

**COMPARATIVE MODELLING OF PATHOGENESIS RELATED 4B PROTEIN (Q6T5J8) OF *ORYZA SATIVA* SUBSP. *INDICA* WITH THE THREE-DIMENSIONAL STRUCTURE OF BARLEY1BW3**Showmy K.S¹, Sinosh Skariyachan² and Yusuf A^{1*}.¹Inter University centre for Plant Biotechnology, Dept.of Botany, University of Calicut, Kerala, India
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ABSTRACT: Pathogenesis related proteins are involved in disease resistance responses in plants. Rice (*Oryza sativa* subsp. *indica*) produces a Pathogenesis Related 4b protein (PR 4b, ID Q6T5J8) one of the vital defensin against fungal and bacterial attack. Purified PR4b inhibits spore germination and mycelial growth of fungi on plants. Use of biological products against bacterial and fungal pathogen attack will be a boost for crop improvement programs. In this study, the structure of PR 4b was modelled by homology modelling using the available structure of 1BW3 from barley. Since the importance of PR 4b in conferring resistance to bacterial and fungal pathogen is well established, a three-dimensional protein structure was modelled by simulation studies to achieve a better understanding of the structure of PR4b. The molecular structure of PR 4b revealed 83% sequence identity to IBW3 A when aligned using BLASTP. Comparative modelling software MODELLER 9v12 has given acceptable values for PR 4b. The structural validity of the derived model was performed by PROCHECK, PROSA1 and three different molecular dynamic force fields viz. Anolea, Qmean and Gromos. The results showed 0.2 Å RMS value, Z-Score of -6.19 indicated that the modelled structure is stable and showed 79.2% of residues in most favoured region in Ramachandran plot. Comparative modelling of PR 4b will help to study the involvement of defence mechanism in *Oryza sativa* and can provide better understanding about its structural integrities.

Key words: *Oryza sativa*, PR4, PR4b, PR proteins, Homology modelling.

INTRODUCTION

Rice (*Oryza sativa*), one of the world's most important food plant and has played a vital role in human nutrition and culture for the past 10,000 years. Yield loss of rice is mainly due to two major diseases; sheath blight and bacterial blight caused by *Rhizoctonia solani* and *Xanthomonas oryzae* pv. *oryzae* respectively [1]. *Xanthomonas oryzae* pv. *oryzae* is a devastating pathogen and has been extensively studied as a model pathogen of monocotyledons to study the host-pathogen interactions, bacterial pathogenesis and defense responses [2]. Fungal diseases like sheath blight, caused by *Rhizoctonia solani* Kühn, is another most destructive disease of rice worldwide [3]. The resistance against pathogen is a heritable trait of plants. In many host-parasite interactions the pathogen produces host specific toxins, responsible for symptoms and disease development. The toxin molecules are attached to specific sensitive sites or receptors in the cell and cause the disease. The hypersensitive reaction to a pathogen is one of the most competent defense mechanisms in nature and leads to the up regulation of numerous plant genes encoding defense proteins. PR proteins are widely distributed in plants and are mainly induced by different stress conditions. They were first discovered and designated as "b-proteins" in tobacco leaves hypersensitized to TMV; since then PR proteins have been described in various plant species [4]. Initial findings justified that these proteins induce resistance in plants, expressing a hypersensitive necrotic response to pathogens of viral, fungal and bacterial origin. "b-proteins" are also induced in susceptible plant – pathogen interactions subjected to abiotic/biotic stresses. A name "pathogenesis-related" protein, or PRs, was proposed by Antoniw *et al* (1980) from different tobacco cultivars [5]. PRs are low-molecular weight proteins (6-43 kDa), extractable and stable at low pH (< 3), thermostable, and highly resistant to proteases [6, 7].

Conversely, studies have shown that there are two main streams of immune system, one uses transmembrane pattern recognition receptors (PRRs) that respond to pathogen associated molecular patterns (PAMPs) and the second acts largely inside the cell uses NBLRR (Nucleotide Binding Leucine Rich Repeat) type R-gene products [4]. Rice defense mechanism with R gene is operational with incompatible interaction against invading pathogen that has corresponding *Avr* genes. The incompatible interaction resulted in the hypersensitive response and the activation of rapid and effective defence responses, including the production of pathogenesis-related proteins, oxidative enzymes and phytoalexins [8, 9]. PR 4b functions as a positive modulator of plant cell death and defense responses [10]. They belong to PR-4 protein family, seen in both monocotyledonous and dicotyledonous plants. The conserved region common to all PR-4 proteins is the barwin domain which was characterized from the barwin protein of barley [11]. The PR-4 family of PR proteins consists of class I and class II chitinases. Class I has a conserved N-terminal cysteine-rich domain and is absent in class II chitinases. The substrate for chitinase (chitin) is the main component of most of the fungal cell walls and its expression is induced by pathogens, hence play an important role in the active defence response of plants. Rice pathogenesis related PR4b protein, OsPR-4b; plays a major role in the disease resistance responses of rice against pathogen attacks through its antifungal activity [12]. This protein expression is up-regulated in rice during *Magnaporthe oryzae* infection. *Xanthomonas oryzae* pv. *oryzae* infection showed increased expression in Xa21-mediated responses at late stages [13]. The PR4b expression decreased in susceptible inoculated (SI) plants from 0 DAI (Day after Inoculation) to 30 DAI and increased in resistant inoculated (RI) plants in the same intervals, adding the relevance of PR 4b protein. Transient expression of PR 4b triggers hypersensitive cell death. Plants contain wide array of pathogen recognition receptors like LRR (Leucine Rich Repeat) proteins to control the defense and cell death signalling. PR 4b is synthesized in the endoplasmic reticulum, interacts with LRR1 in the plasma membrane, and is secreted in to the apoplast via the plasma membrane. Binding of PR 4b to LRR1 requires a chitin-binding domain of PR4b. Purified PR 4b protein inhibits spore germination and mycelial growth of plant fungal pathogens [10]. The lack of 3-D structures of PR 4b of rice is the main constraint in understanding the molecular mechanisms of rice defense against the pathogen. Homology modeling is a novel way of elucidating the structural information of proteins where no crystal structure is available. Computational biology is a powerful tool to predict the structural feature of protein and analyse the conserved domains regardless of primary amino sequence homology within proteins of the same family. Elucidation of the spatial 3D-conformation and/or domain organization(s) of proteins are important prerequisites to better understand protein function. In the present study we report the comparative modelling of PR 4b with 1BW3.

MATERIALS AND METHODS

Retrieval of target protein sequences

The sequence of Pathogenesis Related 4b protein of *Oryza sativa* subsp. *indica* (Rice) (accession no: Q6T5J8; length: 151 amino acid residues) was retrieved from Uniprot [12] in FASTA format.

Proteomic analysis

The primary structure analysis was carried out by ProtParam tool of ExPasy Proteomic Server (<http://expasy.org/cgi-bin/protparam>) [14]. The parameters computed by ProtParam were molecular weight, theoretical pI, amino acid composition, atomic composition, extinction coefficient, estimated half-life, instability index, aliphatic index and grand average of hydropathicity (GRAVY). PSIPRED server [15] was used for the prediction of secondary structure of PR 4b protein. The InterProScan tool (<http://www.ebi.ac.uk/Tools/pfa/iprscan/>) [16] was used to understand the protein family, super family, and domain arrangement within the protein. Conserved domains of PR 4b protein were explored by using Pfam (<http://pfam.janelia.org/>) [17].

Screening of best homologous template for rice PR 4b modelling

Template search for PR 4b was performed using BLASTP program against PDB database (<http://www.rcsb.org/pdb>). The best templates were selected based on the percentage of identity, similarity, expected value, query coverage and alignment scores.

Multiple sequence alignment

Multiple sequence alignment is an essential tool for protein structure and function prediction, phylogeny inference and other common tasks in sequence analysis. Tree-based consistency objective function for alignment evaluation (T-COFFEE) is a multiple sequence alignment (MSA) program [18] and was used to detect the conserved regions of rice PR 4b.

Homology modelling of rice PR 4b

3D model of PR4b protein was built using homology modelling software MODELLER 9v12 [19] on windows operating system by using best template. MODELLER is routinely used for homology or comparative modelling of protein three-dimensional structures. Alignment of the sequence with known related structures provided by the user and MODELLER predicts a model containing all non-hydrogen atoms. The model was visualized by using PyMOL [20].

Model Validation

Quality of the predicted model was assessed using a variety of validation tools, such as PROCHECK [21] and PROSA1 [22]. PROCHECK was used for validation of the structure of protein quality by Ramachandran plot. ProSA1 program (Protein Structure Analysis) is an established tool employed in structure prediction, refinement and validation of experimental protein structures. The generated Z score of model is a measure of compatibility between its sequence and structure. The modelled Z score should be comparable to the Z scores of the template [23]. Knowledge based potentials of mean force will be utilised to evaluate model accuracy and expresses local model quality by plotting energies as a function of amino acid sequence position [24]. Energy minimization for 3D structures was performed using three different molecular dynamic force fields *viz.* Anolea [25], Qmean [26] and Gromos [27].

The pairwise 3-D structure superimposition of the elucidated model of PR4b protein with its closest structural homologue was carried out using DaliLite [28] tool that computes the root mean square deviation (RMSD) between the C α -atoms and all other atoms of the homology model and template.

Model visualization

Final modelled structure of Q6T5J8 was visualized in UCSF Chimera [29].

RESULTS

Retrieval of PR4b sequence of *Oryza sativa* and screening of best homologous template

The sequence of PR 4b (ID. Q6T5J8) was retrieved from Uniprot. A search with BLAST against PDB was assisted to retrieve homologous template structure for the target PR4b protein. BLAST search revealed two putative templates (1BW3 and 4JP7) of best identity of 83% and 72% and percentage similarity of 90% and 79% respectively with the target sequence. The BLAST results are presented in Table 1.

Primary and secondary structure analysis of PR4b protein

The physiochemical properties of PR 4b were predicted by ProtParam [14] (Figure 1). The molecular weight was estimated as 16,474.5 Daltons. The percentage proportion of the amino acids were Ala 12.6%, Val 9.9%, Asp 8.6%, Gly 8.6%, Thr 6.6%, Asn 5.3%, Glu 5.3% and Cys, Leu, Met, Tyr 4.3%. It was calculated that the instability index of PR 4b was 15.64, aliphatic index was 69.80 and the GRAVY index was -0.116.

PSIPRED [15] predicted the secondary structure of PR 4b and is shown in Table 2. Domain prediction by InterPro [16] showed that PR 4b possesses five domains and this domain is observed in Barwin proteins and in some glycoside hydrolases. The functional domain present in PR 4b clarified that the protein has a barwin domain from the region 28 to 148 (Pfam: PF00967). The superimposition and structure annotated pair wise alignment between modelled protein and template was predicted by DaliLite [28]. The superimposition showed that the structural alignment is in the allowed root mean square (RMS) value. The RMSD value between the target and template was estimated to be 0.2 Å which indicate perfect structural alignments and high quality of the model (Figure 6A) The number of superimposed C α carbon atoms found to be 125 with a Z score of 27.1 (Figure 7). The chains A of the template (1BW3_A) consist of 20% helices (6 helices; 25 residues) and 27% beta sheet (5 strands; 34 residues).

Multiple sequence alignment

The multiple sequence alignment performed by T-COFFEE [18] revealed that the amino acids represented with the asterisk symbol are highly conserved and has good identity. The sequence alignment between the target and template showed 99 consensus regions (Figure 2). These regions represented the functional domain of the PR (Pathogenesis Related) protein.

Homology modelling of PR4b protein

Hypothetical 3D model was created since the 3D structure of PR 4b is not available in PDB. The homology model was generated by using FASTA sequence of PR 4b as the target and barwin, basic barley seed protein (PDB ID: 1BW3), chain A from *Hordeum vulgare* as the template sequence. Three input files *viz.* atom file, alignment file and script file are essential for comparative modelling of an unknown protein. The final model was generated by Modeller 9v12 [19]. The modelled structure was uploaded to PyMOL and the 3D structure with stable secondary structures was displayed. The final modelled structure of PR 4b was visualized in UCSF Chimera (Figure 6A & B).

Validation of modelled structure

The quality of PR 4b model was evaluated by a number of tools to test the internal consistency and reliability of the model. In order to select the best model, the structural validity of the model was performed by PROCHECK [19] analysis, PROSA1 [22] and three different molecular dynamic force fields *viz.* Anolea, Qmean and Gromos. Stereo chemical quality of the polypeptide backbone and side chains was evaluated using Ramachandran plot generated by ProCheck (Figure 3). It revealed 79.2% (84) of residues in most favoured region, 18.9% (20) of residues in additional allowed region, 0.9% (1) in generously allowed region and 0.9% (1) residue in disallowed region.

The structure was energy minimised by three different molecular dynamic force fields viz. Anolea, Qmean and Gromos (Figure 4). The energy profile of the model and the Z-score value were obtained using ProSA server that calculates the interaction energy per residue using a distance-based pair potential. The ProSA analysis of the model PR 4b achieved a Z score of -6.19 (Figure 5) which indicates the generated model is of good quality.

Table 1: Templates selected for Comparative Model Building of PR 4b from BLAST Search against PDB

PDB ID	Name of Protein	Source	Chain	Percentage of Identity	Percentage of Similarity	Query Range	E-Value	Score
1BW3	BARWIN, BASIC BARLEY SEED PROTEIN	<i>Hordeum vulgare</i>	A	83%	90%	81%	3e-74	221
4JP7	Papaya Barwin-like Protein	<i>Carica papaya</i>	A	72%	79%	82%	3e-61	188

Table 2: Secondary Structure Comparison of PR 4b and 1BW3

Target/ Template	Number of amino acid residues (%)		
	Helix	Strand	Total number of amino acids
PR 4b (Target)	26 (17)	41 (27)	151
1BW3 (Template)	25 (20)	49 (39)	125

Number of amino acids: 151

Molecular weight: 16474.5

Theoretical pI: 4.61

Amino acid composition:

Ala (A)	19	12.6%
Arg (R)	6	4.0%
Asn (N)	8	5.3%
Asp (D)	13	8.6%
Cys (C)	7	4.6%
Gln (Q)	8	5.3%
Glu (E)	3	2.0%
Gly (G)	13	8.6%
His (H)	2	1.3%
Ile (I)	4	2.6%
Leu (L)	7	4.6%
Lys (K)	4	2.6%
Met (M)	7	4.6%
Phe (F)	3	2.0%
Pro (P)	5	3.3%
Ser (S)	5	3.3%
Thr (T)	10	6.6%
Trp (W)	5	3.3%
Tyr (Y)	7	4.6%
Val (V)	15	9.9%
Pyl (O)	0	0.0%
Sec (U)	0	0.0%
(B)	0	0.0%
(Z)	0	0.0%
(X)	0	0.0%

Figure 1: Primary structure analysis of the protein by ProtParam

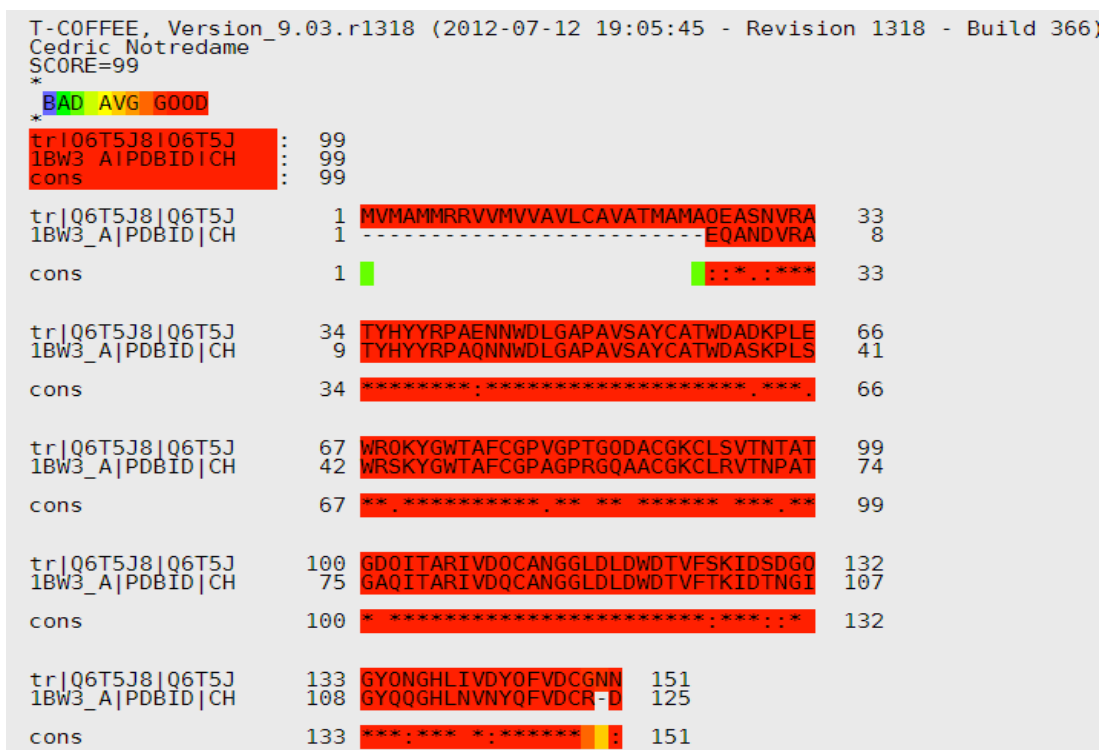


Figure 2: Multiple sequence alignment of the target and best template predicted by T-COFFEE. The symbol “*” indicate highly conserved (identical) residues, “:” indicate high similarity and “.” indicate low similarity.

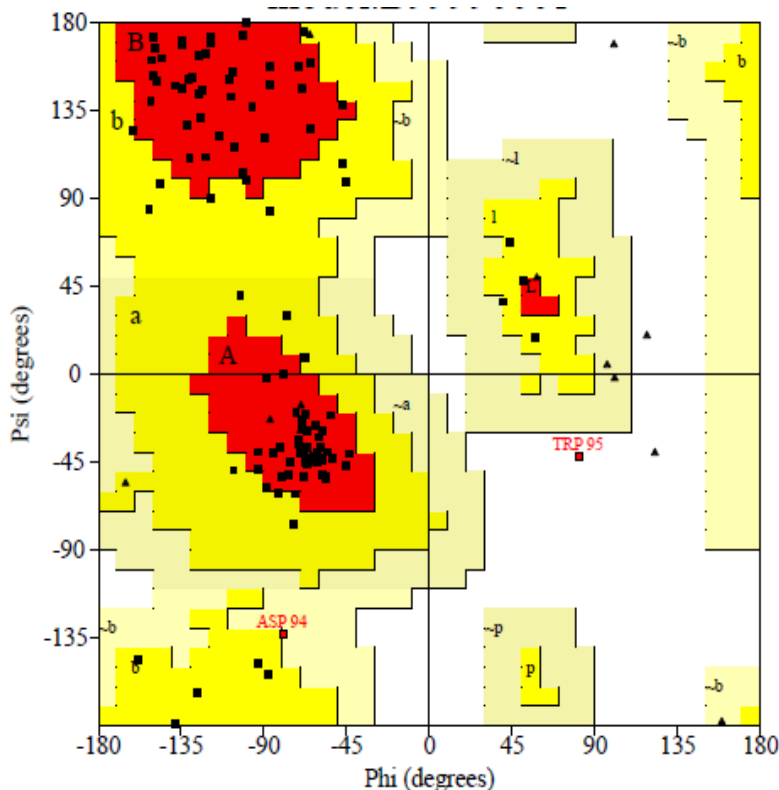


Figure 3: The stereo chemical validation of the hypothetical model by Ramachandran plot generated by ProCheck. Plot indicated that 79.2% of residues in most favoured region and 0.9% in disallowed regions.

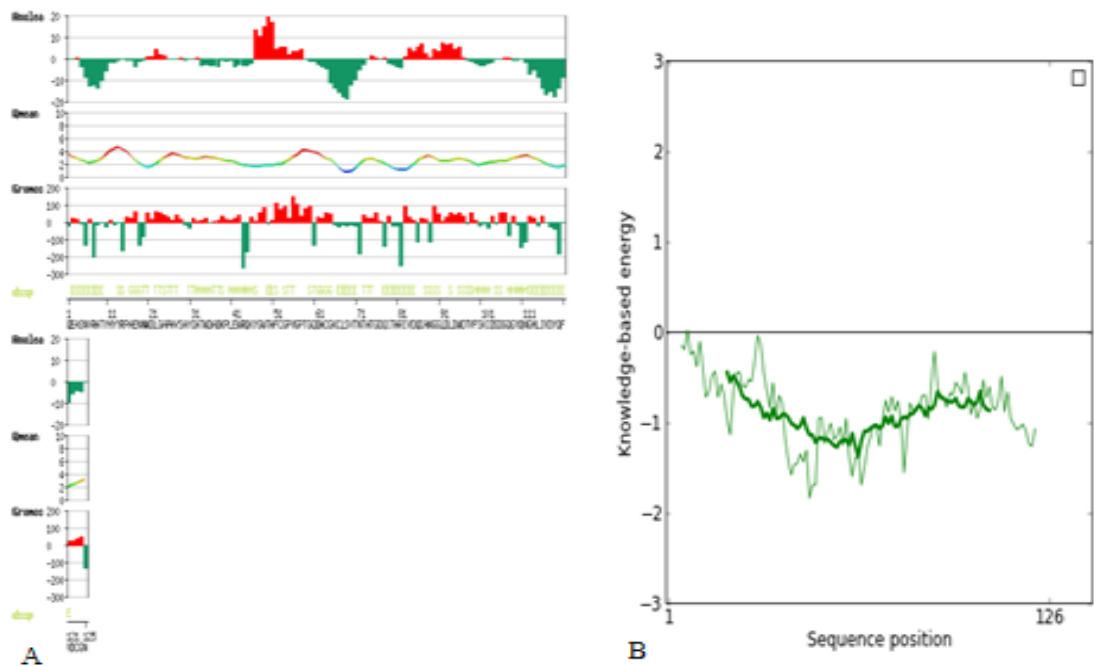


Figure 4: Refinement and validation of the hypothetical structure of PR 4b A) Energy minimized conformation of the modelled protein generated by Anolea, Qmean and Gromos. Green coloured vertical peaks indicated the minimum energy residues and red peaks showed the errors in the residues. (B) Energy plot obtained from ProSAi showed that the modelled protein is a minimum energy conformer.

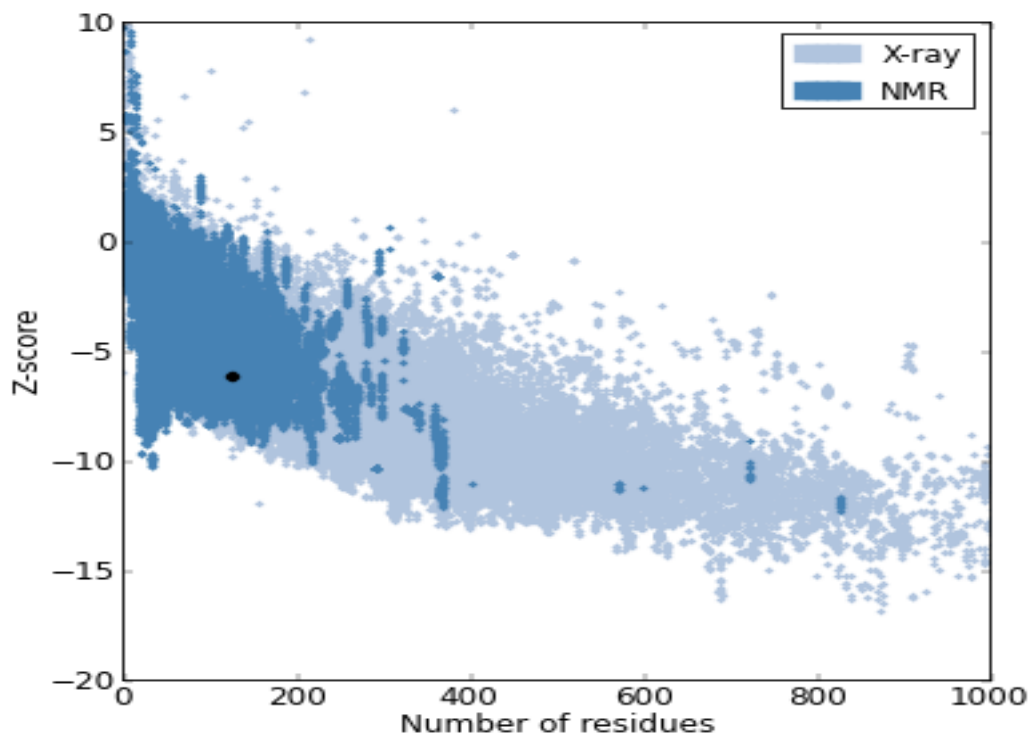


Figure 5: A) The refinement of the hypothetical model by ProSA server. Overall quality of PR 4b model showing a z-score of - 6.19. The plan of Z-Score shows spot of Z score's value of protein determined by NMR (represented in dark blue colour) and by X-ray (represented in light blue colour) using ProSAi program. The black dot represents Z-Score of the model in comparison with NMR structure of the template.

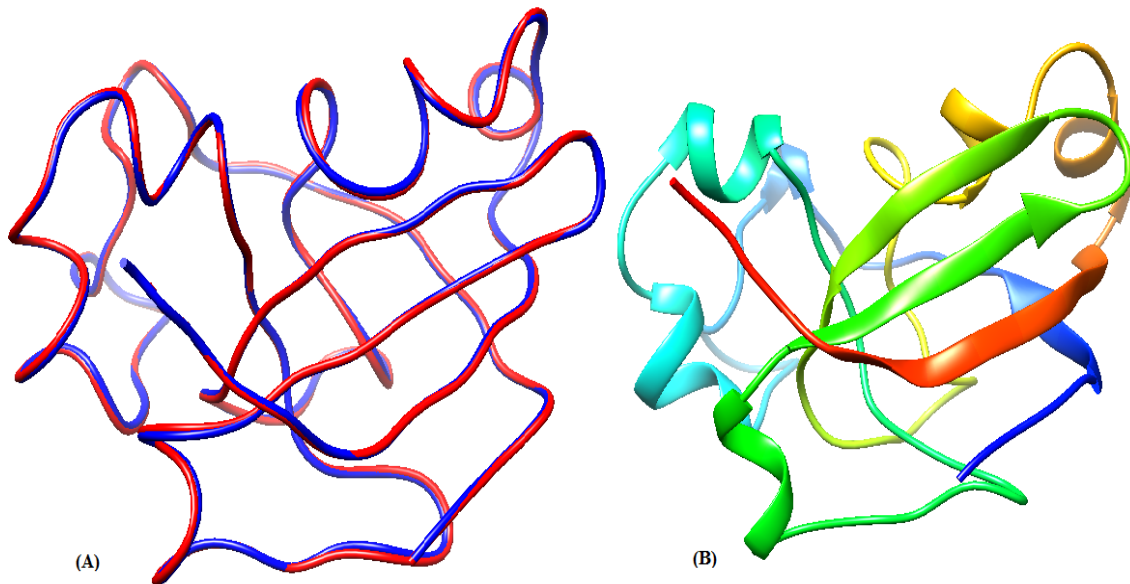


Figure 6: Three-dimensional structural superimposition of PR 4b model with template 1BW3 A) Superimposed image B) Visualisation of hypothetical model of PR 4b

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DSSP LEEEEEEEELLLLLHHHLLLLLLLLLLLLLLLLLLLLHHHLLLLHHHHHLLLELLLLLLLLL
Query QEASNVRATYHYRPAENNWDLGAPAVSAYCATWDADKPLEWRQKYGWTAFCGPVGPTGQ 60
ident | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
Sbjct EQANDVRATYHYRPAQNNWDLGAPAVSAYCATWDASKPLSWRSKYGWTAFCGPAGPRGQ 60
DSSP LEEEEEEEELLLLLHHHLLLLLLLLLLLLLLLLLLLLHHHLLLLHHHHHLLLELLLLLLLLLH

DSSP HHHLEEEEEELLLLLLEEEEEEEELLLLLLLLLLLLLLLLLLLLLHHHLLLLHHHHHLEEEEEEE
Query DACGKCLSVINTATGDQITARIVDQCANGGLDLWDVTFVSKIDSDGQGYQNGHLIVDYQF 120
ident | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
Sbjct AACGKCLRVTNPATGAQITARIVDQCANGGLDLWDVTFVKIDTNGIGYQQGHLNVNYQF 120
DSSP HLLLEEEEEELLLLLLEEEEEEEELLLLLLLLLLLLLLLLLLLLLHHHLLLLHHHHHLEEEEEEE

DSSP ELLLLL
Query VDCGNn 126
ident | | |
Sbjct VDCRD. 125
DSSP ELLLL.
    
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Figure 7: The structure annotated pair wise alignment between the template and target. The DSSP symbol H, E and L indicate helix, sheet and coil respectively.

DISCUSSION

A homology model of PR 4b protein of *Oryza sativa indica* having 151 amino acids is described. An experimentally determined protein structure is essential for comparative modelling. The protein sequence of 1BW3 of Barley was retrieved from UniProt database in FASTA format and used as template for comparative modelling. This template protein had 125 amino acid and explained defense responses against bacteria. This protein is closely related to protein domains encoded by wound induced genes in plants. 1BW3 is a basic seed protein of *Hordeum vulgare* showed 83% identity, 90% similarity, 3e-74 E-value and 221 alignment score with the target sequence. The next similar protein 4JP7 is also a barwin-like protein from *Carica papaya* with 72% identity, 79% similarity, 3e-61 E-value and 188 alignment score with the target sequence. The best templates were selected based on the percentage of identity, similarity, E-value and alignment scores. Lesser E-value, high scores and maximum identity implies good template structures. Hence 1BW3 selected as the best template sequence because the results showed good homology with the target protein.

Proteomic analysis gave detailed information about the target protein. ProtParam [14] calculated the instability index of PR 4b as 15.64, predicts the stable nature of the protein. Aliphatic index was high (i.e., 69.80), indicating its stability at wide range of temperatures. The GRAVY index of PR 4b was very low (-0.116), indicating its high affinity for water.

PSIPRED [15] predicted the secondary structure of PR 4b and the identity of each alpha and beta sheets between target and template. The secondary structure comparison between the target and template showed N-terminal domain with helices, whereas the C-terminal domain contains beta sheets that shared homology across the entire length. InterPro [16] showed that PR 4b had a functional barwin domain comprised of amino acids from 28th to 148th position. A rice PR-4 gene, OsPR-4b was identified and confirmed having significant similarity with the barwin domain and other known PR proteins [12]. Multiple sequence alignment by T-COFFEE [18] confirmed the conserved characteristic of barwin domain and formation of disulphide bonds with six cysteine aminoacids. The barwin domain shares a high level of similarity with the other barwin family proteins. Barwin domain is classified in chitinase class II [30], hence PR 4b can also be included in the same class and confirmed the absence of the N-terminal cysteine rich domain. Comparative modelling of protein is one of the most accurate methods for 3D structure prediction. This method utilises the homology between the sequence of target protein and at least one known structure of protein. Generally this would give reasonable results based on the assumption that the tertiary structure of two proteins will be similar if their sequences are related. Hence 1BW3 A chain structure was downloaded from PDB, has 125 residues revealed by NMR. It consists of 20% helical (6 helices; 25 residues) and 27% beta sheet (5 strands; 34 residues) and eight turns.

ProCheck [21] divides the Ramachandran plot for a protein into four areas: most favoured, additional allowed, generously allowed and disallowed. A good model should have very few residues within the disallowed regions and many in the most favoured regions [31]. The modelled PR 4b has 79.2% of residues in most favoured region and 0.9% in disallowed region. The quality of PR 4b model was evaluated to test the internal consistency and reliability of the model. Z-score value is a measure of model quality as it measures the total energy of the structures. ProSA1 [22] analysis showed that protein folding energy of the modelled structure was in good agreement with that of the template. The Z score of 6.19, PR 4b achieved is in the range of native conformation and that of template was -5.62. The negative value of energy reflects reliability of the model reflecting the good quality of the model. In this plot, groups of structures from different sources (X-ray, NMR) are distinguished by different colors [22]. Superimposed structure of target and template and RMSD assessment indicated the generated model is reasonably good and quite similar to template. These results revealed that the model generated through modeller is most acceptable and it can be used for further investigation. Validation results also suggested that the predicted model is a reliable 3D structure of PR 4b.

CONCLUSIONS

In this study, 3D structure of PR 4b was developed using available plant defensin as templates which revealed most identity with the PR 4b. Molecular model of PR 4b protein was predicted using homology modelling method which is reliable and it can be used for evaluating the defensin mechanism in plants. The proposed model is useful to understand rice defensin protein against pathogen challenge and chemical induction. Thus, PR 4b structure explained has excellent similarity structure of barwin domain which contains six cysteine residues that combine to form three disulfide bridges. Sequence of barwin showed high similarity with the 122 amino acid stretch in the C-terminal end of the products of two wound induced genes (win1 and win2) from potato, the product of the *havin* gene of rubber trees and PR protein 4 from tobacco. The high levels of similarity to these proteins suggest that the PR 4b may be involved in a common defense mechanism in plants. Similarity with barwin domain and absence of N-terminal cysteine residues also shows that PR 4b can be classified in the classII chitinase group. Comparative modelling of PR 4b with the three dimensional structure of Barwin seed protein is a reliable 3D structure. Elucidation of the 3D structure of PR 4b will lead to the functional studies of the protein and to predict the structure of novel defensin proteins.

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