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In Silico and In vitro Investigation of Plant Defensin as potent agents to combat antipathogen applications

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Abstract
Plant health is significantly impacted by pathogen diseases, which has led to the development of complex defense mechanisms, such as the production of defensins, which are cationic antimicrobial peptides essential to a plant's innate immune system. Plant defensins are a primary source of innate immunity in plants. Structurally, they are highly stable, small, cysteine-rich antimicrobial peptides. Fungal cells have been reported as the most invading pathogen among biotic flora. The fungal cell wall is rich in receptors such as MAP Kinase receptor and Phosphatidic acid receptor. In this study, a bioinformatic approach has been made to study the interactions of different plant defensins with the fungal cell wall receptors. Around 20 plant defensins have been identified, and their protein sequences were retrieved from biological databases. Docking studies showed that plant defensins showed stronger interactions with cell wall receptors coding for MAPK and phosphatidic acid pathways in the cytosol with the lowest binding energies and zero rmsd values. The in vitro studies aimed to clone the MsDef1 plant defensin gene from Medicago sativa into E. coli to enable large-scale production and additional investigation. Using the phenol-chloroform technique, genomic DNA was extracted from Medicago seeds and analyzed using agarose gel electrophoresis. Gradient PCR was used to amplify the MsDef1 gene with an optimal annealing temperature of 59°C, producing a unique 252 bp DNA band. For quantitative RT-PCR analysis, total RNA was separated, subjected to DNase treatment, and converted into cDNA. Defensin gene expression was verified in comparison to the internal control, β-actin. The heat shock transformation method was used to insert the MsDef1 gene into E–coli after it was cloned into the pCambia1301 vector. The growth of white colonies served as an indicator of a successful transformation, and transformed colonies were chosen on an LB medium containing IPTG and Kanamycin. The recombinant bacterial cell was transfected into Arabidopsis plant and selected transgenic plants were selected on MS media supplemented with kanamycin. The ability to clone plant defensin genes into E. coli and its transfection into the host plant has been demonstrated by this work, offering a valuable method for mass-producing defensin proteins for use in agriculture and medicine.
Keywords: *Plant defensins, cloning, useful in agriculture and medicine*

Abbreviations:
MAPK- Mitogen Activated Protein Kinase
PA- Phosphatidic Acid
PCR- Polymerase Chain Reaction
IPTG- Isopropyl β-D-1-thiogalactopyranoside
MS media- Murashige and Skoog media
MsDef1- *Medicago sativa* Defensin 1
Rs-AFP- Radish seed Anti-Fungal Protein
CSα/β- Cysteine-Stabilized α-helix β-sheet
NCBI- National Center for Biotechnology Information
BLAST- Basic Local Alignment Search Tool
PDB- Protein Data Bank
TAE- Tris base, Acetic acid and EDTA
DEPC- Diethyl Pyro carbonate
EDTA- Ethylene Diamine Tetra Acetic acid
DNA- Deoxy ribonucleotide
RNA- Ribonucleotide
cDNA- Complementary DNA
qRT-PCR- Quantitative reverse transcription polymerase chain reaction
UDG- Uracil-DNA Glucosylase

pg-Picograms
EcoR1- *Escherichia coli* R strain I
CaCl2- Calcium Chloride
LB- Luria Bertani
ml- millilitres
OD- Optical Density
MTCC- Microbial Type Culture Collection
rpm- Revolutions per minute
RMSD- Root Mean Square Deviation

INTRODUCTION

Microbial pathogens consistently threaten the plant kingdom, prompting the evolution of protective strategies. Defensins, cationic antimicrobial peptides essential toessential to plants' innate immune system, are the basis of their defense mechanism. Defensins are pervasive across both the plant and animal realms. (Colilla FJ et al., 1990). Defensins and related peptides have a variety of uses. They lead to the breakdown of microbial membranes and act as ligands for cellular recognition and signaling. Plant defensins were first identified from wheat and barley grains in the early 1800s, making these grains the pioneer sources of these chemicals (Mendez E et al., 1990; Bruix M et al., 1993). When these defensin proteins were first identified, they were called "γ-thionins," a term that was influenced by the size of these proteins (about five kDa) and their cysteine content (usually 4–8 residues (Osborn RW et al., 1995). Defensins exhibit varying functions across plant, insect, and mammalian species. Notably, some plant defensins lack antibacterial activity. (Lay F.T. et al., 2005). They contribute to the defensethe defense against a wide range of fungal infections. Plant pathogenic fungi,,fungi, including *Fusarium culmorum*, *Botrytis cinerea*, and human Candida albicans, are resistant to defensins (Terras FRG et al., 1995).

Additionally, defensins from seeds have been isolated and thoroughly structurally, biochemically, and molecularly characterized. A notable example is Rs-AFPs (radish seed antifungal protein), constituting approximately 0.5% of the total seed protein content. This seed-derived defensin eliminates soil microorganisms by shedding its protective coat during germination. Roughly 30% of Rs-AFPs are released from the disrupted seed coats, effectively hindering fungal growth in the soil. These seed-targeted defensins are the plants' initial defense against soil-borne pathogens, in addition to seeds, various plant defensins are distributed within plant tissues like leaves, tubers, pods, roots, fruits, floral components, and bark. (Carvalho AO et al., 2009; Lay F.T. et al., 2005). The functions of these defensins include theirtheir role in providing innate immunity to plants. They impart anti-proliferative activity and anti-parasitic activity, provide protection to reproductive parts of the plant, help the plants combat abiotic stress and heavy metal stress, have growth inhibitory effects against bacterial, viral, fungal, and yeast pathogens, and enhance the herbicide properties of BAR gene. Table 1 details the name of the defensin gene present in the plant, its targeted tissue expression, and its biological activity.

Structural conformation and mode of action of plant defensins:

The characteristic structural pattern of plant defensins is a compact, stabilized tertiary structure with an alpha helix, antiparallel beta sheets, and a triple-stranded arrangement. The resulting structure is referred to as the CSα/β (Cysteine-Stabilized α-helix β-sheet) motif (Kobayashi et al., 1991; Zhu et al., 2005). Disulfide bridges enhance this arrangement. Two further conserved motifs, the α-core, and the γ core, are found in addition to the CSα/β motif. The core that includes the hairpin loop joining β-strands 2 and 3 (Lβ2β3) is called the γ core, and the loop that joins the first β strand to the α helix is known as the α-core (Yount et al., 2004; Wilmes et al., 2011). Because 6–8 is present, the amino acid sequence Cys1–Cys8, Cys2-Cys5, Cys3–Cys6, and Cys4–Cys7 does not change.

This conserved amino acid sequence introduces unique features and roles upon any modification, whether numerical or structural (Picart et al., 2012). The carpet and pore models are the two main ideas developed to explain the antibacterial or antifungal effect of these plant defensins. Defensins interact with negatively charged ionic molecules of invasive pathogens on the plasma membrane in these models. This intercellular connection increases the plasma membrane's permeability, which lets cell contents leak out and eventually causes necrotic cell death.

The pore model shows how oligomers are created to create pores in the cell membrane; the carpet model highlights how pores are formed. According to a different idea, plant defensins interact with phospholipids on the cell membrane, which increases ion permeability and allows the peptides to enter the cell. Reactive oxygen species (ROS) are produced inside the cell as a result of this process, which also causes increased PCD (Programmed Cell Death) (Hegedus & Marx, 2013).

DEFENSIN	**SOURCE OF DEFENSIN**	**TARGET ORGANISM**	**TISSUE DEFENSIN IS EXPRESSED**	**BIOLOGICAL ROLE**	**REFERENCES**
PgD5	*Piceaglauca*	*Verticilliumdahliae*	Leaf, roots	Antifungal activity	Wong,J.H et al., 2012
Defensin-like peptide	*Phaseolus vulgaris*	*Mycosphaerellaarachidicola*	Leaf, roots	Antifungal activity	Portieles et al., 2010
NmDef02	*Nicotianamegalosiphon*	*Fusarium oxysporum*	Leaf	Inhibits oomycetes	Kant, P et al., 2009
Pdc1	*Zea mays*	*Fusarium graminearum*	Fruit	Antifungal activity	Wang S et al., 2009
Limyin	*Phaseoluslimensis*	*Fusarium solani*	Roots	Antifungal activity	Vijayan,S et al., 2008
TvD1	*Tephrosiavillosa*	*Pheaoisariopsispersonata*	Leaves	anti-insect and antifungal activity	Ramamoorthy,V et al., 2007
MtDef4	*Medicagotruncatula*	*Fusarium graminearum*	root, seeds	Antifungal and antipathogenic activity	Spelbrink et al., 2004
MsDef1(alfAFP)	*Medicago sativa*	*Fusarium graminearum*	Seeds	antifungal activity	Almeida et al., 2001
Psd1	*Pisumsativum*	*Neurosporacrassa*	Pods	antifungal activity	Gao et ., 2001
alfAFP	*Medicago sativa*	*Verticilliumdahliae*	Seeds	antifungal activity	Osborn et al., 1995
HsAFP1	*Heucherasanguinea*	*Septoriatritici*	Coral bells	antifungal activity	Terras et al., 1992
AhAMP1	*Aesculushippocastanum*	*Leptosphaeriamaculans*	Seed	antifungal activity	Terras et al., 1992
RsAFP2	*Raphanussativus*	*Pyriculariaoryzae*	Seed	antifungal activity	Park et al., 2002
NaD1	*Nicotianaalata*	*F.oxysporum*	Flower	Protecting reproductive parts	Amanda I et al., 2016
PhD1 and PhD2,	*Petunia hybrid*	*F.oxysporum*	flowers	antifungal activity	Ariane F. Lacerda et al., 2014
DEF2	*Solanum lycopersicum*	*F.oxysporum*	seeds	antifungal activity	Pedro Da Silva et al., 2002

wasabi defensin (WT1	*rice, potato, and orchid*	*Magnaporthegrisea*, *Erwiniacarotovora*and *Botrytis cinerea*	Root	antifungal activity	Gao et al., 2007
cdef1	*Capsicum frutescens*	*Phytophthorainfestans*and *Fusarium sp*	Seeds	Antifungal	Pedro Da Silva et al., 2002
Dm-AMP1	*Dahlia merckii*	*Magnaportheoryzae*and *Rhizoctoniasolani*	flower	antifungal activity	Siddhesh B et al., 2018
AlfAFP	*Alfalfa*	*V.Dahliae*	seeds	antifungal activity	Wang,Y.P et al., 1999
Spil	*Norway spruce*	*Erwiniacarotovara*, *Heterobasidionannosum*	seeds	antifungal activity	MalinElfstrand et al., 2001
DRR230 a DRR230-b	*Pisumsativum*	*Leptospaeriamaculan*, *F.oxysporum*, *Asochtapinodes*, *Trichoderma reesei*, *AScochytalentis*, *F.Solani*, *L.maculans*, *Ascochytapisi*, *Alternaria alternate*	Leaves, stem, flowers	antipathogenic activity	Karin Thevissen et al., 2003
BSD 1	*Chinese cabbage*	*P.parasitica*	Flower	Protecting reproductive parts	Anuradha et al., 2008
BjD	*Brassica nigra*	*F.moniliforme*, *P.parasitica*	seeds	antifungal activity	Cary et al., 2000
D4E1	*Synthetic*	*Aspergillus flavus*, *Verticiliumdalhia*	seeds	antifungal activity	Kanzaki,H et al., 2002

TABLE 1: Table displaying an inventory of plant defensins, including their source of origin, specific tissue localization, targeted pathogenic organisms, and distinctive biological roles.

Dynamics of fungal cell wall:

The cell wall of fungal pathogens has a dynamic structure that aids in performing diverse functions like cell viability, pathogenesis, and cell morphogenesis. The cell wall of the fungi is a dynamic organelle whose composition highly impacts the ecology of the fungi and regulates the interactions with the surrounding environment (NEIL A. R. GOW, 2017). Plant defensins target several cell wall receptors like MAP kinase receptors, GlcCer, and phosphatidic acid receptors, which abide on the fungal pathogen cell wall. These receptors play a significant role in signaling pathways vital to the cell's physiology and development, like cell cycle control, response to stress, resistance to harmful radiations, temperature changes, and fungal-plant interactions (Domingo Martínez-Soto, 2017). Computational biology tools like homology modeling can be used to study the interactions between the plant defensins and the receptors on the fungal cell wall. Defensins target the cell wall of the invading pathogens to kill them and protect the plant against fungal infection.

Fungal and mollusc-representative compounds are also present (P.H. Mygind, 2005). At micromolar quantities, the 45-amino acid protein MsDef1, derived from the seed of Medicago sativa, inhibits the growth of *Fusarium graminearum*, a filamentous fungus. The two categories of antifungal plant defensins, known as morphogenic and non-morphogenic, are separated by their impact on the morphology of fungal hyphae (Terras FR, 1992). While non-morphogenic defensins suppress hyphal development without creating noticeable morphological changes, morphogenic defensins suppress hyphal growth and a corresponding increase in hyphal branching (Broekaert WF, 1995). A morphogenic defensin called MsDef1 causes fungal hyphae to extensively hyperbranch (Gao A, 2000). In this study, we have successfully cloned the plant defensin gene MsDef1 isolated from the Medicago plant into E. coli cell is

an essential research tool for producing large quantities of the defensin protein to research its characteristics and use in medicine or agriculture.

MATERIALS AND METHODS

1. **Homology modeling:**

The defensin protein was selected using biological databases such as NCBI, BLAST, PDB, and and ExPasy, and their FASTA sequences werewere retrieved.

2. **Model Generation and validation:**

The model generation and validation were done using SWISS-MODEL, which serves as an automated server dedicated to the comparative modeling of three-dimensional (3D) protein structures. Discovery Studio (It is a software suite designed for simulating systems of small molecules and macromolecules. This suite is developed and distributed by Dassault Systems BIOVIA.), SAVES v6. 0 (SAVES v6.0 (available at https://saves.mbi.ucla.edu/) constitutes a comprehensive toolkit encompassing five distinct tools (ERRAT, VERIFY3D, PROVE, PROCHECK, and WHAT CHECK). These tools collectively forecast various categories of stereochemical attributes about protein structures.), PROSA (It helps in the recognition of errors in the protein model)

3. Selection of Ligands:

The Ligands for Molecular Docking, the fungal cell wall receptors, were selected from PubChem. This repository is a public resource for data concerning chemical compounds and their biological functions. It was established as part of the U.S. National Institutes of Health (NIH) Molecular Libraries Roadmap Initiatives.

5**. Docking of Ligand and Macromolecule**

Docking was performed using PyRx. PyRx is a virtual molecular screening tool for docking small-molecule libraries with macromolecules, aiming to identify lead compounds with specific biological functionalities. Autodock Vina command prompt was used to split ligands—visualize interactions between ligands and protein using BIOVIA Discovery Studio Visualiser.

6**. Isolation and characterization of genomic DNA**

The genomic DNA was isolated from the seeds of the Medicago plant using the phenol-chloroform method and run on 0.8% agarose gel.

7**. Gradient PCR of Defensin gene**

1XTAE and agarose were obtained from Himedia (India), whereas DEPC, TRIZOL reagent, Tris, EDTA, EtBr, and acetic acid were obtained from Sigma (USA). The Def forward, actin, and reverse primers were acquired from GCC Biotech Pvt. Ltd. in India. The First Stand of cDNA synthesis kit was purchased from Roche in Switzerland. Table 2 illustrates the primers used for MsDef1 gene amplification. Table 3 demonstrates the PCR conditions for the amplification of the MsDef1 gene. After amplification, the PCR products were run on 1% agarose gel.

S.No	Primers Name	Oligo Sequence (5' to 3')	PCR Product Length
1	Def1-F	GCTTATGCTTCCTCTTCCTCGT	252
	Def1-R	TGCCGCTAACTGCATTCTCTT	

Table 2: Primer sequence of MsDef1

Step	Temperature	Time	No. of PCR cycles
Initial denaturation	94°C	3mins	30 PCR cycles
Denaturation	94°C	30sec	
Annealing	55°C, 56°C, 57°C, 58.1°C and 59°C	30sec	

Extension	72°C	2mins	
Final extension	72°C	10mins	
Hold	4°C		

Table 3: PCR conditions for the MsDef1 gene

8. Defensin gene expression analysis using quantitative RT-PCR

Isolation of RNA

The TRIZOL technique was used to isolate total RNA and a 1% agarose gel was used, and the quality and quantity of RNA were estimated using the Labman UV Vis Spectrometer followed by DNase treatment and gene level detection of defensin gene using qRT-PCR

Gene level detection of defensin gene

Reverse transcriptase (MMLV) was added to a reaction mixture to convert 1.5 µg of total RNA to cDNA. For Oligo (dT), cDNA synthesis was performed by incubating at 42 °C for 60 minutes, heating at 70 °C for 5 minutes, and then holding at 4 °C to stop the reaction. The produced cDNA was employed as a template to identify gene expression in the target. Quantitative Real-Time PCR (qRT PCR) using Quantstudio 5 and the PowerUp Syber Green kit (Applied Biosystems, CA, USA). To measure the expression of the gene, qRT-PCR was done for chosen genes using the relative quantification (2^-ΔΔCT) method. The endogenous control (βActin) control cells were utilized as the calibrator to normalize the expression.

Quantitative RT PCR

The following materials were obtained to perform quantitative RT-PCR. 1X TAE, agarose, and DEPC were from Sigma, USA; TRIZOL reagent and Himedia, India. The forward and reverse primers for βactin were acquired from GCC Biotech Pvt.Ltd. in India,, Defensin specific primers obtained from Barcode Biosciences Pvt. Ltd., the First Stand cDNA synthesis kit from Roche in Switzerland. Applied Biosystems, CA, USA PowerUP Syber Green kit Quantstudio Version 5. Table 4 illustrates the primers used. The gene amplification was done in a PCR thermal cycler (Quant Studio 5, Applied Biosystems).

Gene	**Direction**	**Sequence (5' – 3')**
Def	Forward	GCTTATGCTTCCTCTTCCTCGT
	Reverse	TGCCGCTAACTGCATTCTCTT
Actin	Forward	TCCATCTTGGCATCTCTCAG
	Reverse	GTACCCGCATCAGGCATCTG

Table 4: Primers used for qRT-PCR

PCR conditions- UDG activation -50°C for 2 minutes, Dual-Lock DNA polymerase-95 C for 2 minutes, Denaturation-95°C for15 sec, Annealing -60°C for 1 minute, and extension- 72°C for 2 minutes. The real-time data was captured at the end of each extension stage, and a a melting curve analysis was performed.

9. Cloning of MsDef1 into pCambia1301 vector

pCambia vector was purchased from Abcam, USA, EcoR1 and T_4 DNA Ligase was from SRL, India.

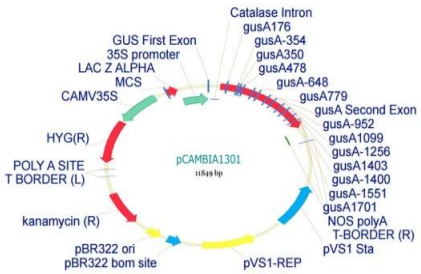


Figure 1: Plasmid map of pCambia 1301 vector

Restriction digestion and ligation

Restriction digestion was done by adding 5µl of 50pg pCambia in one tube and 5µl EcoR1 (10U/ml). 5µl of PCR product and 5µl of EcoR1 (10U/ml) were added in another tube. The two tubes were incubated at 37 C for 4 hours. Both contents of the tubes were mixed after incubation, and 5 µl of (5 U/µl) T_4 DNA ligase was added. The two tubes were incubated for 10 minutes at 65 oC.

Preparation of Calcium chloride competent E. coli cells and heat shock transformation

Competent cell preparation: *E. coli* was inoculated into LB broth, placed on a shaking incubator at 37°C and 200 rpm and incubated for 12–16 hours. 1 mL of overnight E.coli culture was added to 99 mL of fresh LB (1:100 dilution, no antibiotics). Shake incubated at 37°C and 200 rpm for 3–4 hours or until OD reaches 0.4. The reagents were stored at ice-cold or 4 °C. The culture was separated into multiple Oakridge tubes and placed on ice for 20 minutes. Further, the tubes were centrifuged at 4°C at 4000 p.m. for 10 minutes. The supernatant was discarded by tipping tubes over a discard bin and then aspirating any remaining media. The pellet was resuspended in 20 mL of ice-cold 0.1 M $CaCl_2$ and incubated on ice for 30 minutes, followed by centrifuging the tubes at 4°C at 4000 rpm for 10 minutes. The supernatant was discarded, and the pellets were combined by resuspending in 5 mL of ice-cold 0.1 M $CaCl_2$ with 15% glycerol and preserved in a -80°C freezer for future use.

Heat shock transformation:

Following the generation of competent cells, the heat-shock treatment introduced plasmid DNA into cells. The cells, after transformation, were plated on the appropriate LB plates supplemented with the reagent for selection or screening. The plates were incubated overnight, and the colony counts were recorded the next day.

Heat-shock: The competent cells were thawed, and 1–5 ul (10 pg–100 ng) of plasmid (pCambia, pCambia with a cloned gene) (do not exceed 5 uL for a 50 pL cell aliquot) was added separately in two tubes and incubated on ice for 30 minutes. Heat shock treatment was done by placing the cells in a 42°C water bath for exactly 30 seconds, then put on ice for 2 minutes. 1 mL of pre-warmed LB medium was added, shaken, and incubated at 37 °C, 200 p.m., for 1 hour for outgrowth.

Plating and incubation: The culture was spread on warm selective and screening plates. The selective medium is an LB medium containing 50 µg/ml of Kanamycin and 1mM/ml of IPTG. After plating, the plates were further incubated at 37°C for 12-16 hours to observe transformed colonies.

10. Transformation of pCambia Vector to *Agrobacterium tumefaciens* cells

Preparation of competent *Agrobacterium tumefaciens* ((MTCC No. 33970) was purchased from MTCC, Chandigarh, India) cells: Inoculate 250 mL of LB medium with 750 µL *Agrobacterium tumefaciens* and incubate at 28°C (Shaking incubator) with vigorous shaking for 11 hours until the OD is approximately 0.75. Pellet the cells by centrifugation (5000 rpm). Wash the pellet with 2 mL sterile tubes and centrifuge at room temperature for 10 minutes at 5000 rpm. Resuspend the pellet in 20 mL LB medium and aliquot 250 µL of this suspension into 1.5 mL microcentrifuge tubes.

Transformation and recovery of *Agrobacterium tumefaciens* cells: 20 µL of the pCambia Vector (5 µg) was added to the competent *Agrobacterium tumefaciens* sample (250 µL) in LB medium and incubated on ice (0°C) for 5 minutes and further incubated in liquid nitrogen (-80°C) for 5 minutes, followed by incubating at 37°C (water bath) for 5 minutes. LB medium (1 mL) was added to each vial and incubated at room temperature (with rotation) for 4 hours,, then plated the bacteria by streak plate method onto LB Agar plates containing appropriate selection antibiotics and incubated at 28°C for 3 days. After 3 days of incubation, a a single colony was picked and re-streaked onto an LB Agar plate containing 50 µg/ml of kanamycin selection antibiotics and incubated at 28°C for 2 days.

11. Transfection of Arabidopsis plant with *Agrobacterium tumefaciens*

15 – 20 mL of MS medium was pipetted into 25 x 250 mm sterile test tubes for each plant leaf to be grown after dip. One colony of transformed bacteria in LB media containing 50 µg/ml of Kanamycin selection antibiotics (2 mL) was grown overnight at 28°C (Shaking incubator) with vigorous shaking. Inoculate 225 mL of LB medium with 1.25 mL of preculture from above. Incubate overnight at 28°C (shaking incubator) with vigorous shaking. The bacteria were pelleted by centrifugation at 6000 rpm at 25°C for 10 minutes. Resuspend the bacteria in an infiltration medium (50 mL) and swirl to ensure a complete mixture of the bacteria. Transfer the bacterial suspension to a sterile dipping box. This process is best done in a laminar flow hood under sterile conditions. The plant leaves were dipped in the bacterial

suspension for 30 seconds. (*Agrobacterium tumefaciens* used to infiltrate five leaves of 5-weeks-old soil-grown Arabidopsis plants). After dipping, the plants were placed on agar plates (MS medium), and the lids were sealed with parafilm. The lids were then set overnight at room temperature. After overnight storage, the plant material was rinsed with sterile water, and the transformed plants were placed in sterile tubes containing the MS Agar Gel for growth. The plants were grown under long-day light conditions (16-hour daylight, 8-hour darkness) for several weeks until flowering and seed pod production occurred.

RESULTS AND DISCUSSION

1. 3D structure of plant defensins:

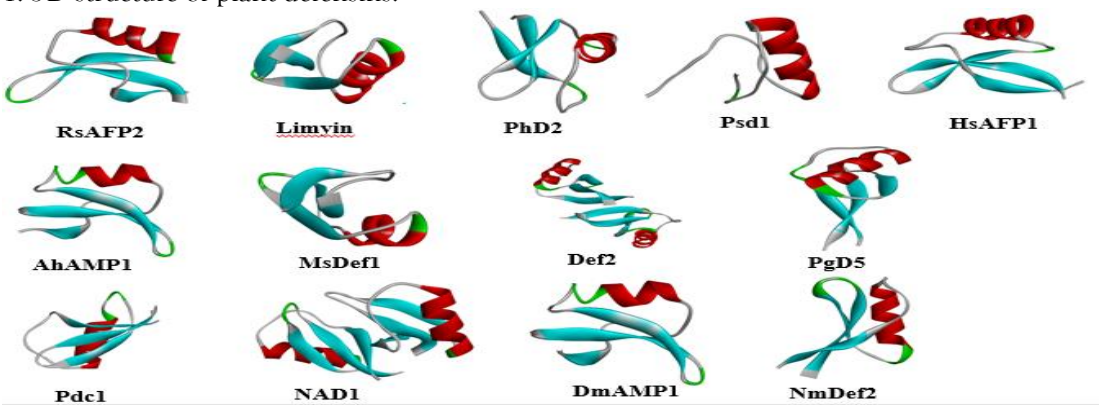


Figure 2: 3D structures of plant defensin proteins

Figure 1 shows the 3D structures of the plant defensin proteins obtained from the SWISS model. The structure revealed the presence of alpha helices and beta sheets. The Ramachandran plot illustrates the distribution of residues as percentages across favored regions, supplementary allowed areas, generously permitted regions, and the absence of residues within the disallowed region.

2. Molecular Docking:

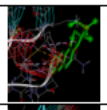
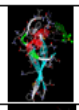
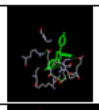
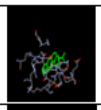
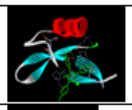
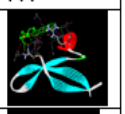
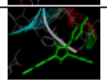
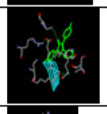
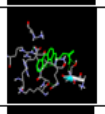
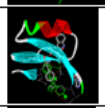
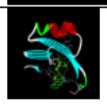
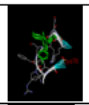
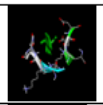
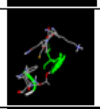
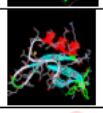
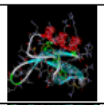
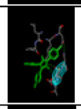
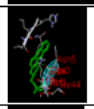
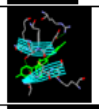
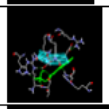
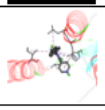
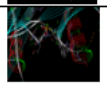
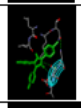
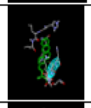
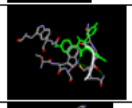
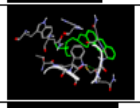
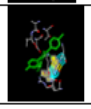
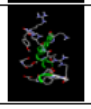
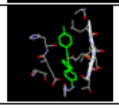
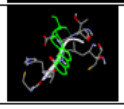
DEFENSINS	FUNGAL CELL WALL RECEPTORS		DEFENSINS	FUNGAL CELL WALL RECEPTORS		DEFENSINS	FUNGAL CELL WALL RECEPTORS	
	MAPK	PA		MAPK	PA		MAPK	PA
PgD5			NAD1			HsAFP1		
NmDef2			PhD1			AhAMP1		
Pdc1			PhD2			RsAFP2		
Limyin			Def2			MtDef5		
TVD1			DmAmp1					
MsDef1			Psd1					

Table 5: Interactions between fungal cell wall receptors and plant defensin proteins

DEFENSINS	FUNGAL CELL WALL	DEFENSINS	FUNGAL CELL	DEFENSINS	FUNGAL CELL WALL RECEPTORS

	RECEPTORS (Binding affinity /rmsd value)		WALL RECEPTORS (Binding affinity /rmsd value)		(Binding affinity/rmsd value)			
	MAPK	PA		MAPK	PA		MAPK	PA
PgD5	-6.2/0	-5.7/0	NAD1	-7.2/0	-6.9/0	HsAFP 1	-5.6/0	-7.2/0
NmDef2	-5.2/0	-5.6/0	PhD1	-6.5/0	-6.5/0	AhAMP 1	-5.7/0	-6.4/0
Pdc1	-6.4/0	-6.4/0	PhD2	-6.4/0	-6.8/0	RsAFP2	-5.4/0	-6.1/0
Limyin	-7.0/0	-7.2/0	Def2	-7.0/0	-7.6/0	MtDef5	-7.5/0	-6/0
TVD1	-6.8/0	-7.1/0	DmAmp1	-5.9/0	-5.7/0			
MsDef1	-6.4/0	-6.7/0	Psd1	-7.1/0	-7.4/0			

Table 6: Binding affinity and rmsd value of plant defensins against fungal cell wall receptors MAPK and PA

PyRx was used to dock ligand and macromolecules. A grid surrounded the entire structure, and Vina was then used to calculate the ligands' bond affinities and RMSD values. PyRx is used for docking investigations. The protein is docked as the macromolecule, while the cell receptors are ligands. A Grid with the dimensions and a center position is created using Vina Wizard to enclose the complete protein comprising the active sites. A table listing the ligands, their RMSD values, and their binding affinities is produced when the program has been run a second time. Only proteins with RMSD values of 0 should be taken into consideration. RMSD stands for root mean square deviation. The degree of the binding relationship between a protein and its ligand is measured by binding affinity. The binding affinities must be negative, and the lower the number, the better the outcome; the more significant the binding affinity, the lesser the affinity between the target molecule and ligand, and the more subdued their attraction and binding become.

Splitting of ligand files: By using the command prompt and Vina software, the ligands are split into different files based on their RMSD values. The ligand files in their PDBQT format are separated using the Auto Dock vina software according to their RMSD values and binding affinities. The command "vina_split" is used by the command prompt to execute the splitting operation. Each ligand file is divided into 10 conformations that may be further examined to determine the ligands with the binding affinities and RMSD values. These ligands are then employed as ligands in the final phase to research the interactions between ligands and proteins. The Discovery Studio showed the ligands and the defensin protein interactions in 2D and 3D. Table 5 and 6 illustrate the interaction between defensin proteins and fungal cell wall receptors and their respective binding affinity and rmsd value.

3. Isolation and characterization of genomic DNA

The phenol-chloroform method was used for the isolation of genomic DNA from the germinated seeds of *the Medicago* plant. The isolated genomic DNA was run on a 0.8% agarose gel and visualized for DNA bands under a UV transilluminator. Figure 3 illustrates the result of the isolation and characterization of genomic DNA. Lane 1 is loaded with a DNA ladder, followed by DNA bands observed in lanes 2 and 3.

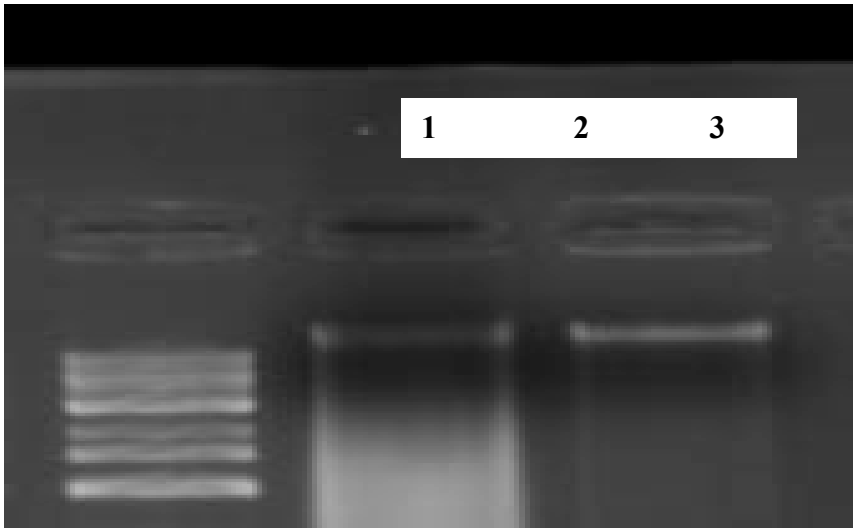


Figure 3: Gel picture showing amplification of genomic DNA isolated from germinated seeds of Medicago plant. Lane 1 denotes the DNA ladder of 1kb, and lanes 2 and 3 show the appearance of DNA bands.

4. Gradient PCR of defensin gene

Gradient PCR was for the MsDef1 defensin gene, and it was observed in figure 4 that a distinct amplified DNA band of 252bp in length was seen at an annealing temperature of 59 C.

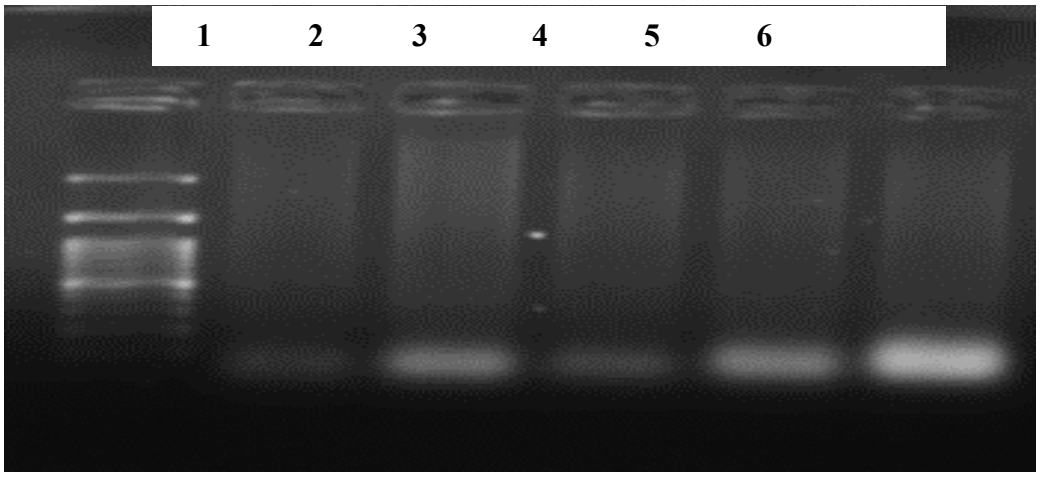


Figure 4: Optimization of Defensin gene expression analysis by gradient PCR method.
Lane 1-Molecular ladder of 100kb, 2- Annealing temp (55°C), 3-Annealing temp (56°C), 4-Annealing temp (57°C), 5-Annealing temp (58.1°C) and 6- Annealing temp (59°C).

5. Defensin gene expression analysis using quantitative RT-PCR

The isolated method used for total RNA was theTrizol, followed by DNase treatment to remove any DNA contamination. RNA obtained was converted to cDNA for qRT-PCR analysis. The defensin expression was analyzed using qRT-PCR against βActin, which served as an internal control for the experiment. The number of PCR cycles is displayed on the X-axis of the amplification plot, and the Y-axis shows the fluorescence from the amplification reaction, which is proportionate to the amplified product in the tube. Two phases have been seen in the amplification plot: an exponential phase and a non-exponential plateau phase. The amount of PCR product roughly doubles in each cycle during the exponential phase. However, while the reaction continues, some of its constituents are consumed, and eventually, one or more of them become limited. The reaction now slows and moves into the plateau phase. Relative defensin gene expression and fold variation were analyzed using the delta Ct values (actin=0.1395; defensin= -3.751,

expression=14.83, and fold variation= 1.17) obtained. It was observed that the defensin proteins were expressed in the seeds of the Medicago plant as observed in figure 5 and 6.

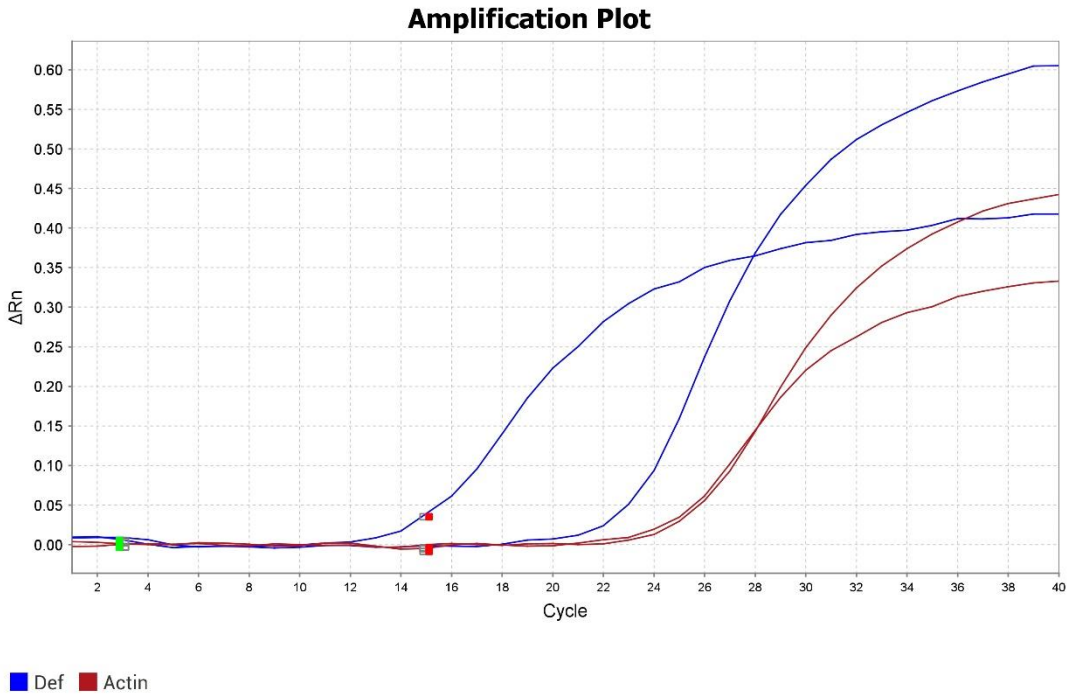


Figure 5: Amplification plot of defensin protein

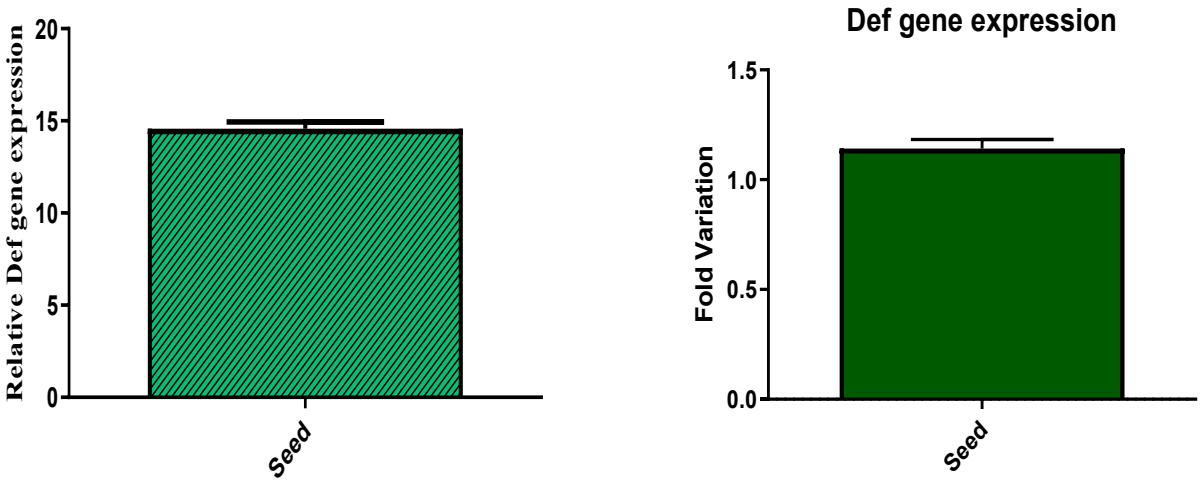
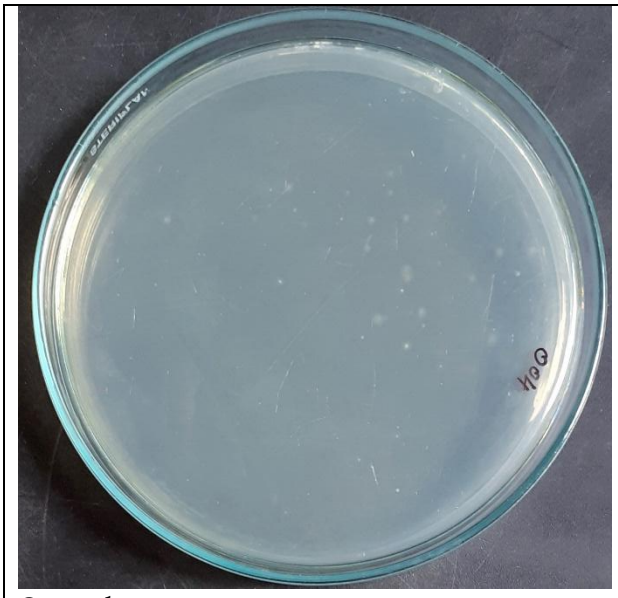


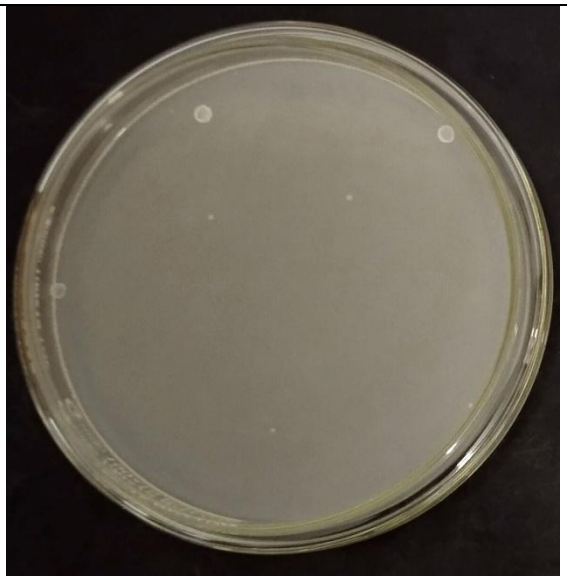
Figure 6: Defensin gene expression in Medicago plant

6. Cloning of MsDef1 into pCambia1301 vector

The PCR product of the MsDef1 gene and the pCambia1301 vector was cut using the EcoR1 restriction enzyme and ligated using T_4 DNA ligase. This recombinant vector was transformed into the E. coli cell using the heat shock method, and the transformed cells was plated on a selective medium, i.e., LB medium, containing Kanamycin and IPTG. Figure 7 demonstrates that white-colored colonies were observed in selective media containing the transformed E. coli competent cell with the pCambia vector and cloned DEF gene, and no growth was observed in selective media containing the E. coli competent cells in the control plate.



Control
No colonies were observed in the 50 µg/ml kanamycin-added LB medium.



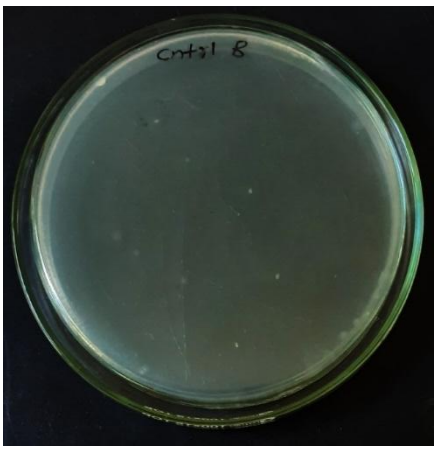
Clone-transformed colonies
observed in the 50 µg/ml kanamycin-added LB medium

Figure 7: Plate images of transformed colonies

7. Transformation of pCambia Vector to *Agrobacterium tumefaciens* cells

Recombinant pCambia Vector was successfully transformed into *Agrobacterium tumefaciens*. The appearance of colonies on the LB-Kanamycin plates showed in figure 8 demonstrates that the Agrobacterium has successfully taken up the recombinant vector.

A



B

Figure 8: Transformation and recovery of *Agrobacterium tumefaciens* cells in 50 µg/ml of Kanamycin selection LB Agar medium. A. Agrobacterium transformed cells, B. Negative control.

8. Transfection of Arabidopsis plant with *Agrobacterium tumefaciens*

The transformed *Agrobacterium tumefaciens* was transfected into Arabidopsis thaliana through the leaf dip. The transfected plants were selected onto MS media supplemented with Kanamycin as observed in figure 9 and 10.

Figure 9: Transfection of pCambia vector to plant leaves through Agrobacterium

Figure 10: Selection of transformed plant on Kanamycin selection medium

CONCLUSION

Homology modeling and other computational biology tools are crucial for understanding the interactions between plant defensins and fungal cell wall receptors. Defensins attack the invaders' cell walls to destroy them. Plant defensins bind to various cell wall receptors, including phosphatidic acid, GlcCer, and MAP kinase receptors. The current work investigated the interactions between several plant defensins and fungal cell wall receptors using an in-silico method. This study found that because defensins have zero rmsd values and low binding energies, they all bind to fungal cell wall receptors with great affinity. It has been noted that plant defensins exhibited affinity for both fungal cell wall receptors to lyse the invasive pathogen and start an innate immune response. The use of these plant defensin genes as a transgene to create plants resistant to biotic stressors is confirmed in large part by this in-silico investigation. Plant defensins have biological activity that makes them appealing to biotechnology. Antimicrobial, insecticidal, and even anti-parasitic plant actions are some of these qualities. These characteristics make the peptides a promising choice for the transgenic creation of agronomically significant plants that may fight off infections and for application in protein engineering. The defensin from R. sativus Rs-AFP2, which prevents numerous fungal pathogens like C. lindemuthianum, F. culmorum, F. oxysporum, N. haematococca, B. cinerea, V. dahliae, and M. fijiensis, further demonstrates the antifungal activity, which offers an additional benefit. These are, thus, the clear benefits of using R gene-mediated transformation in plants. Defensins can also confer or improve resistance when combined with other antimicrobial substances. Lastly, defensins may provide abiotic stress adaptability in addition to their antibacterial properties. In this study, successful isolation and characterization of the MsDef1 gene using the phenol-chloroform technique, genomic DNA was extracted from the Medicago plant's germinated seeds. The DNA was examined with a UV transilluminator to look for DNA bands. Using a gradient PCR technique, the defensin gene was amplified, and at 59 C, a clear 252 bp band was seen. Quantitative RT-PCR was used to study the expression of the defensin gene in comparison to an internal control, βActin. Two stages were visible in the amplification plot: an exponential phase and a non-exponential plateau phase. Delta Ct values were used to analyze the fold variation and relative defensin gene expression. After MsDef1 was cloned into the pCambia1301 vector, E. coli cells were transformed into the recombinant vector. Selective medium containing the transformed E. Coli competent cell with the pCambia vector and cloned DEF gene showed white-colored colonies. In contrast, the competent E. Coli cells in the control plate showed no growth at all in any of the selective media, which paves a way to induce E. coli to manufacture large amounts of plant defensin protein for a variety of uses, such as researching the protein's function and possible use in agriculture or medicine.

Conflict of Interest

The author(s) confirm that this article content has no conflict of interest.

Acknowledgements

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