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Molecular interaction of replicase protein geminivirus from Pesisir Selatan isolate with *Ankyrin-NPR1* domain

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Abstract. Knowing the effect of geminivirus protein Rep interaction with ankyrin domain of the *NPR1* [Non-expressor of pathogenesis-related 1] gene was aimed to understand how the suppressing of expression resistance genes associated with the event of a pathogen attack. Interactions that occur will affect the work of the NPR1 protein as a transcription factor. EMSA [Electrophoretic Mobility Shift Assay] technique was applied to study their interaction. The ratio of proteins with nucleic acids was 7.6 ng/µL Rep x 100 ng/µL ankyrin [also in the mutant] and 7.6 ng/µL Rep x 5 ng/µL ankyrin was able to visualize the binding activity. Modeling, simulations, and interaction with NPR1 protein mutations were also carried out to understand the effect of the interactions. Three-dimensional analyses of NPR1 and mutant NPR1 proteins showed different binding positions and interactions. The complex interaction formed between non-mutant NPR1 protein and Rep protein had docking score -542.04 and -523.56 respectively. The mutant sequence showed no binding.
Keywords: Ankyrin, EMSA, Geminivirus, *NPR1*, Replicase.

1. Introduction

Rep [C1] protein is a replication and transcription regulator in geminivirus. It functions at the beginning of replication and initiates DNA replication [1]. The Rep [C1] has a multifunctional character which is important in the rolling circle replication [RCR] mechanism and interacts with the retinoblastoma protein [RBR] [2].

Rep protein has the capability to interact with Retinoblastoma-Related Protein which is involved in the cell death program regulation [3]. This interaction triggers transcription factor extrication and activates Proliferating Cell Nuclear [PCNA] to build the replication machinery of the virus. By doing this mechanism the infected cell will undergo cell cycle reprogram and trigger cell replication. Moreover, the virus blockades the macromolecule trafficking and interferes with hormone signaling which prone to plant defense paralysis [4].

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The ankyrin is a domain located in NPR1 protein which has a size of 747 bp and classified as a transcription factor of the resistance gene. Understanding the molecular mechanism between Rep protein and Ankyrin is one of the effective ways in order to prevent or increase the capability of the plant against PepYLCV infection. Furthermore, this can also be associated to determine whether the interaction will interfere with the Pathogen-related resistance [PRs] expression because of NPR1 inactivation. In order to elucidate such mechanism, Electrophoretic Mobility Shift Assay [EMSA] [5] technique was applied.

2. Material and Method

2.1. Rep gen preparation

The *Rep* gene was Amplified and cloned in pGEM-Teasy as described previously by Nova *et al*., [6]. To confirm the existence of the gene fragment, the recombinant plasmid was amplified by using T7SP6 and C1-TD21-*Sma*/*Bam*HINT [FR]. The *Rep* gene was cloned into the pGEM T-Easy vector [Promega-USA] and transformed into a competent cell of *Escherichia coli* DH5α. The extraction of recombinant plasmid was conducted using the protocol described by Bimboim and Doly [7]. Plasmid pGEM-Rep and pET-28a[+] were restricted using two restriction enzymes namely *Bam*HI FD and *Sac*I [Thermofisher scientific-USA]. This process was performed by using a PCR-adapted program with the following program for *Bam*HI FD [37°C for 15 min and 80°C for 80 min] and for *Sac*I by following the program at 37°C in 2 hours for enzyme activation and inactivated at 80°C for 20 minutes. The *Rep* gene fragment was purified using the GeneJET Gel Extraction Kit [Thermo scientific-USA] based on the company protocol. Rep gen was ligated to pET-28a[+] and cloned based on the recommended procedure from Novagen protocol with cocktail composition as calculated in the Promega BioMath Calculators program [https://www.Promega.com].

2.2. Protein expression and purification

The procedures were started by the induction of transformant bacteria using isopropyl β-D-1-thiogalactoside [IPTG]. Purification was carried using MagneHisTM Protein Purification System [Promega, USA] following the recommended procedure. The concentration of purified protein was determined using Bradford [8] and Visualized by 15% SDS-PAGE.

2.3. EMSA assay

Rep-Ankyrin domain interaction was carried using EMSA Kit E33075 [Invitrogen]. Samples were run with 200 Volt for 35 min in 6% Native SDS-PAGE.

2.4. In silico assay

The analysis was conducted by several online and offline software. Modeling of proteins was carried out using the Swiss Model program [9]. Analysis of the surface position of the interactions was carried out using the CASTp program [10]. Simulation of protein interaction and docking with nucleic acids was carried out using the HDOCK program [11]. Whereas the interaction tests between Rep protein and Npr1 protein was carried out using the Hex 8.0.0 program [12].

3. Result and Discussion

3.1. Vector Construction

Plasmid recombinant [pGEM-Rep] was inserted inside the former plasmid particularly in the Lac Z region. This was subjected to the selection process after transformation known as blue-white selection. In the second construction pET-28a[+], described in Figure 1B, Rep and pET-28a[+] were equally restricted by endonuclease restriction enzymes *Bam*H1 and *Sac*1 due to the closest region to downstream and T7 Terminator respectively.

After reassuring plasmid pET-28a[+] transformation was performed into *E. coli* strain BL21. The heat shock method was carried out at 42°C for 45 seconds as described in the method. The positive bacteria were examined by using Primer T7/PFTR with an estimated product 1520 bp. Figure 1C is the visualization of amplification where it was clearly seen that all of the bacteria tested are positive.

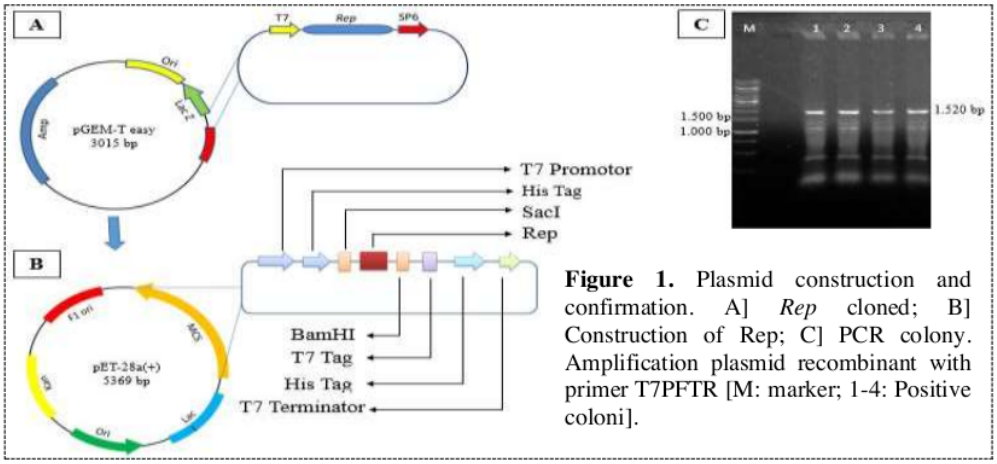


Figure 1. Plasmid construction and confirmation. A] *Rep* cloned; B] Construction of Rep; C] PCR colony. Amplification plasmid recombinant with primer T7PFTR [M: marker; 1-4: Positive coloni].

3.2. Replicase expression

The positive colony containing the Rep gene was processed to expression and purification. Fifteen percent SDS-PAGE was used to visualize Rep protein as shown in Figure 2.

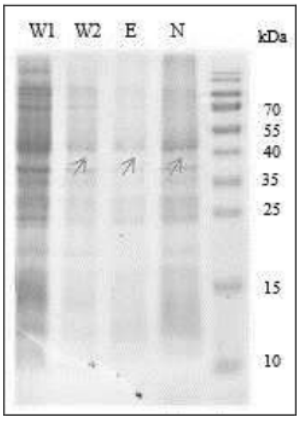


Figure 2. Expression and purification of Rep protein. W1-W2: washing protein; E: Elusion; N: Nickel-Bound Protein; M: Marker. Rep protein pointed by arrows

Based on Figure 2, the protein was predicted 41 kDa. The size was correlated with the length of the inserted Rep gene fragment. Both W1 and W2 show the purification process. It was convinced that the purification kit has successfully separated protein during the purification process, thus in the second washing [W2], the protein concentration was lower than the previous [W1] washing. In the elution [E],

Rep protein should be detected in high intensity indicating a high concentration of protein. Nevertheless, a large number of proteins were detected in Nickel including Rep protein.

3.3. Replicase-Ankyrin domain interaction

Three-dimensional analysis and prediction of the binding site were analyzed using the SWISS-MODEL [https:/ /swiss model.expasy .org/interactive/] and presented in figure 3.

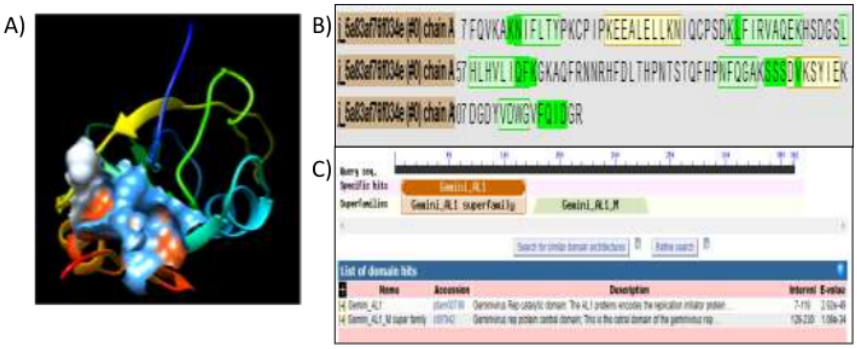
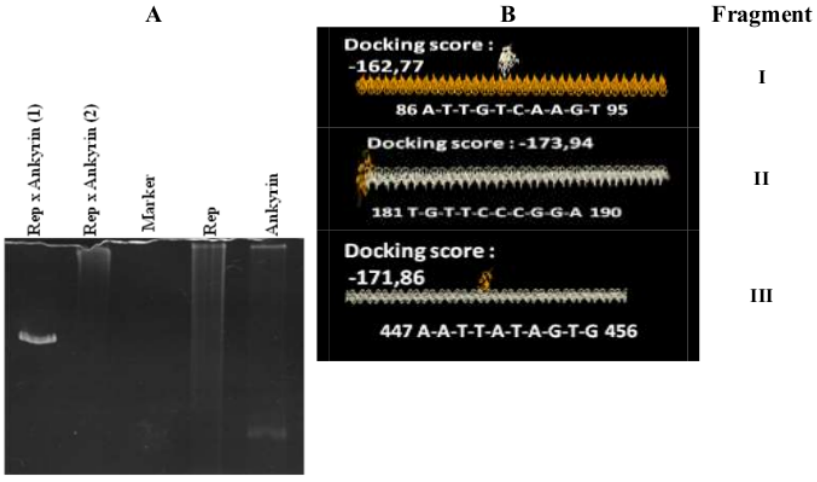
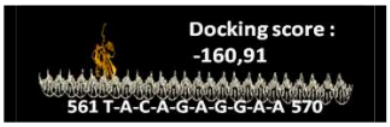


Figure 3. Prediction of the binding site. A] Three-dimensional structure of Rep protein; B] Predicted amino acid that could bind; C] Conserved region.

Conserve domain analysis using the tool provided at the NCBI website exhibited that the Rep protein has two conserve domains conferring different function namely Rep catalytic domain and Rep protein central domain. The former has a coding region of Rep initiator which imitates specific initiator enzyme and replisome which is an important element while the latter is the core of conserved domain. Visualization of interaction was obtained by Hex.

The estimation of the binding area was determined by the size of the surface area. This means that the greater the value of the surface area of the predicted binding region, the higher the likelihood of binding occurrence. These amino acids covered 12-K [glutamine], 13-N [asparagine], 42-L [leucine], 63-Q [glutamine], 64-F [phenylalanine], 65-K [lysine] , 96-S [serine], 97-S [serine], 98-S [serine], 100-V [valine], 116-F [phenylalanine], 117-Q [glutamine], 118-I [isoleucine], 119-D [aspartic acid]. All amino acids are located in the Catalytic domain.





IV

Figure 4. Rep protein x ankyrin interaction. A] EMSA. B] Docking score in each position

In figure 4, protein and DNA were loaded separately. The first line was 7.6 ng/µL Rep x 100 ng/µL ankyrin and the second line was 7.6 ng/µL Rep x 5 ng/µL ankyrin. The first line shows the best visualization indicating good ratio concentration. Conformation changing of interacted Rep protein with Ankyrin was determined by Nova et al., [13]. Figure 4B shows the binding region that occurs in the *Npr1* gene. The fragments have different atomic values. The first fragment of the interaction occurred at 86-95 bp with a docking score of -162.77. The second fragment at 181-190 bp with a docking score of -173.95, while the third fragment of the interaction occurred at 447-456 bp with a docking score of -171.86. In the last fragment occurred at 561-570 bp with a docking score of -160.91. The Binding point is strongly suggested in the middle of the Ankyrin fragment. Ankyrin fragments were divided into 4 fragments which contain deference atoms value [7380, 7390, 7380 and 5207 atoms]. Prediction of interaction was based on the range of protein from Ankyrin fragment which summarized in Tabel 1.

Table 1. Summary of Rep binding point along the four split Ankyrin fragments

Fragment	Range	Binding Surface [Å^2]	Atom	Bound Sequences	Time [s]	Number of Binding Point	Docking Score
I	15.71 Å	60955.85	7380	ATTGTCAAGT	0.14	34	-162.77
II	15.73 Å	60963.37	7380	TGTTCCCGGA	0.16	34	-173.94
III	15.79 Å	60968.28	7380	AATTATAGTG	0.16	34	-171.86
IV	14.38 Å	40132.83	5207	TACAGAGGAA	0.16	33	-160.91

All of the fragments might have interaction with Rep. In Table 1, every fragment had nearly the same number for every parameter. Range and docking scores were used as a parameter to determine the highest binding probability. Data showed that all those four fragments exhibited nearly a similar number, particularly in range and docking scores. The lowest energy is shown by fragment II [-173.94] while the highest one is shown by fragment IV [-160.91]. Although there was no significant difference, the best probability for binding should be fragment II.

3.4. Mutagenesis binding assay

Ascertaining the position of interaction, the ankyrin sequence has been mutated by using site-directed mutagenesis, started by designing mutagenesis primers. The position and interaction of deletion are shown in Figure 5.

Figure 5. Ankyrin mutagenesis binding assay. A] EMSA of mutant Ankyrin, B] Schematic mutation position. Red: Primers; Blue: deleted sequence.

Figure 5 shows the deletion position on the ankyrin binding site. EMSA assay [Figure 5A] was analyzed by the native SDS-PAGE method, showing no binding between Rep protein and Ankyrin mutants [2nd line] [7.6 ng/µL Rep x 100 ng/µL ankyrin]. The first line is Rep protein that doesn't bind so it spread. DNA is seen at the lowest position on the 2nd line. This is because it's molecular weight and is not influenced by the protein that should be bound.

4. Discussion

The constructed *Rep* gen was introduced into *E. coli* strain BL21 cell using pET28a[+] plasmid vector [14]. The targeted gene is expressed through the regulation of the T7 promoter in which relay on T7 RNA polymerase of *E. coli* as it's the host. The gene is inserted in the MCS prior to being restricted using restriction enzyme *Sac*I and *Bam*HI [Figure 1B]. According to Primrose and Twyman [15], the critical point to express the gene in plasmid is the orientation. Using primer C1-TD21-*Sma*/*Bam*HINT and T7-PFTR for amplification we successfully confirmed by PCR producing a fragment of 1,120 bp and 1,520 bp respectively. Figure 1C shows the consistency of transformation in every single colony we analyzed, indicating 100% transformation efficiency.

The expression of the *Rep* gene in *E. coli* strain BL21 was induced by 0.4 mM isopropyl-β-D-thiogalactopyranoside [IPTG]. According to Berini *et al*., [16], 0.4 mM IPTG is sufficient in inducing kinase protein with a medium level of expression. Based on that, we assumed that the Rep protein produced by our system is sufficient for further steps. Furthermore, our purification step seemed successful to provide quality for the EMSA assay. Figure 2, line E indicated that target protein was bound in nickel particle. The purity of the targeted protein is represented by the intensity of its band visualized in electrophoresis gel [17].

In many biological processes, molecular interactions are very crucial. Analysis at the molecular level should provide a brief insight into what actually happens is. In this manner, the EMSA essay is regarded as a powerful and effective way to investigate the existence of such interaction [18]. Based on our data [Figure 4B] there are some possible binding positions along with the Ankyrin domain. Despite, Rep-Ankyrin complex formation was remarkably the most likely than the Ankyrin complex itself.

The ratio between protein and nucleic acid to be successfully forming complex interaction seemed very important. This parameter could be visualized by the intensity of the produced complex fragments. In our study, the ratio of 7.6 ng/µL Rep protein and 100 ng/µL Ankyrin domain [line 1] seemed to be optimal. Research conducted by Heffler and Walters [19] showed that lower protein concentrations [1 ng/µL] and 50 ng/µL of nucleic acid showed the best interaction results. Boelt *et al*., [20] obtained a protein concentration of 1 µg to be able to display visual interaction results. Although

the interaction can be examined by EMSA, the detail aspects such as strong and transient interaction can not be determined. The solution to that problem can be overcome by means of some bioinformatics tools.

5. Conclusion

The interaction had been confirmed by EMSA and bioinformatics analysis with the binding score ranging from -173.94 to -160.91 and around 15 Å. Replicase geminivirus from Pesisir Selatan had the ability to bind with the ankyrin Npr1 domain. The interaction position occurs at 181-190 bp, this was evidenced by the absence of interaction after this part was removed.

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