NPR1 Cloning

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Molecular Cloning and Characterization of Npr1 Ankyrin Domain from Capsicum Annum L.

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ABSTRACT

The study aims to determine the genomic structure of the NPR1 ankyrin domain isolated from Capsicum annum cv. Cabai Berangkai and predict its secondary and tertiary structure by homology protein modeling. The three weeks old chili leaves were taken as samples for DNA isolation. The NPR1 gene was amplified using specific primers by Nested and Touch-Down PCR for two rounds. The second-round PCR products were cloned into pGEM T-Easy vector and transformed into E. coli DH5α via heat-shock method. The transformant were verified by colony PCR and sequencing. The sequencing data was used for genomic analysis and to determine the 3D structure of the NPR1 ankyrin domain. The sequence analysis of ankyrin domain between CbNPR1 and AtNPR1 resulted in 62.5% identity and 78.1% similarity. The conserved important amino acid of Cys216 and His334 were also observed. In the secondary structure ankyrin repeat containing helix-and β sheet conformation was observed. These conformations were confirmed by tertiary structures using Ankb 24 protein as a template. In conclusion, all of the results suggested

CbNPR1 and AtNPR1 are predicted for having similar structural conformation and biological function in the plant defense system.

Keywords: *Capsicum annum; NPR1; Ankyrin; Homology Modeling Protein*

INTRODUCTION

A pepper plant (*Capsicum annum* L.) is an important and high demand horticultural product in Indonesia. In 2015, pepper production in Indonesia was approximately 63.402 tons [1]. Pepper fruits are considered as cultural identity in Indonesia, especially in West Sumatera as one of the central of pepper production. Unfortunately, pepper crops are susceptible to be infected by pathogenic microorganisms. One of those caused by DNA virus known as Pepper Yellow Leaf Curl Virus (PepYLCV) [2].
The efforts to cure diseases caused by PepYLCV have been developed based on the molecular interactions between plants and *Geminivirus* during virus infections. Recent studies have shown a number of genes expression level was up and down regulated while infection process occurred [3]. Based on those different of expression level, all of the genes which associated with plant defense system were studied further to predict how *Geminivirus* manipulating the immune system of plants.

During *Geminivirus* infection, plants synthesize Salicylic acid (SA), a key signaling molecule for plant disease resistance, and accumulation of pathogenesis-related (PR) proteins is induced to establish both local and systemic acquired resistance (SAR) against virus invasion [4]. In plant defense, SAR is playing an important role, providing long term and broad-spectrum resistance against a wide variety of fungus, bacteria, and virus pathogens [4].

Extensive studies have shown that the *non-expressor of pathogenesis related* genes (NPR1) functions as the regulator of SA-mediated SAR in Arabidopsis [5]. Overexpression of NPR1 in plants shows increased PR gene expression and pathogen resistance [6-7] . Overexpression of arabidopsis NPR1 (*AtNPR1*) in a different type of plants has enhanced bacterial and fungal resistance [8–10]. Functional analysis of NPR1 confer disease resistance against pathogens but may also have various biological functions in different plant species[6,11]. Our research focus to analyze ankyrin domain in NPR1, which is predicted to be very important for many biological functions and protein–protein interactions in plant [12]. The ankyrin repeat was demonstrated functionally important by studying the conserved His334 residue in the ankyrin repeat, this residue is crucial for hydrogen bonds that are necessary for 3D structure [13]. Therefore, characterization of NPR1 ankyrin domain in various species is important for understanding its role, especially in plant defense system.

This study aimed at discovering and characterization of the NPR1 ankyrin domain in *Capsicum annum cv. Cabai Berangkai* (labeled as CbNPR1) compared with *Arabidopsis thaliana* NPR1 ankyrin repeat domain (labeled as AtNPR1) for prediction its structural conformation and biological function similarity.

MATERIALS AND METHODS

Plant Material

Capsicum annum cv. Cabai Berangkai was used to retrieve the NPR1 ankyrin domain gene. This cultivar was obtained from a local plantation, located in 50 Koto, Payakumbuh, West Sumatera, Indonesia. Seeds were grown in Green house at Andalas University. Tissues of young leaves were collected from healthy plants and its genomic DNA was directly extracted on the same day.

Genomic DNA Extraction

Genomic DNA was extracted from the young leaves of *Capsicum annum cv. Cabai Berangkai,* using a *cetyltrimethylammonium bromide* (CTAB) method as previously described by Jamsari [14]

Isolation of NPR1 Ankyrin Domain

To obtain NPR1 ankyrin domain gene, the specific primers; outer forward-reverse and inner fforward-reverse were used for amplification of CbNPR1 ankyrin domain gene from the extracted *Capsicum annum cv. Cabai Berangkai* genomic DNA (Table 1). All primers were designed at the NCBI database (http://www.ncbi.nlm.nih.gov/) by using *Capsicum annum cv. Zunla* as a genomic reference. KAPA 2G Robust Hot Start Ready Mix 2X was used as PCR mixture kit. The 25 uL PCR reaction volume contained 0.5 uM of each primer, 1 ng/uL genomic DNA, 0.2 mm of each dNTP, 2mM of $MgCl_2$, and 1 U of DNA polymerase.

Table-1: Primers used in amplification of CbNPR1 ankyrin domain gene

Primer ID	Sequence (5' - 3')	Length (nt)	Size of PCR product (bp)
Outer Forward	TTCCCCCTTTGCAGAGACAC	20	2626
Outer Reverse	CCTGCACCCACTTTGAGCTT	20	
Inner Forward	AACAAAGCTGCAGCAGACGA	20	687
Inner Reverse	CGCCTGCCATAGCAAGAGAT	20	

PCR amplification was carried out by a combination of Nested PCR [15] and Touch-Down PCR [16]. For outer primers, touch-down PCR was performed under the following conditions: in the first phase, DNA was denatured at 94°C for 3 min, followed by 20 cycles at 94°C for 30 s, 68°C for 45 s, 72°C for 60 s, with the annealing temperature decreasing by 1°C per cycle. In the second phase, 25 cycles at 94°C, for 30 s, 58°C for 45 s, 72°C for 60 s, and final extension at 72°C for 5 min. For inner primers, Touch-down PCR was performed under the following conditions: In the first phase, DNA was denatured at 94°C for 3 min, followed by 20 cycles at 94°C for 30 s, 76°C for 45 s, 72°C for 60 s, with the annealing temperature decreasing by 1°C per cycle. In the second phase, 25 cycles at 94°C, for 30 s, 66°C for 45 s, 72°C for 60 s, and final extension at 72°C for 5 min.

The PCR product was cloned into pGEM T-Easy Vector (Promega) and transformed into chemically competent DH5α *E. coli* cells using heat-shock at 42°C for 45 s. Colony PCR products were separated by electrophoresis on a 1% agarose gel stained with ethidium bromide and visualized under UV light using an image analysis system GelDoc (Bio-Rad). Sequencing was conducted on transformants to determine the validity of NPR1 insert.

Bioinformatics Analysis and Homology Protein Modeling

Similarity searches were performed in the GenBank (http://www.ncbi.nlm.nih.gov) using the BLASTP algorithms [17]. The presence of ankyrin domain was detected using NCBI Conserved Domain Database (*https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi*) [18]. Amino acid sequences and percent amino acid identity were alignment and determined by the Pairwise Sequence Alignment program (http://www.ebi.ac.uk/Tools/psa/emboss_needle/) [19,20]. The ssecondary structure of ankyrin domain was determined using PDB Sum Database (http://www.ebi.ac.uk/thornton-srv/databases/pdbsum) [21]. Homology protein modeling was performed on the SWISS-MODEL workspace server using user template modeling (http://swissmodel.expasy.org/) [22]. The 3D models were visualized and edited with the UCSF Chimera PDB viewer [23].

RESULTS AND DISCUSSION

Results

Isolation of CbNPR1 ankyrin domain

CbNPR1 ankyrin domain was identified in pepper plant by PCR amplification with specific primers. Two pairs of specific primers, previously designed for Nested PCR were used. In addition, Touch- down PCR was used to give optimum amplification. For the first round, using a pair of outer primers, a single band of the predicted size (~2626 bp) was amplified from genomic DNA (**Figure 1A**), other bands were also observed in low quantity. Then, the first-round PCR products were used as a template for second round Nested PCR. A Single band of the predicted size (~ 687 bp) was amplified (**Figure 1B**), representing the NPR1 ankyrin domain gene. No other bands were observed in this round. The second-round PCR product was cloned into pGEM T Easy Vector and transformed into chemically competent DH5α *E coli* cells.

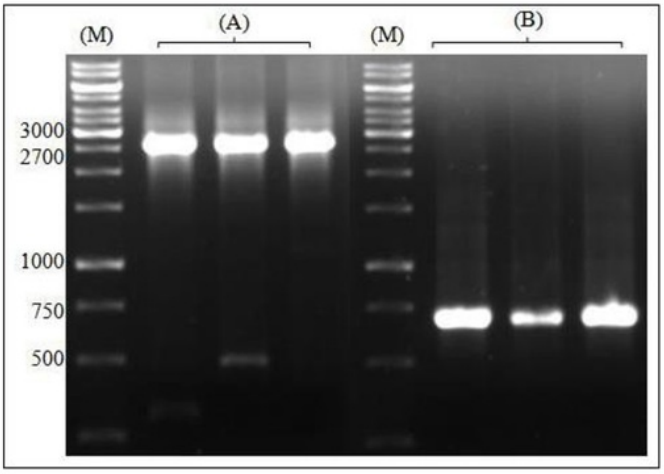

Figure-1: Amplification of the NPR1 ankyrin gene extracted from leaves of 3 weeks old. Amplification was done by two rounds, (A) Amplification using Outer Primer with genomic DNA as a template (B) Amplification using Inner Primer with first round PCR product as a template. M: 1kb Ladder was used as marker.

Colony PCR confirmed the insert gene fragment (~ 687 bp) from culture cells. A strong band was shown and none of the unspecific band was observed. These results have indicated that the combination of Nested and Touch-Down PCR amplification is alternatively effective for isolating high quality of CbNPR1 ankyrin domain gene from *Capsicum annum*.

Sequence analysis

Codon translation of the CbNPR1 ankyrin domain nucleotides revealed that it consists of 206 amino acid residues, BLASTP searches revealed that the amino acid sequence has a high identity (99%) with known NPR1 *Capsicum annum* regulatory protein (Accession NP_001312028.1).

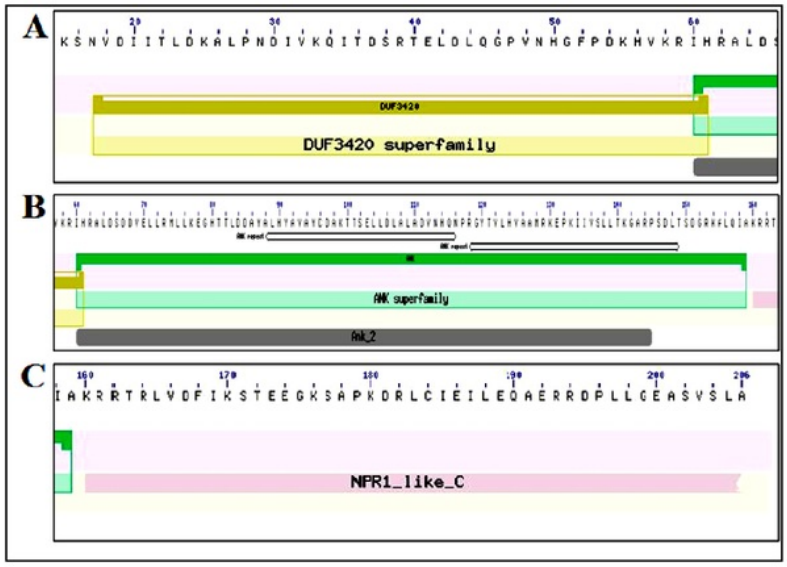

Figure-2: Graphical representation of CbNPR1. (A) Amino acids sequence encoding DUF3420 domain. (B) Amino acids sequence encoding Ankyrin domain. (C) Amino acids sequence encoding NPR1 like C domain.

Further analysis indicated that the sequence contains *DUF3420* domain in the N-terminal region and some parts of *NPR1 like C* in the C-terminal region (Figure 2) with ankyrin domain is located between those domains. These results confirm that complete sequence of CbNPR1 ankyrin domain has successfully obtained from *Capsicum annum cv. Cabai Berangkai*.

Genomic comparison

Multiple alignments between the amino acid from the sequence result of CbNPR1 with AtNPR1 resulted in 56.8% identity and 74.3% similarity. Then, multiple alignments for ankyrin domain sequences resulted in 62.5% identity and 78.1% similarity. Further analysis also revealed conserved important functional amino acids defined by Cys216 and His334 (Figure 3).

```
CbNPR1          1 AMERLLSSCIEIIVKSNVDIITLDKALPNDIVKQITDSRTELDLQGPVNH    50
                  |.:||..|.|||||||||:::|:|:||.::||:|.|.|.||.|:.|.
AtNPR1          1 AMMKLLDRCKEIIVKSNVDMVSLEKSLPEELVKEIIDRRKELGLEVPK--    48
                          Ankyrin ──►
CbNPR1         51 GFPDKHVKRIHRALDSDDVELLRMLLKEGHTTLDDAYALHYAVAYCDAKT    100
                    ..|||..:|:|||||:||:::||||.||.||||.|||:|||||:.||
AtNPR1         49 --VKKHVSNVHKALDSDDIELVKLLLKEDHTNLDDACALHFAVAYCNVKT    96

CbNPR1        101 TSELLDLALADVNHQNPRGYTVLHVAAMRKEPKIIVSLLTKGARPSDLTS    150
                  .::||.|.||||||:|||||||||||||||||::|:|||.|||..|:.|.
AtNPR1         97 ATDLLKLDLADVNHRNPRGYTVLHVAAMRKEPQLILSLLEKGASASEATL    146

CbNPR1        151 DGRKALQIAKRRTRLVDFIKSTEEGKSAPKDRLCIEILEQAERRDPLLGE    200
                  :||.||.|||:.|..|:.....|:.|.:.|.|||:|||||.::|:.:..:
AtNPR1        147 EGRTALMIAKQATMAVECNNIPEQCKHSLKGRLCVEILEQEDKREQIPRD    196

CbNPR1        201 ASVSLA    206
                  ...|.|
AtNPR1        197 VPPSFA    202
```

Figure-3: Multiple alignments of amino acids between partial sequence of CbNPR1 and AtNPR1. Cys216 and His334 are marked by blue and green color, respectively. Ankyrin domain is underlined.

Secondary structure of CbNPR1 ankyrin domain shows 7 helices and 3 helices for AtNPR1 (**Figure 4**). This study observed the region 6-10 (H1), 15-20 (H2), 43-53 (H3), 67-72 (H4), 78-85 (H5), 101-104 (H6), and 109-113 (H7), which are responsible to form helices. The helix structure of H4, H5, and H6 are similar in AtNPR1 with the region 67-72 (H1), 78-85 (H2), and 101-105 (H3).

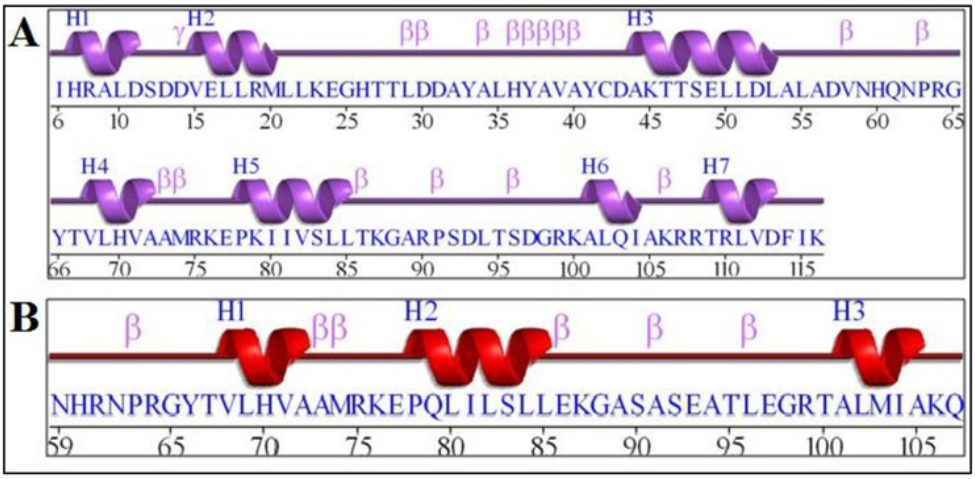

Figure-4: Graphical representation of NPR1 ankyrin domain secondary structure. (A) CbNPR1. (B) AtNPR1. β = Beta sheet. H= Helix.

Homology Modeling

The CbNPR1 ankyrin domain sequence was used to predict its tertiary structure using homology protein modeling. The crystallographic data Ankb 24 Ankyrin Repeats (PDB Accession No. 4RLV.A) was used as a template (Figure 5).

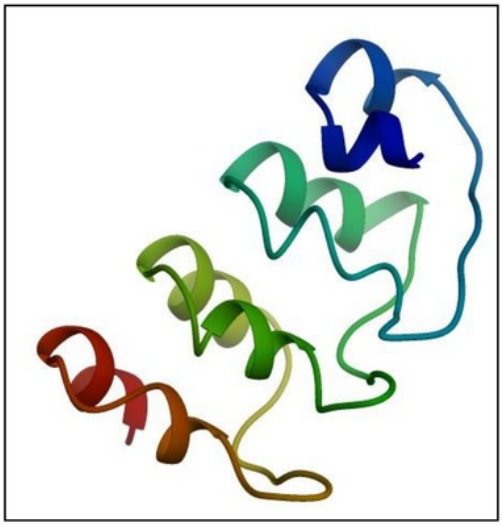

Figure-5: Ribbon view of the predicted three-dimensional structures of CbNPR1 ankyrin domain by homology protein modeling.

DISCUSSION

NPR1 is an important regulatory component in plant defense system, specifically for the activation of SAR [24]. It has predicted as a cofactor for inducing PR gene expression by a binding mechanism to TGA2 transcription factor [13,25,26]. Recently, NPR1 gene has been isolated and characterized in several plant species, although only several cultivars have been studied in *Capsicum annum*, especially its functional domains. In this study, NPR1 ankyrin domain gene was cloned from genomic DNA of *Capsicum annum cv. Cabai Berangkai*.

In sequence analysis, conserved Cys216 was observed. In the recent study, this amino acid was shown to play important role in oligomerization of NPR1 in Arabidopsis [13]. That prediction was confirmed in a mutation experiment, where Cys216 was mutated to Asp216, the efficiency of oligomer formation was reduced as compared to the wild-type protein [13]. Then, Cys216 is crucial in forming protein structure. In this study, Cys216 of CbNPR1 is conserved with AtNPR1 (Figure 3), suggesting that CbNPR1 and AtNPR1 may have similar structural conformation.

Another important residue, His334, which conserved in CbNPR1, has been shown to play a critical role in the interaction with transcription factor [13]. There is a report of AtNPR1 substitution of His334 by tyrosine in the ankyrin domain impairs the interaction with a transcription factor TGA2 [13]. These suggested that CbNPR1 may have similar biological functional with AtNPR1 since the His334 of CbNPR1 is conserved with AtNPR1 (Figure 3).

The ankyrin helix-turn-helix repeat conformation was observed in CbNPR1 secondary structure (Figure 4) and tertiary structures (Figure 5). The ankyrin repeats for CbNPR1 contains 7 antiparallel α-helices followed by β-sheets. These ankyrin repeats have similarity with AtNPR1 ankyrin repeats pattern. These data convinced that CbNPR1 may really have similar structural conformation with AtNPR1.

CONCLUSION

CbNPR1 ankyrin domain shares 62.5% identity and 78.1% similarity with AtNPR1, suggested that CbNPR1 gene is predicted to be homologous with Arabidopsis AtNPR1. The conserved residue of Cys216 and His334 are suggested that they may have similar in structural conformation and biological function.

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