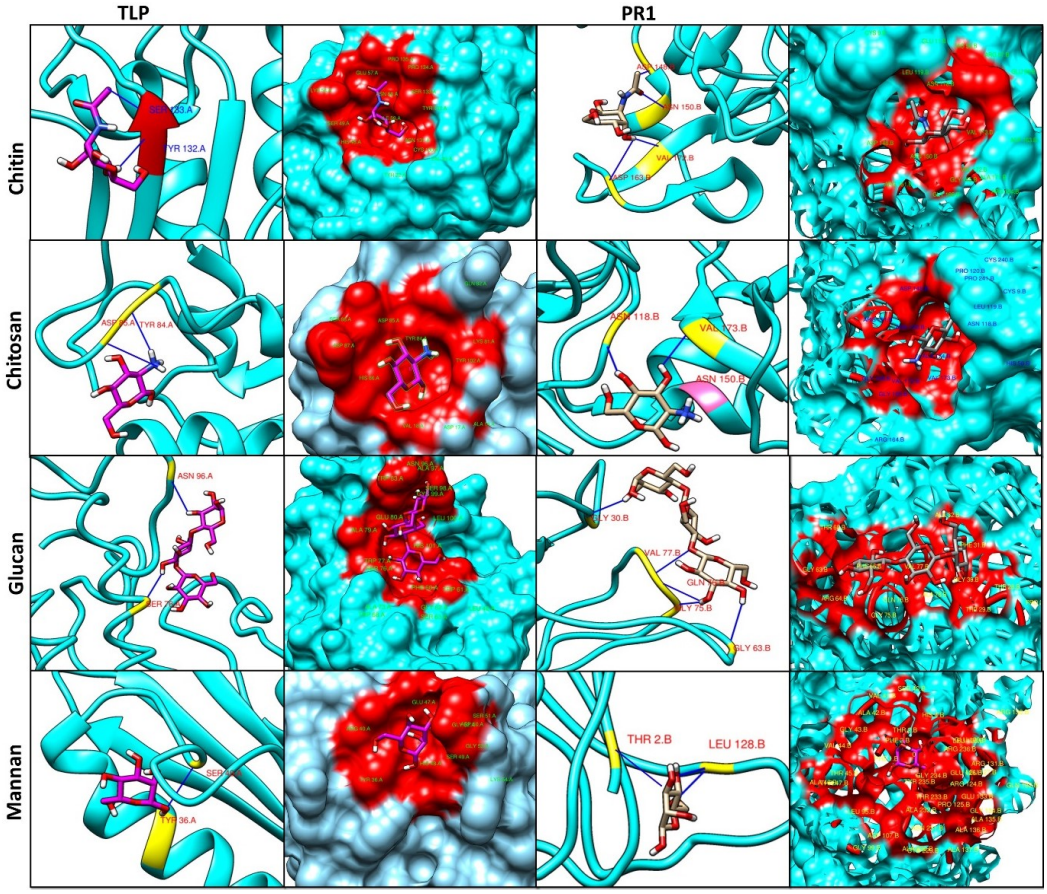

Fig. 5: Interacting residues of TLP and PR1 are represented in 1st and 3rd column. Active site residues are shown in 2nd and 4th column (Red area represents the active site). Protein is shown in cyan color and hydrogen bonding is shown in blue lines.

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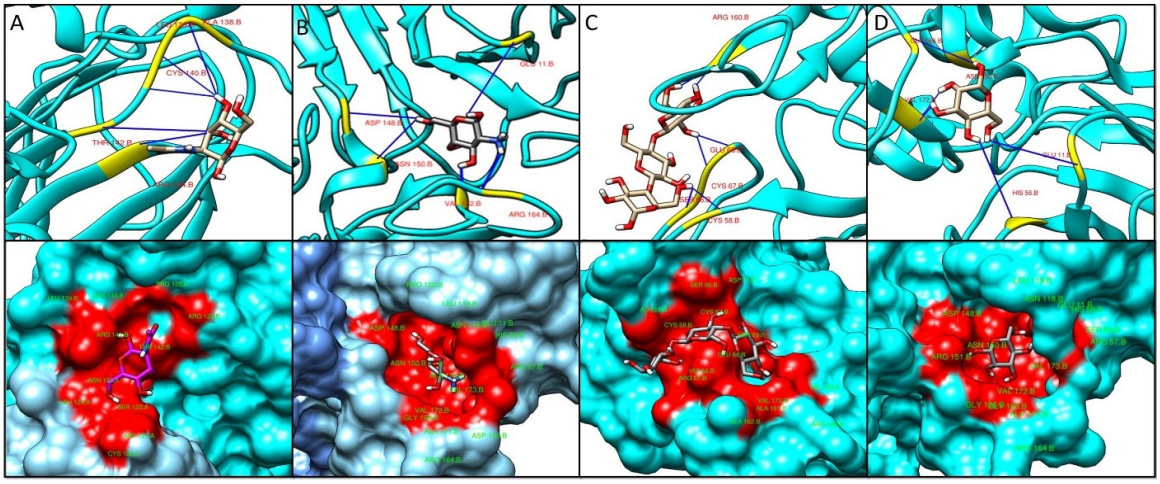

Fig. 6: (A) Interacting residues of TLP-PR1 complex with chitin. (B) Interacting residues of TLP-PR1 complex with chitosan. (C) Interacting residues of TLP-PR1 complex with glucan. (D) Interacting residues of TLP-PR1 complex with mannan. Second row shows the active sites of TLP-PR1 complex (Red area represents the active site). Protein is shown in cyan color and hydrogen bonding is shown in blue lines.

4. DISCUSSION

Pathogenesis related proteins are key constituents of plant defense system and play crucial role in devising protective measures to withstand fungal pathogen infection. A wide variety of PR proteins have been reported so far to play direct or indirect role in plant defense system (Han & Schneiter, 2024). Thaumatin-like protein is an important PR protein involved in resistance against biotic and abiotic stress in plants (Sharma et al., 2022). Literature review suggests that TLP proteins exhibit glucanase activity by binding with β-1,3-glucan (Gorjanović et al., 2007). So it may degrade fungal cell wall by penetrating into membrane or by hydrolyzing β-1,3-glucan (Gorjanović et al., 2007). Anyhow, exact role of pathogenesis-related (PR) proteins in plant defense system and their coordination with each other is not clear yet. Studies showed that TLPs and PR1 proteins interact with pathogen receptors and may depict higher level of anti-pathogenic activity (Wang et al., 2020; Javed et al., 2025).

Plants respond to pathogenesis by a complex network of defense system. Defense related proteins enhance antifungal activity in plants thus work synergistically (Kumar et al., 2025). *In vitro* assay proved the enhanced synergistic effect of barley endosperm RIP with barley chitinase proteins. The tobacco plants co-expressing barley endosperm RIP and barley chitinase showed better level of resistance against *Rhizoctonia solani* infection as compared with the plants expressing single barley protein (Jach et al., 1995). Another study also revealed the enhanced antifungal activity in wheat co-expressing β-1,3-glucanase and chitinase gene compared to wheat expressing single PR protein (rice TLP protein) against *Fusarium graminearum* (Anand et al., 2003). Enhanced resistance against fungal pathogens was also observed in transgenic pea by stacking chitinase and glucanase proteins. Synergistic effect of these proteins was observed as a result antifungal activity was increased in tobacco and tomato plants against *Cercospora nicotianae* and *Fusarium oxysporum* f.sp. *lycopersici*, respectively. Contrarily, transgenic plants expressing single PR protein did not show that much level of resistance. Hence it has been revealed that synergistic expression of PR proteins results in enhanced antifungal activity (Amian et al., 2011).

The current study focused on *In-silico* analysis of thaumatin-like proteins and PR1 proteins including physico-chemical properties, prediction of subcellular localization, topological analysis, domain analysis, conserved motif analysis, phylogenetic analysis, secondary and tertiary structure prediction and docking analysis with fungal cell wall components (Figueiredo et al., 2024). Subcellular localization and solubility depend on isoelectric point and charge on the protein. TLP is a neutral protein whereas PR1 is a negatively charged protein with acidic nature. Both of the proteins are unstable in nature as their instability index is greater than 40 (Guruprasad et al., 1990). They are thermostable as is evident from their aliphatic index. GRAVY values indicated that TLP is hydrophobic in nature and PR1 is hydrophilic in nature. The positive GRAVY values depicts that protein is hydrophobic in nature and vice versa (Kyte & Doolittle, 1982). The said GRAVY value (for a protein or peptide) is determined as the summation of hydropathy values of all of the amino acid residues, divided by the number of residues in that particular sequence.

Subcellular localization was predicted by various tools. According to Cello and Euloc prediction TLP and PR1 both are extracellular proteins. Transient expression of recombinant TLP proteins; CkTLP-GFP (Wang et al., 2011)

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and TaPR5-GFP (Labarrere et al., 2011) proved that they are extracellular proteins. Another study showed that some other TLP proteins like CsTL1 and RlemTLP were found to be localized in both the cytoplasm and periphery of plasma membrane instead of extracellular spaces and are involved in anti-pathogenic activity (Kim et al., 2009). The subcellular localization analysis of recombinant AdTLPGFP protein also exhibited its extracellular localization (Singh et al., 2013). Similarly some previous studies reported that PR1 is localized extracellularly (Gu & Innes, 2012; Watanabe et al., 2013). Other tools like PrediSi and SignalP 4.1 predicted that TLP has signal peptide of 27 amino acid and this protein was predicted for secretion. Other researchers (Singh et al., 2013) also reported that TLP has signal peptide of 21 amino acids. PR1 has not been predicted for secretion and (Pečenková et al., 2017) validated that no exosome secretion was observed in time series scanning of PR1. Topological analysis of TLP proteins using numerous online tools predicted the existence of transmembrane helices. According to previous studies, some ORFs of TLPs are indicated as transmembrane protein (Blouin et al., 2018; Ruiz-García et al., 2020). Topological analysis of PR1 predicted it as outer protein and previous studies showed that PR1 vesicles are amassed just underneath the outer surface of cotyledon epidermal cells (Pečenková et al., 2017).

Autodock vina was used for docking analysis, both the ligands and protein are reflected as rigid during docking procedure. The results having RMSD (Root-mea-square-deviation) less than 1.0 Å were grouped together and signified with the most favorable free energy of binding. Further analysis were carried out by alignment of receptor structure with the pose having lowest energy of binding or binding affinity (Azam & Abbasi, 2013). Both TLP and PR1 showed interaction with chitin, chitosan, glucan and mannan molecules, the fundamental components of fungal cell wall and showed stronger interaction in combination (Fig. 5 and 6). Hence, these proteins have critical role in anti-pathogenic activity but may work better in combination as compared with the individual proteins. Further, this information will be used to target active sites of TLP and PR1 proteins involved in anti-pathogenic activity which might be of great help for the development of fungal pathogens resistance plants.

5. CONCLUSION

This study provides a comprehensive in silico framework elucidating the molecular basis of the synergistic antifungal action between thaumatin-like protein (TLP; PR5) and pathogenesis-related protein 1 (PR1). Detailed sequence, structural, and physicochemical analyses revealed that although TLP and PR1 differ markedly in size, charge, hydropathicity, and membrane topology, both proteins share extracellular localization and conserved functional domains consistent with their established roles in plant defense. These intrinsic differences appear to be complementary rather than redundant, creating a favorable context for cooperative interactions against fungal pathogens. Molecular docking analyses demonstrated that both TLP and PR1 individually interact with major fungal cell-wall components—chitin, chitosan, β-glucan, and mannan—through specific hydrogen-bonding residues. Importantly, the TLP–PR1 complex consistently exhibited stronger binding affinities and a broader spectrum of interacting residues than either protein alone, indicating enhanced molecular recognition and stability of the protein–ligand complexes. This strengthened interaction provides mechanistic insight into previously observed synergistic antifungal effects reported in transgenic and co-expression studies, and suggests that combined action may facilitate more effective disruption of fungal cell-wall integrity. Overall, the findings support the concept that co-expression or stacking of PR1 and TLP genes could confer superior and broad-spectrum resistance against fungal pathogens compared with single-gene strategies. The identification of key interacting residues and active sites further offers valuable targets for protein engineering and rational design of disease-resistant crops. While the conclusions are derived from computational analyses, they establish a strong theoretical foundation for future experimental validation through biochemical assays, mutational studies, and transgenic approaches. Collectively, this work advances our molecular understanding of PR-protein synergism and highlights its practical potential for sustainable crop protection strategies

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Ethics Statement: This work involved only in-silico molecular modelling and simulations. No human or animal

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