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Partial isolation of distal promoter sequence of the *NPR1* gene from local chilli Pepper [*Capsicum annuum* L.] genotype berangkai

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Abstract. Understanding of *NPR1* gene expression is a crucial aspect in improvement of chilli pepper resistant. The *NPR1* gene is known to confer broad spectrum resistance toward many phytopathogens by activating SAR mechanism. For that reason, characterization of the *NPR1*-gene promoter is necessary. The distal promoter of the *NPR1*-gene in general is characterized by the presence of enhancer, silencer, and cis-acting elements, located 5,950 bp from the ATG starting point. Therefore this study is focused to the isolation of the distal promoter sequence of the *NPR1* gene from a local *Capsicum annuum* L. Genotype *Berangkai*. Specific primer pairs designated as PD_CbNPR1-F1 [FR] and PD_CbNPR1-F2 [FR] were designed to amplify the whole targeted segments. However, only 1,873 bp in total [31.5 %] of sequences could successfully be elucidated from the first round, covering 970 and 993 bp from its upstream and downstream segments respectively. Homology analysis using BLAST tool successfully confirmed 99 and 100 % homology with its reference sequence derived from cv. *Zunla-1*. These results indicated that the *NPR1* distal promoter sequence is successfully isolated from chilli pepper [*Capsicum annuum* L.] genotype *Berangkai*. However, further analysis using primer walking strategy should be undertaken in order to identify the entire sequence of its promoter region.

1. Introduction

Chilli pepper [*Capsicum annuum* L.], a member of the Solanaceae family, is an important spice crop worldwide. However, most commercially grown chilli pepper cultivars are susceptible to various pathogens infection. Jamsari and Pedri [1] reported that Pepper Yellow Leaf Curl Virus [PepYLCV] is one of the main pathogens in chilli plants. Some genes were also successfully elucidated from some isolates reflecting different pathogenic level [2-4]. PepYLCV causes leaf curl disease [PepYLCD] which is characterized by the fading of the leaf bone (vein clearing) which then develops into yellowish, thickening of the leaf bone, and cupping. Long-term infection can cause shrinking, bright yellowing, stunting and falling off the flowers.

Conventionally, control of leaf curl disease is only able to reduce whitefly [*Bemisia tabaci*] population as the vectors. Despite a decline in population and whitefly attack, it cannot control viral infections that occur in plant tissues, especially in phloem. Molecular techniques through genetic



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engineering platform is also being developed as an alternative solution. One of them is by utilizing plant resistance genes known as pathogenesis related [PR] genes that are capable of producing anti-microbial proteins [5]. Activation of the PR gene is induced through the interaction of the transcriptional factor with the non-express or of pathogenesis-related 1 [NPR1] protein.

The *NPR1* protein acts as the key regulator in plant resistance system, namely Systemic Acquired Resistance (SAR) [6 - 9]. SAR can respond to various pathogen attacks and control infections in all plant tissues [10-12]. The study conducted by Zhang et al. [13] showed over expression of *Arabidopsis thaliana NPR1* in *Citrus paradisi* var. *Duncan* transgenic increases 10 folds resistance to *Xanthomonascitri* subsp. *citric* [Xc]). The results of study by Zhong et al. [14] showed that over expression of *Gladiolus hybridusNPR1* in *Arabidopsis thaliana* plant was able to increase resistance by 3.8 folds against *Pseudomonas syringaepv. Tomato DC3000*. NPR1 is also effective against viruses, which are *Tobacco mosaic virus* [15] and *Euphorbia mosaic virus* [16]. The results of the study concluded that *NPR1* in each plant has the same function as the regulator in plant defence systems. Therefore, NPR1 in chilli pepper plants also potent in plant resistance systems to against pathogens.

One component that plays an important role in the regulation of gene expression is the promoter. Promoter is regulatory sequences located in upstream of the open reading frame [ORF] [17]. The structure of the gene promoter consists of the distal promoter and the core promoter [18]. The distal promoter region contains enhancers, silencers, and cis-acting elements [17]. These elements have an important role during the process of transcription initiation, so information about the type, position, and number of these elements in a promoter can be used as a reference in optimizing gene expression. Increasing expression level of the resistance genes is also able to improve crop resistance mechanism.

So far, interaction between *NPR1* promoters and molecules produced by the virus during infection has not yet been described elsewhere. To elucidate the role of the PD_CbNPR1, we isolated the PD_CbNPR1 from chili pepper [*Capsicum annum* L] genotype *Berangkai*. This study is expected to be the first step as a reference in understanding the regulation of NPR1 promoter and to improve the resistance of chili pepper to PepYLCV.

2. Materials and Methods

2.1. Plant materials and isolation of genomic DNA

Capsicum annum L. genotype *Berangkai* was prepared as previously described by Nova et al. [4]. Genomic DNA was isolated from the young leaves using a cetyltrimethylammonium bromide [CTAB] method as previously described by Doyle and Doyle [19].

2.2. Specific primer designing

The putative promoter of the *NPR1* gene in chilli pepper is located 10,000 bp from ATG which consists of a distal promoter and a core promoter. The distal promoter of the *NPR1* gene in chilli pepper genotype *Berangkai* [namely PD_CbNPR1] have 5,950 bp in length and located at 4,051 to 10,000 bp in upstream of ATG. The reference sequence used in this study based on the genomic data of *Capsicum annumcvZunla-1* chromosome 7 [NC_029983.1] [20] accessed via NCBI at <http://www.ncbi.nlm.nih.gov>. Specific primers were designed to amplify the distal promoter sequence of the *NPR1* gene consisted of two primer pairs (forward-reverse) flanking two fragments. Each fragment is then named PD_CbNPR1-F1 [FR] and PD_CbNPR1-F2 [FR] with the estimated products size are 3,597 bp and 3,351 bp respectively. The primers location scheme can be seen in Figure 1.

The specific primers were analysed with the Oligo Analyser 3.1 software [<https://sg.idtdna.com/calc/analyser>] and the Multiple Primer Analyser software [<https://www.thermofisher.com/id/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/multiple-primer-analyser.html>].

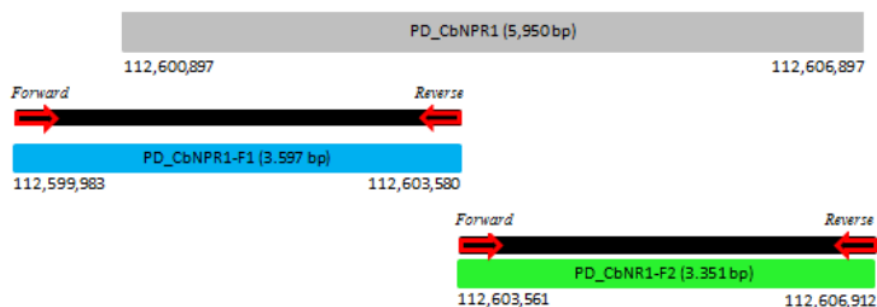


Figure 1. Schematic diagram of specific primer location.

2.3. Isolation of *PD_CbNPR1*

The 5,950 bp fragment of *PD_CbNPR1* was isolated from chilli pepper genomic DNA using 2 *PD_CbNPR1* specific primer pairs. KOD-Plus-Neo kit [Toyobo, Japan] was used as PCR master mix kit. The 50 μ L PCR reaction volume contained 5 μ L 10 x KOD-Plus-Neobuffer, 5 μ L 2 mM dNTPs, 3 μ L 25 mM $MgSO_4$, 1.5 μ L of each primer (10 ng/ μ L), 1 μ L genomic DNA (10 ng/ μ L), 32 μ L nuclease free water, and 1 μ L KOD-Plus-Neo. A touchdown PCR reaction was used in this study with temperature ranged 70-55 $^{\circ}C$ as described previously [4].

2.4. Sequencing and BLAST analysis

Successful isolation of the *PD_CbNPR1* promoter sequence was confirmed by sequencing technique. The amplicon with 3,597 bp in size was sequenced from both termini. BLAST analysis [21] was used to verify the sequence integrity by comparing with the *C. Annuum cv Zunla-1* genomic data.

3. Results and Discussion

3.1. Isolated DNA of *Capsicum annuum L.* genotype *Berangkai*

Capsicum annuum L. genotype *Berangkai* genomic DNA was extracted from the young leaves. The concentration of λ DNA marker that used in this study is 100 ng/ μ L. The visualization of the isolated DNA band is twice brighter than λ DNA, so it can be stated that the concentration of the isolated DNA is 200 ng/ μ L. The isolated DNA then was used as a DNA template for amplification step.

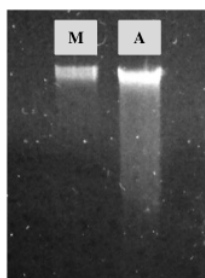


Figure 2. Genomic DNA isolation from *C. annuum L.* genotype *Berangkai*. (M: Lambda (λ) DNA marker; A: *C. annuum* isolated DNA.)

3.2. *PD_CbNPR1* specific primers

The length of specific primers used in this study is around 18-21 bp, regarded as ideal primers amounted to 18-26 bp. Specific primers designed can be seen in Table 1.

Table 1. The specific primers of PD_CbNPR1

Name	Nucleotide Sequences	Length (bp)	Tm (°C)	GC (%)	Product length (bp)
PD_CbNPR1-F1 (F)	5' TGA TCG CAC TCA CCG AAC 3'	18	64.3	55.6	3,597
PD_CbNPR1-F1 (R)	3' CCG TAC CTT GTT AAC CCC ATC 5'	21	64.4	52.4	
PD_CbNPR1-F2 (F)	5' CAT GGG GTT AAC AAG GTA CC 3'	20	61.0	50.0	3,351
PD_CbNPR1-F2 (R)	3' CCC AAG GCG TAA CTA TTG AAC 5'	21	62.6	47.6	

3.3. *PD_CbNPR1* amplification products

Two PD_CbNPR1 fragments were identified in chilli pepper plant by PCR amplification with specific primers. Two pairs of specific primers for each fragment, previously designed were used. A single band of the predicted size from each primer pairs were amplified from genomic DNA (Figure 3), and no other bands were observed in this step.

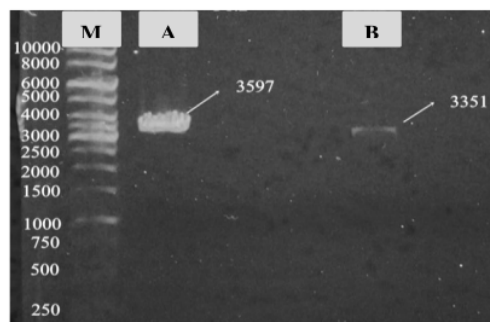


Figure 3. Visualization of the PD_CbNPR1 sequence amplification (M: 1 kb marker; A: PD_CbNPR1-F1; B: PD_CbNPR1-F2.)

3.4. Sequencing of *PD_CbNPR1-F1* and BLAST analysis

To verify the successful isolation of the PD_CbNPR1 fragments, bi-directional sequencing method was used. In this study, sequencing only for the PD_CbNPR1-F1 fragment with its specific primer pair. The chromatogram sequencing results can be seen in figure 4.

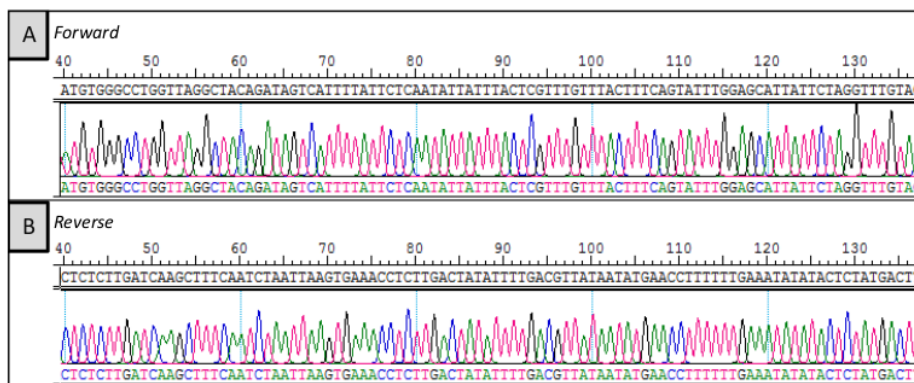


Figure 4. Chromatogram of sequencing result of PD_CbNPR1-F1. A: Upstream reading with forward primer. B: Downstream reading with reverse primer.

The results of the upstream reading with forward primer [PD_CbNPR1-F1-[F]] is about 970 bp in size, while the downstream reading with reverse primer [PD_CbNPR1-F1[R]] is 993 bp in size. Between PD_CbNPR1-F1[F] upstream reading and PD_CbNPR1-F1[R] downstream reading, there are no overlap areas with each other, but leaves the gap areas in between [Figure 5]. The bi-directional sequencing of PD_CbNPR1-F1 using its specific primer could not cover the whole fragment sequence with *C. annuum* cv. *Zunla-1*. The location of the PD_CbNPR1-F1[F] upstream reading sequence in the genomic data is in the 112,600,031-112,601,000 bases. While the results of BLAST PD_CbNPR1-F1[R] downstream reading show the level of similarity with *C. annuum* cv. *Zunla-1* is 100 %. The sequence location is in the 112,602,544-112,603,536 base sequence in the genome data

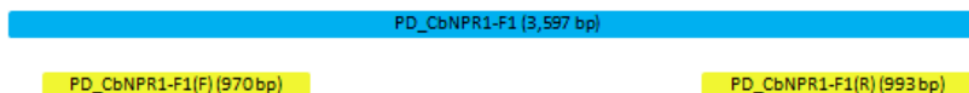


Figure 5. Schematic diagram of PD_CbNPR1-F1 sequencing result. BLAST results of PD_CbNPR1-F1 [F] upstream reading sequence showed 99 % similarity.

Plant defence system, especially SAR, NPR1 is one of the main regulatory components. The NPR1 protein could induce the PR genes activation through the interaction with the transcriptional factors. Regulation of NPR1 gene expression is regulated by its promoter. Recently, the NPR1 promoter has been isolated and characterized in several plant species such as *Oryza sativa* [7], *Arabidopsis thaliana* [22], and *Gladiolus hybrids* [14], but so far has not been reported in *Capsicum annuum* yet.

In this study, isolation and identification of NPR1 promoter was focused on the distal promoter region. Distal promoter region is a segment harboring silencer, enhancer, and cis-acting elements that play an important role during transcription regulation. Two pair specific primers were designed and successfully amplified two fragments of PD_CbNPR1. Sequencing result of PD_CbNPR1-F1 was only successful from the upstream and downstream region covering 970 bp and 993 bp segments in length, respectively. This indicated that not the entire sequence was successfully elucidated. A gap which is around 1,634 bp is existing. Therefore, the primer walking strategy [3] was applied to identify the entire sequence using some new primer pairs designed from the internal flanking region in vicinity of the previously designed primer.

4. Conclusion

Two fragments of PD_CbNPR1 were successfully isolated from *Capsicum annuum* genotype *Berangkai* genomic DNA with length 3,597 bp [PD_CbNPR1-F1] and 3,351 bp [PD_CbNPR1-F2]. The 970 and 993 bp fragments from the upstream and downstream region of PD_CbNPR1-F1 were successfully confirmed. In order to identify the entire sequence of PD_CbNPR1, further activities using primer walking strategy is recommended.

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