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In-Silico Modeling of Pattern Recognition Receptor EFR and Molecular Interaction with Pathogen Associated Molecular Pattern elf18

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ABSTRACT

Plants depend entirely on pattern-triggered immunity (PTI) system to protect them from various pathogenic bacteria. It is activated by pathogen-associated molecular pattern (PAMP) of the host plant by pattern recognition receptor (PRR) with co-receptor. Elongation Factor Receptor (EFR) is one of the PRR used to protect against Brassica species disease. Although research on transgenic approach have been carried out to analyze the EFR protein, but the full ectodomain interactions of EFR with PAMP elf18 protein and co-receptor Brassinosteroid Insensitive 1-associated receptor kinase (BAK1) through in-silico has not been accomplished yet. The purpose of this study was to determine the interaction of EFR protein with elf18 protein through in-silico analysis. In this study, PRR EFR and PAMP elf18 was constructed by homology modeling using HHpred Modeler, followed by docking and molecular dynamics (MD) simulations of EFR and elf18 protein using Z-Dock and GROMACS 5.0.4 respectively. Modelling results showed that multiple template modeling (MTM) generated best models compared to single template modeling (STM) due to their best quality of the protein structure obtained by HHpred Modeler generate best-validation results of 71.123 ERRAT, 95.67% Verify3D and 92.8% in favoured region of the Ramachandran Plot. Docking results showed that the complex interaction of BAK1 and elf18 binds at leucine-rich-repeat (LRR) EFR (LRR 1-8 and LRR 12-14). After 50ns MD Simulation, the results showed that the docked complexes have significant reduction of H-bonds. For EFR-elf18-BAK1 (normal) complex, 20 hydrogen bonds were sustained compared to EFR-elf18-BAK1 (mutated) complex that only sustained 16 hydrogen bonds, proved that the mutated protein have less interaction after simulation. This study may contribute significantly towards understanding the early event of Pattern Triggered Immunity mechanism of EFR-elf18-BAK1 protein complex.

Keywords:
PAMP, EFR, MD Simulation, elf18

1. Introduction

Brassica plant species such as Cabbage (*Brassica oleracea* var. *capitata* L.) is one of the main vegetables consumed in Malaysia, not only as fresh market vegetable but also eaten fresh in food consumption such as coleslaw, sauerkraut and cabbage roll. The *Brassica* species mainly affected by

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diseases such as *Xanthomonas campestris* and *Plamodiospora brassicae* causes by the most prominent insect pest, *Plutella xylostella.* There is a lot of research has been already done to control the disease such as transgenic crops as well as the usage of the biological insecticide and insect-resistant genetically modified crop, which is ineffective due to the some factors such as short-lived of plant species, non-environmental friendly and cost effective.

Many of the research recently focussing on *in-silico* analysis study where all the data of biological experiments are being carried out entirely in computer. Pattern recognition receptor (PRR) plays an important role in *in-silico* study for rapid detection of potential danger caused by pests and microbes by pathogen-associated molecular pattern (PAMP). EFR is one of the most-characterized membrane protein used for *in-silico* study. Previous research on transgenic expressions of LRR-RK EFR protein with different receptor protein of Flagellin Sensitive2 (FLS2) has been carried out through experiments such as binding assay, co-immunoprecipitation, conservation mapping and others. Although most of PRR/PAMP are already been identified, however the full ectodomain analysis of EFR protein and its interaction with PAMP has not been carried out yet. Therefore, this current study make an attempt to interact PRR of full domain EFR protein without trans-species transfer with elf18 protein (PAMP specified for EFR) and co-receptor BAK1 protein, to analyse the interaction at atomic level between PRR/PAMP through *in-silico* analysis using bioinformatics approach.

2. Literature Review

2.1 Pattern Triggered Immunity (PTI)

PTI is the first layer defense mechanism in plant innate immune system to protect plants from wide range of pathogens like bacteria, fungi and viruses [16]. Plants do not have antibody or special cell to destroy pathogens, yet they only have various pattern recognition receptors (PRRs) which recognized specific PAMPs in specific pathogenic bacteria. EFR is the most recognised protein identified in *Arabidopsis* plant. Apart from EFR, FLS2 receptor also has been identified in the model plant *Arabidopsis thaliana* [29]. Other receptor such as Peptidoglycans (PGNs) which perceive to LYM1 and LYM3 protein also identified in *Arabidopsis* plant, whereas PGN that perceive to LYP4 and LYP6 protein has been identified in rice plant. As well, the *Arabidopsis* ReMAX receptor perceived by yet-unknown PRR from *xanthomonads* in *Brassicaceae* plant. However, the recognition of the receptors have been thoroughly proved in recent wet lab research.

2.2 Role of EFR Receptor

EFR (EF-Tu Receptor) is a pattern-recognition receptor (PRR) that binds to the prokaryotic protein EF-Tu (elongation factor thermo unstable) in *Arabidopsis thaliana* plant (Pfeilmeier *et al.*, 2018). Based on UniprotKB server, EFR constitutes the pattern-recognition receptor (PPR) that determines the specific perception of elongation factor Tu (EF-Tu), a potential elicitor of the defence response to pathogen-associated molecular patterns (PAMPs). EFR receptors have a high affinity for the EF-Tu PAMP. This has been proven analytically through competitive binding assays.

2.3 Bioinformatics Approach in Protein 3D Structure Prediction

An important observation in 3D prediction is that the proteins that share similar sequences often share similar protein structures tool [14]. Protein structure significantly termed as arrangement of atoms in three-dimensional. Based on this concept, although bioinformatics approach techniques does not generate equivalent results, as those from X-ray crystallography, but the methods are

comparatively fast and inexpensive to build a close approximation of a structure from a target sequence, without the time and costs of experimental procedures.

2.4 Homology Modelling

Homology modelling, which is also known as comparative modelling, mainly indicates the build of an atomic-resolution model of the target protein initially from its amino acid sequence and three-dimensional structure of similarly related homologous protein known as template [3]. This method begins from selection of homologues with known structures from the PDB and if the query template sequence has high sequence percent identity which is more than 30% to the structure, thus the homology detection is more straightforward which is usually done by comparing the query sequence with all the sequences of the structures in the PDB [27]. There are few list of homology modelling tools of Modeller 9.10, SWISS-MODEL, HHpred Modeller, Geno-3D and more. Homology modelling can produce high-quality structural models when the target and template are closely related.

2.5 Multiple Template Modelling (MTM) Approach

3D modelling protein structures can be improves if more than one fold template was used for the modelling method. There is a significant analysis of the multiple-template model, which has the appropriate combinations of templates that enhance the model quality compared to single-template modelling [5]. Perhaps multiple-template modelling emphasise on not to be only in mix and match segments of templates in order to achieve more coverage yet, there must be an improvement in model quality. Since multi –domain-modelling template difficult to find, thus each domain from a respective protein has been model and combined to one full protein model. There is a multi-template combination algorithm for protein. The algorithm chooses and combines full template and target alignments where the score is approximate to the top template and target alignment within a threshold given.

3. Methodology

3.1 Modeling of Pattern Recognition Receptor EFR

For single template modelling, NCBI Blastp tool and HHpred tool has been used to analyse EFR protein amino acid sequence, in which through NCBI Blastp the Protein Data Bank (PDB) using default parameter values was used in order to find for one suitable template for EFR protein. Basic single template modelling done for EFR protein using few homology modelling tools which are HHpred Modeller, CPHmodels-3.2, Geno3D, SWISS-MODEL and Local-meta threading server **(**LOMETS) modelling tools of PRC, pGenTHREADER, Prospect2, FFAS-3D, FFAS03, SP3, SPARKS-X, MUSTER [13]. Basic modelling for EFR protein using few tools shown in Table 1.

Multiple template modelling started through multiple sequence alignment, which was carried for full EFR protein by using Praline tool, in which top five template chosen based on lowest e-value resulted from NCBI Blastp to observe the relationship of the template sequence coverage with target sequence [13]. All the selected templates in its respective domains to model EFR. From all the selected templates, the best templates for LRR domain and K domain has been proceed to permutation and combination method to make sure the best combination of templates proceed for modelling. The modelling of the entire paired template has been done using HHpred tool.

Table 1
Single Template Modelling Approach for EFR protein

Tools used	Modelling method	Template used
HHpred Modeller	Homology	4mn8A
CPHmodels-3.2	Homology	4mn8A
Geno3D	Homology	4mn8A
SWISS-MODEL	Homology	4mn8A
PRC	LOMETS	4mn8A
pGenTHREADER	LOMETS	4mn8A
PROSPECT2	LOMETS	5gijB
FFAS-3D	LOMETS	5gijB
FFAS03	LOMETS	5gijB
SP3	LOMETS	5gr9b
SPARKS-X	LOMETS	5gijB
MUSTER	LOMETS	5gijB

3.2 Structure Validation

The structural validation method has been done mainly to analyse and evaluate the structural consistency and reliability of the protein model [11]. The method has been done specifically to generate the validation score after each modelling method to analyse the best tool, which produce a good structure of protein model. The model has been validate using tools such ERRAT (B. Wallner & Elofsson, 2006), Verify3D and RAMPAGE server. ERRAT is mainly to verify protein structures determined by crystallography and study the statistics of non-bonded atom-atom interactions, Verify3D functions to determine the compatibility of an 3D atomic model with its own amino acid sequence, whereas the Ramachandran plot from RAMPAGE server is used to analyse the consistently of the protein model. The final model in multiple template modelling also validate by using MolProbity, ProQ ProSA to prove the quality of the model in detail. The protein structure also has been visually analysed in maximum quality by PyMOL tool.

3.3 Molecular Interaction of EFR, elf18 and BAK1

The complexes has been proceed to docking with the protein-protein docking tool ZDOCK 3.0.2 [7]. For multiple protein docking first EFR was docked with PAMP elf18, then the best docked complex structure was proceed to further docking with co-receptor BAK1/BAK1M (mutated). From the top 10 predicted models, the best predicted protein were analysed in PyMOL. Besides, the protein validation has been done in Molprobity Ramachandran Analysis [10] to choose the stable complex from all the predicted docked complexes and measure the polar contacts using PyMOL. Also the comparison for binding mechanism of hydrogen bond [4], hydrophobic interactions, ionic interactions, aromatic-aromatic interactions, aromatic-sulphur interactions, cation-pi interactions [12] bonding for before simulation and after simulation has been done for the docked complexes. On top of that, the polar contact measurement of protein by PyMOL tool and the comparison picture before simulation and after simulation has been described in detail for all the five complexes.

3.4 Molecular Dynamics Simulation of Docked Complexes

After protein-protein docking, the best predicted docked complexes were subjected to run molecular dynamics (MD) simulation using GROMACS 5.0.4 [19]. The Gromacs96 54a7 united force field was used to run the simulation. Before running the simulation, the system were solvated,

neutralized, energy minimized and equilibrated. For solvation procedure, the proteins were taken into a cubic box with minimum distance of 1Å between protein surface and its edges. Then the protein complexes in the cubic boxes were solvated with simple point charge (SPC) water model. The systems were neutralized with genion tool of GROMACS before proceed to energy minimization. Then the systems were equilibrated for 2ns NPT ensemble followed by 1ns NVT ensemble maintaining a constant 1atm pressure and 300 K temperature respectively. Finally, 50ns MD simulation were subjected to run for each system and root mean square deviation (RMSD), root mean square fluctuation (RMSF) was done. Also after 50ns of simulation, the complexes were subjected to further analysis by PyMOL tool.

4. Results and Discussion

4.1 Modeling of Pattern Recognition Receptor EFR

NCBI BLASTp analysis of 1031AA sequence of EFR protein resulted several templates according to e-value, and its each identity. Among the template, 4MNA_A (Chain A, Crystal structure of the free Fls2 Ectodomains has the maximum score of (363) with lowest e-value 2e-112 and 37% identity. For HHpred, 4mn8_A (LRR receptor-like serine/threonine-protein kinase FLS2; FLS2, BAK1, flg22, Flagellin, plant; HET: SO4, NAG; 3.062A {Arabidopsis thaliana}) was found to be the best template for the modelling of the EFR protein, which gives highest percentage identity, which is 100% probability and maximum score of 782. Homology modelling tool of HHpred Modeller produce the best model with less structure deviation compared to CPHmodels-3.2, Geno3D, and SWISS-MODEL which couldn't able to produce full 1031 AA model. Multiple template modelling has been done using few main tools of different modelling methods, such as HHpred modeller (homology) [1], AIDA (homology) [28], Phyre2 (Ab initio), Raptor-X (Threading). HHpred modeller generate model for each domain of EFR protein ,where it results a clear view of the model using best templates of LRR domain, TM domain and kinase domain, in which the model has been analysed in detail in Figure 1. For AIDA and Raptor-X, the model generated for both tools are moderately superposed and specially model the LRR domain and TM domain only, which kinase domain hasn't been generated and remains open as undefined structure, thus the model remain incomplete. Moreover, the model also not consistent to the secondary structure and not same to the predicted template. Phyre2 intensive modelling only generated LRR domain, in which the kinase domain has not resulted a clear view and TM domain has not been generated and left a gap in the structure model, which causes the structural deviation in the full model, proved that Phyre2 doesn't produce good quality models compared to HHpred modeller.

4.2 Structure Validation

The tools that gives results for the validation of single template modelling using ERRAT are HHpred Modeller, SWISS-MODEL, CPHmodels-3.2, Geno3D and cdPPAS. Whereas other modelling tool such as PRC, PROSPECT2, pGenTHREADER, FFAS03, FFAS-3D, SPARKS-X, MUSTER, SP3, cdPPAS gives error value. This is mainly because these tools are based on threading method which has high chances to produce less quality models than homology modelling tools (Akansha Saxena, 2013). Among the tools which generates results for ERRAT, the tools which gives results with acceptable value (>50) are (HHpred) Modeller (68.858), SWISS-MODEL (74.223), and Geno3D (85.417). For model validation of combined domain in multiple template modelling, the entire protein model which was generated from all the tools, resulted ERRAT for (>50) for the accepted value. For Verify3D, only HHpred modeller (95.67%) generated results (>80), which indicated as the model resulted from HHpred has good quality structure and have high AA compatibility structure.

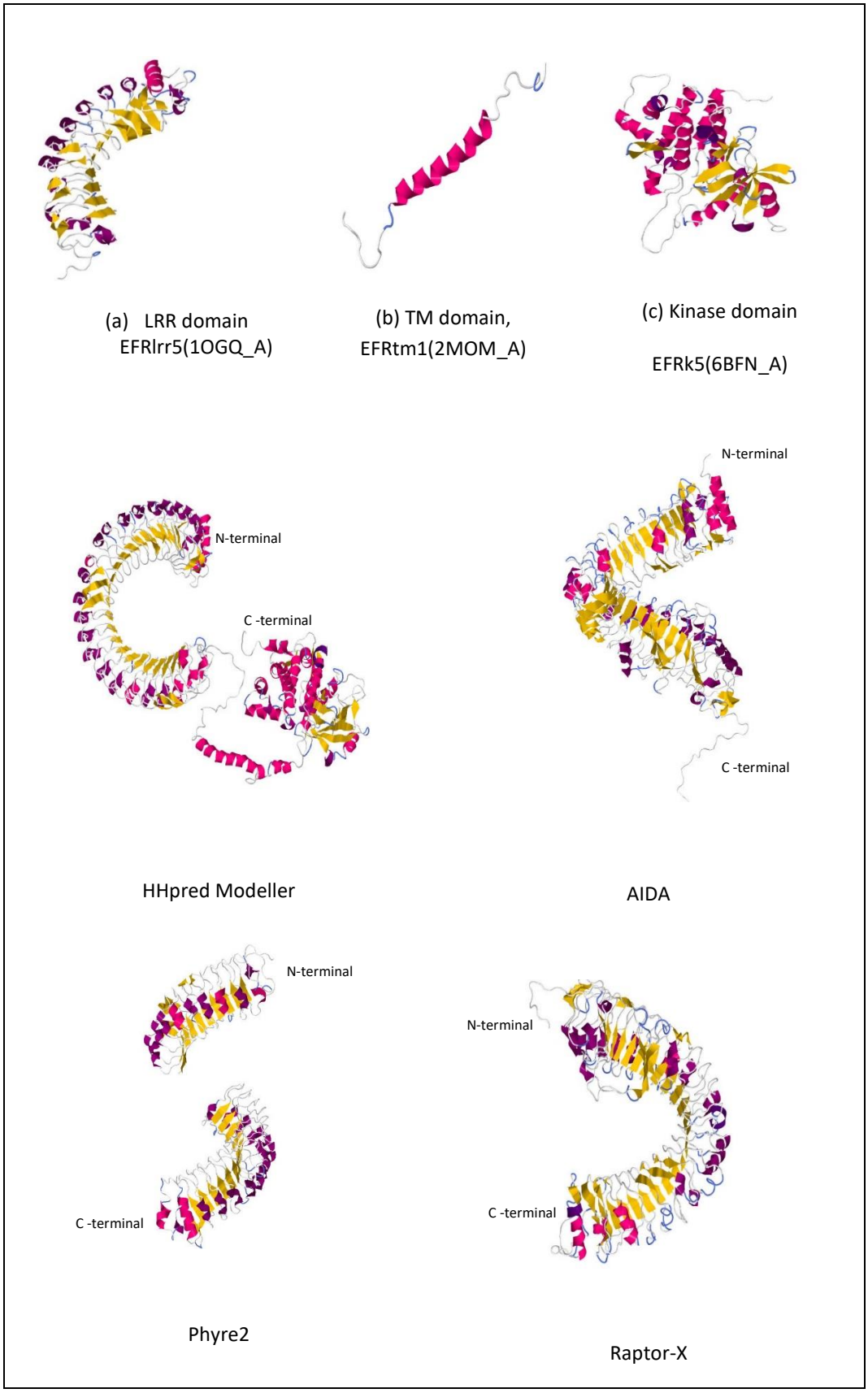

Fig. 1. Multiple template modelling approach of EFR by different modelling tools

Ramachandran plot Summary from RAMPAGE resulted that protein model that has been generated from HHPRED modeller has more residues in favoured region (92.8%) and has the lowest residues in Outlier region (0.4%), which is nearly 0%, as shown in Table 2.

Table 2
Validation results of models of EFR protein by different tools for Multiple Template Modelling

Tools used	Errat	Verify3D (%)	Ramachandran Plot Summary from RAMPAGE (%)		
			FR	AR	OR
HHpred server	71.123	95.67	92.8	6.8	0.4
AIDA	79.175	74.04	92.5	6.2	1.3
Phyre2	67.512	75.2	89.0	7.7	3.3
Raptor-X	69.589	76.67	91.3	6.6	2.2

FR, Favoured region; AR, Allowed region; OR, Outlier region.

4.3 Molecular Interaction of EFR, elf18 and BAK1

The significant bonding pattern observed in EFR with elf18 and BAK1 and mutated BAK1 are Hydrogen bonding, hydrophobic interactions within 5 Angstroms, ionic interactions within 6 Angstroms, Aromatic-Aromatic Interactions within 4.5 and 7 Angstroms, Aromatic-Sulphur Interactions within 5.3 Angstroms, Cation-Pi Interactions within 6 Angstroms.

4.4 Molecular Interaction of EFR with elf18 and Co-Receptor BAK1

Hydrogen bonds formed between EFR ARG21 with BAK1 ARG146 and GLU98 respectively. In this case, measure 3 in Table 3 proved that H-bond between ARG21 and GLU98 is the shortest measurement with the distance of 1.8 Å through PyMOL tool. There are also few hydrogen bond sustained in the interaction between EFR and elf18 such as EFR TYR398 with elf18 LYS4, and EFR ASN422 with elf18 ARG7. In this case, polar contact measurement through PyMOL proved that TYR398 and LYS4 has the shortest distance of 1.4Å.

4.5 Molecular Interaction of EFR with elf18 and Co-Receptor (utated) BAK1

The binding interactions for EFR with elf18 and mutated BAK1 has generated and sustained different residues compared to complex with normal BAK1. There are high number of hydrogen bond generated majorly between EFR and mutated BAK1, and in total of 11-hydrogen bond sustained after simulation. In this case, measure 5 in Table 4 proved that H-bond between ALA174 and GLN527 is the shortest measurement with the distance of 1.2Å, which indicates the strongest bonding between the respective residues using PyMOL tool. Measure 13 in Table 4 proved that H-bond between TYR398 and LYS4 is the shortest measurement with the distance of 1.4Å and Measure 17 proved the shortest measurement distance between elf18 SER1 and mutated BAK1 ASP507 which is 2.8Å.

Table 3
Polar contact Measurements of EFR with elf18 and co-receptor BAK1 through PyMOL tool

Protein	Measurement	Donor Atom	Acceptor Atom	Distance (Å)
		LRR EFR	**BAK1**	
LRR EFR_elf18_BAK1	Measure 1	SER-26_O	GLN-166_O	2.2
	Measure 2	ARG-21_O	ARG-146_N	2.4
	Measure 3	ARG-21_N	GLU-98_O	3.4
		ARG-21_N	GLU-98_O	1.8
		ARG-21_N	ASP-74_O	3.1
	Measure 4	GLY-55_O	ARG-72_N	2.8
	Measure 5	LYS-57_N	TYR-100_O	2.8
	Measure 6	GLN-196_N	GLU-2_O	2.9
		SER-220_O		3.4
	Measure 7	ASP-269_O	ARG-4_N	2.9
	Measure 8	GLN-369_N	LEU-15_O	3.5
		elf18	**BAK1**	
	Measure 9	SER-1_O	PHE-11_N	2.1
			PHE-11_O	2.1
		LRR EFR	**elf18**	
	Measure 10	GLU-374_O	LYS-4_N	2.7
	Measure 11	SER-372_O	LYS-2_N	2.4
		ASP-396_O		2.7
	Measure 12	TYR-398_O	GLU-3_O	3.3
			LYS-4_O	1.4
	Measure 13	SER-399_O	GLU-6_O	2.3
	Measure 14	SER-423_O	ARG-7_N	2.1
	Measure 15	ASN-422_N	ARG-7_O	3.4

Table 4
Polar contact Measurements of EFR with elf18 and co-receptor (mutated) BAK1 through PyMOL tool

Protein	Measurement	Donor Atom	Acceptor Atom	Distance (Å)
		LRR EFR	**BAK1(M)**	
LRR EFR_elf18_BAK1(M)	Measure 1	LYS-57_N	GLU_426_O	2.0
		LYS-57_N	ASN-368_O	3.4
		LYS-57_N	GLU-426_O	3.5
		ARG-21_N	GLU-426_O	2.7
	Measure 2	ASP-79_O	LYS-389_N	2.7
	Measure 3	SER-127_O	ASN-529_N	3.3
	Measure 4	SER_126_O	GLY-528_O	2.1
	Measure 5	ALA-174_O	GLN-527_N	1.2
	Measure 6	GLN-196_N	GLU-519_O	2.9
		SER-220_O		3.0
	Measure 7	ARG-267_N	LYS-516_O	3.2
	Measure 8	ASP-269_O	LYS-516_N	2.7
	Measure 9	TRP-291_N	LYS-514_O	3.0
	Measure 10	PHE-268_O	LYS-516_N	1.5
		LRR EFR	**elf18**	
	Measure 11	ASP-396_O	LYS-2_N	2.7
		SER-372_O		2.4
	Measure 12	GLU-374_O	LYS-4_N	2.7
	Measure 13	TYR-398_O	GLU-3_O	3.3
			LYS-4_O	1.4
	Measure 14	SER-399_O	GLU-6_O	2.3
	Measure 15	ASN-422_N	ARG-7_O	3.4
	Measure 16	SER-423_O	ARG-7_N	2.1
		elf18	**BAK1(M)**	
	Measure 17	SER-1_O	ASP-507_O	2.8
	Measure 18	SER-511_O	LEU-464_O	3.3

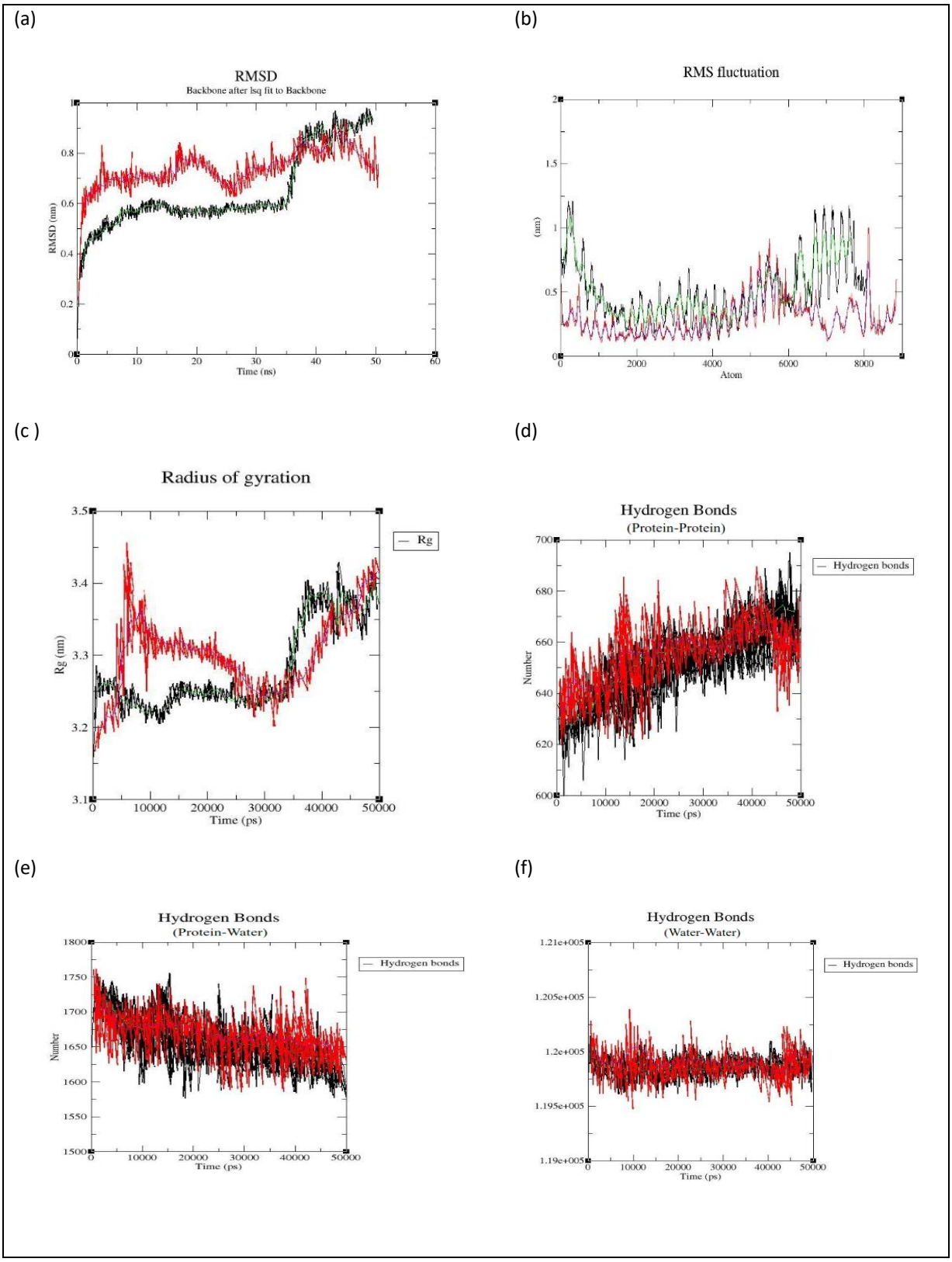

Fig. 2. (a) RMSD ; (b) RMSF ; (c) Rg ; (d-f) Hydrogen bond formed over simulation between protein-protein, protein-water and water-water for EFR, elf18 and BAK1 docked complex. Black curves shows the results for EFR, elf18 and BAK1 docked complex where the red curves shows the results for EFR, elf18 and (mutated) BAK1 docked complex

4.6 Molecular Dynamics Simulation of Docked Complexes

Analysis on molecular dynamics on the docked complexes is mainly on Root mean square deviation (RMSD), root mean square fluctuation (RMSF), radius of gyration (Rg) and additionally on number of hydrogen bond, which was calculated using GROMACS tools.

4.7 Molecular Dynamics Simulation of EFR with PAMP elf18 and Co-Receptor BAK1 Complexes

RMSD value for both complexes increase up to 10ns period of time and complex with mutated BAK1 showed an average fluctuation until 50ns, whereas complex with normal BAK1 maintained a steady graph up to 35ns period and showed an upward trend consequently till 50ns. For the complex with mutated BAK1 initially showed higher RMSD value yet for the complex with normal BAK1 showed higher RMSD value towards 50ns period as shown in Figure 2. RMSF for both complex showed almost similar level of fluctuation, yet complex with normal BAK1 showed higher RMS fluctuations mainly at the terminal regions compared with complex with mutated BAK1. Rg for C-alpha atom, complex with normal BAK1 showed mild fluctuation till 35ns period of time, followed by a sharp increase and Rg value maintained at 3.4nm by 50ns period of time. Complex with mutated BAK1 showed higher Rg value at 5ns and a downward trend till 35ns and consequently showed an upward trend till 50ns. Overall Rg value observed is lower and maintain a steady value for 30ns for complex with normal BAK1 compared to the complex with mutated BAK1, which also showed minimum fluctuation and less Rg value change over time. The protein-protein hydrogen bond number graph showed an upward trend for both of the complexes. The interactions between proteins increases over simulation time.

5. Conclusion

This study is the first approach to *in-silico* analysis to find the best complex interaction of EFR protein and PAMP elf8 with the aid of co-receptor BAK1. This result proved that HHpred Modeller generated the best modelled of EFR protein with good validation score. Following that, multiple template modelling method generates good models compared to single template modelling method with less structure deviation and gaps. The molecular dynamics simulation analysis proved the structure stability of the most reliable interaction occurred between EFR, elf18 with normal BAK1 supported by RMSD graphs. Further research needed to be done to understand the comparative analysis of FLS2-flg22-BAK1 crystal structure as template and EFR-elf18-BAK1 as protein model complex and proper mechanism of inside membrane activity of EFR using bioinformatics approach.

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