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Isolation and characterization of the NPR1 gene from highly susceptible pepper cv. Berangkai.

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Nonexpressor of pathogenesis-related-1 (NPR1) is one of the important proteins involved in plant defense systems. This protein activates systemic acquired resistance (SAR) and induces expression of Pathogenesis-related (PR) gene. In this article, the NPR1 gene has been isolated from *Capsicum annum* cv. Berangkai which are susceptible to infection of geminivirus. The putative full gene *NPR1* Berangkai sequence, abbreviated CaNPR1.Berangkai, was obtained by assembling the sequencing results of 3 exons of the *NPR1* gene. The putative full gene CaNPR1.Berangkai has a nucleotide length of 2,786 base pairs and 390 amino acids. The results of protein domain analysis show that CbNPR1 has a BTB domain at residues 60-130, ankyrin domain at residues 245-344, and NLS domains at residues 345 – 390. It is indicated that CaNPR1.Berangkai is similar to CaNPR1 Zunla and AtNPR1.

Keywords: Nonexpressor of pathogenesis-related-1 (NPR1), Systemic acquired resistance (SAR), PCR, Geminivirus, *Capsicum annum*

INTRODUCTION

Chilli pepper is economically important in Indonesia and many countries worldwide. There are various chili pepper genotypes used in Indonesia. One of the most popular is Cabai Berangkai. This genotype is highly susceptible to PepYLCV (Pepper Yellow Leaf Curl Virus), members of the Geminiviridae family, and become an epidemic in Indonesia region (Jamsari and Pedri, 2013; Solahudin et al., 2015; Jamsari et al., 2016). Although conventional methods are often effective in disease management, unfortunately, it has some drawback aspects. For that reason, an alternative approach to the conventional method has to be developed. One of them is investigating the genes which are responsible for virus resistance and characterize them in depth aspects.

The genomic aspects dealing with the regulation of the plant's immune system has been studied to understand the mechanism and genes involved in defense response. Activation of systemic acquired resistance (SAR) triggers the defense reactions when the plant was infected by pathogens (Durrant and Dong, 2004). In some cases, the defense reactions are regulated by salicylic acid to induce the expression of pathogenesis-related (PR) genes via non-expressor of pathogenesis-related 1 (NPR1) proteins. Activation of the PR genes expression

can promote stronger resistance to certain pathogens and it depends on NPR1 proteins as the master regulator (Wally et al., 2009). Previous research has shown that the *NPR1* mutants caused in failing to express some certain PR genes (Fan and Dong, 2002). Overexpression of the *NPR1* could up-regulate some PR genes expression level and enhance resistance to certain pathogens. Homologous overexpression has been shown to enhance resistance against various pathogens (Zhang et al., 2010).

The *NPR1* genes have been found to be homologous among plants, yet there are certainly different in nucleotide sequence. Therefore, it is important to understand the implication of those difference in defense responses. In this paper, we report the characteristic of the *NPR1* gene sequence isolated from *C.annum* cv. Cabai Berangkai and its genomic structure are deeply discussed.

MATERIALS AND METHODS

Plant material and genomic DNA extraction

A 21 days old *Capsicum annum* cv. Cabai Berangkai plant grown in a green house was used in this study. The young leaves were collected, washed with sterilized distilled water, and cut into small pieces. Genomic DNA was isolated using the CTAB extraction method described by Dellaporta et. al. (1983). Genomic DNA was resolved on 1.5% agarose gels containing ethidium bromide and purified with GeneJET Gel Extraction and DNA Clean-Up kit (Thermo Scientific-USA).

Amplification of CaNPR1.Berangkai gene from genomic DNA

Specific primers were designed in the exon regions referring to the genomic sequence of *Capsicum annum* cv. Zunla (NC_029983.1). Both 5 and 3 ends of all primers were flanked by introns to ensure a full amplification of each exon (Table 1).

CaNPR1.Berangkai gene structure analysis

The conserved domains search (CDS) at NCBI was used to determine functional domains [https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi]. Multiple sequence alignment was performed with CLUSTALWinBioedit 7.2.5.

Table 1. List of primer and their sequences used in the study

ORF	Forward Primer (5' -> 3')	Reverse Primer (5' -> 3')	Product Size
Exon 1	CGCTCAGTCAGAGGAAGCAA	GAGACGCTCGAGATTGCGTA	1.633
Exon 2	AACAAAGCTGCAGCAGACGA	CGCCTGCCATAGCAAGAGAT	687
Exon 3	TCCGTTTTAATGGTGGGTGGT	TTGCCAGCCCTAGTGATCAAG	405
Exon 4	CAAAACCATGAACACGGCG	AGGCTCACAAGATTTGCTGC	952

The thermal cycler (BioRad-USA) was programmed for touchdown PCR as follows: 25 cycles of 96^{o}C for 60 s, 96 ^{o}C for 15 s, 70 ^{o}C for 45 s (-1 decrement each cycle), 72 ^{o}C for 60 s, and followed by a final extension at 72 ^{o}C for 5 min. The PCR products were resolved on 1.5% agarose gels containing ethidium bromide and purified with GeneJET Gel Extraction and DNA Clean-Up kit (Thermo Scientific-USA).

RESULTS

Isolation of a putative CaNPR1.Berangkai gene

Touch down PCR was performed to obtain three exons of the *NPR1* gene from the genomic template. The primers were designed based on the genomic sequence of *Capsicum annum* cv. Zunla (NC_029983.1). Three fragments were isolated; 1,489 bp, 203 bp, and 398 bp. The genomic DNA sequence of CaNPR1.Berangkai was 2,768 bp in length. The CaNPR1.Berangkai genomic sequence revealed that there were four exons and three introns, which is similar to the genomic structure of reference model CaNPR1.Zunla and AtNPR1 (Fig. 1).

Genomic structure of putative CaNPR1.Berangkai gene

The genomic alignment between Berangkai and Zunla as the reference showed a mutation on 4 exons (Table 2). Computational translation of the CaNPR1.Berangkai nucleotides to amino acids revealed that it consists of 390 residues.

The CaNPR1.Berangkai protein showed domain of BTB/POZ from amino acid residues 60 to 130, ankyrin from amino acid residues 245 to 344, and NLS from amino acid residues 345 to 390 (Fig.2).

Alignment of the full-length CaNPR1.Berangkai and AtNPR1 amino acid sequences shared 39.46% identities and 55.14% similarities.

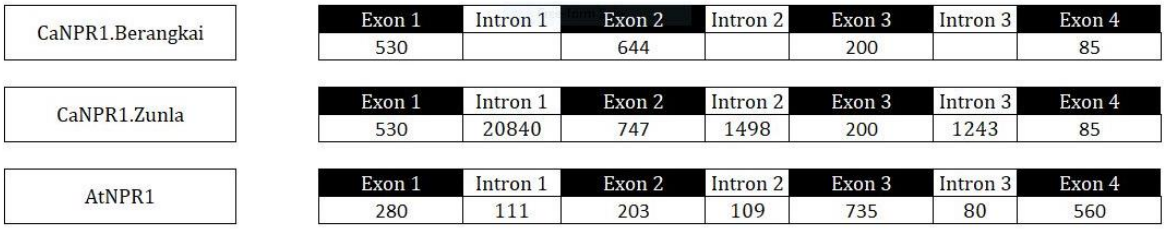

Figure 1. Gene structures of CaNPR1.Berangkai. Diagram of CaNPR1.Berangkai compared with CaNPR1.Zunla as reference and AtNPR1. Below the box represents the size of nucleotides.

Table 2. Mutation distribution along the exons of the CaNPR1.Berangkai sequence

Location	Mutation Type	Base	Location
Exon 1	Substitution	G>A	112.611.032
Exon 2	Deletion	T	112.632.324
	Deletion	A	112.632.333
	Deletion	A	112.632.334
	Deletion	A	112.632.347
	Insertion	A	112.632.964---965
	Substitution	A>G	112.632.966
	Substitution	T>G	112.632.967
	Substitution	A>G	112.632.971
	Substitution	T>A	112.632.977
	Substitution	G>C	112.632.978
	Substitution	A>C	112.632.979
	Substitution	T>A	112.632.980
	Substitution	T>C	112.632.981
Exon 3	-	-	-
Exon 4	Substitution	T>A	112.636.034
	Substitution	A>C	112.636.036
	Substitution	A>C	112.636.040

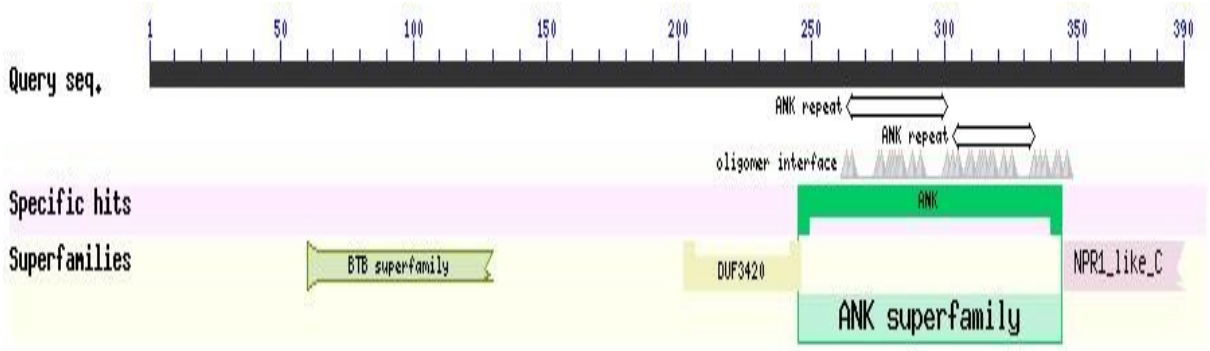

Figure 2.Protein domain of CaNPR1.Berangkai.
Schematic representation of predicted BTB/POZ, ankyrin, and NPR like C (NLS) domain. The domain was analyzed using NCBI conserved domain search (CDS).

Although the amino acid sequences were not similar in both of the NPR1 gene, the putative amino acids critical for the NPR1 function; Cys^{82}, Cys^{216}, Cys^{150}, and His^{334} were conserved in CaNPR1.Berangkai. However, the LENRV and NIMIN motifs are not observed in CaNPR1.Berangkai.

DISCUSSION

We have isolated the *NPR1* gene sequence from chili pepper *Capsicum annum* cv Cabai Berangkai. In this study, the *NPR1* gene was isolated directly from genomic DNA using specific primers and designated as CaNPR1.Berangkai. The CaNPR1.Berangkai contained three predicted BTB/POZ, Ankyrin, and NLS domain. All the three domains are particular in the*NPR1* genes that are highly conserved among many species (Backer et al., 2015). The BTB/POZ domain is located near the N-terminal end and the Ankyrin domain is located after BTB domain, while the NLS domain is located near the C-terminal end. The BTB/POZ domain has been shown to have a role in homo-dimerization of NPR1 protein (Fobert et al., 2009) and the ankyrin repeat region mediates interaction with TGA transcription factors (Peraza-Echeverria et al., 2012). The C-terminal region of NPR1 has been shown to contain a nuclear localization signal (NLS) that directs NPR1 monomers into the nucleus upon induction (Weigel et al., 2001).

The BTB/POZ domain has two important residues Cys^{82} and Cys^{150}. Mutations in the Cys^{82} can cause protein monomerization, localization of cell nuclei and continuous expression of PR genes (Mou, et al., 2003). While mutations in the Cys^{150} can abolish the function of NPR1. The ankyrin domain has two important residues Cys^{216} and His^{334} acting in NPR1 monomer binding process with the TGA2 transcription factor (Cao et al., 1997; Sandhu et al., 2009). Under normal circumstances, the TGA2 is a repressor in the PR gene promoter region, when it binds to the NPR1 monomer via ankyrin domain, TGA2 will conform and act as an activator (Johnson et al., 2008). The mutations on these residues may result in no protein-protein interaction between NPR1 monomers and TGA2. The NLS domain has two important residues Cys^{521} and Cys^{529}, serves as the binding site for NPR1 with salicylic acid (Maier et al., 2011). The mutation occurs in both residues will disrupt the interaction of NPR1 with several transcription factors interacting with the PR1 promotors. Almost all the important residues were found in both of CaNPR1.Berangkai and AtNPR1, except Cys^{521} and Cys^{529}. However, the LENRV and NIMIN motif known as the positive regulator for PR1 (Maier et al., 2011) are also not existing. Hence the absence of those important amino acids and motives could be the cause of reduced effectiveness of NPR1 in the SAR system against biotic stress.

CONCLUSION

As a conclusion, this present study has successfully isolated and analyzed the complete *Capsicum* cv Berangkai *NPR1* ORF sequence. The CaNPR1.Berangkai sequence has a high level of similarity with CaNPR1.Zunla and slightly similar to AtNPR1. Furthermore, four important amino acids in CaNPR1.Berangkai were conserved with AtNPR1 so that the functions associated with these amino acids can be assumed to be no different. A further experimental investigation is needed to identify if Cys^{521}, Cys^{529}, the LENRV, and NIMIN motifs were responsible for the low effectiveness of NPR1 in SAR.

CONFLICT OF INTEREST

The authors declared that the present study was performed in the absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

BN: Methodology, Investigation, Software, Data analysis, and Writing Original Draft. RH: Exon 2 gene isolation and Data Analysis, MF: Exon 3 and 4 gene isolation and Data Analysis. LS: Data Curation, Project Administration. AA: Funding Acquisition. JJ: Conceptualization, Funding Acquisition, Methodology, Resources, Supervision, Validation, Review, and Editing.

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