Distal Promoter-F1000Research

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RESEARCH ARTICLE

REVISED Conserved structure of the *NPR1* gene distal promoter isolated from a chili pepper (*Capsicum annuum* L.) in West Sumatera [version 2; peer review: 3 approved with reservations]

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Abstract

Background: The non-expressor of pathogenesis related gene 1 (*NPR1*) protein is one of the key regulators in the systemic acquired resistance plant defense system. The *cis*-acting elements of its distal promoter gene are characterized by salicylic acid inducing elements such as the W-box, RAV1AAT and ASF1, accompanied by enhancer and silencer elements. This study was aimed to isolate and characterize the distal promoter sequence of the *NPR1* gene (*PD_CbNPR1*) from the chili pepper (*Capsicum annuum* L.) genotype *Berangkai*, a local genotype known to produce large yields, but is susceptible to viral infection. Elucidating its sequence structure will open a broad range of possibilities to engineer the *NPR1* gene expression which is important to improve chili pepper resistant.
Methods: PCR-based cloning combined with a primer walking strategy was applied in this study. The BioEdit tool was used to edit the sequence and verify sequence integrity, while homology analysis was conducted with BLASTn searching. Identification of a *cis*-acting element was detected by PLACE, PlantCare, and PlantPAN.
Results: Isolation of the complete distal promoter sequence of *PD_CbNPR1* produced a fragment 5,950 bp in size. BLASTn search analysis indicated that *PD_CbNPR1* sequence is highly conserved (99% identity) showing only a single nucleotide polymorphism (SNP) (base substitution) compared with its reference sequence. Analysis using PLACE tools successfully identified nine *cis*-acting elements containing a W-box, WLE1, RAV1AAT, TATA-box, CAAT-box, GARE and GT1 with multi repeats and diverse motives, as well as enhancer and silencer elements, which is characterized by a CCAAT-box and GAGAAATT pattern, respectively.
Conclusion: The distal promoter of the *NPR1* gene is highly conserved, showing only one SNP caused by one base substitution event.

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Keywords
CCAAT-box, distal promoter, NPR1 gene, RAV1AAT, W-box

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REVISED **Amendments from Version 1**

Some changes in our 2nd version dealt with the comments and suggestions addressed by the two reviewers Minghui Lu, and Santy Peraza-Echeverria.

In the **Abstract**: two bioinformatic tools: PlantCare and PlantPAN are added. The term "homology" has been changed to "identity".

In the **Methods**: The *"Genomic DNA isolation"* subsection has been expanded. PlantCARE (Lescot *et al.*, 2002) and PlantPAN are mentioned in the "Bioinformatic tools and sequence analysis" subsection.

In the **Results and Discussion**: Figure 1 has been replaced and now shows the transcription start site and the length of core promoter.

In the *"Characteristics of the NPR1 distal promoter"* subsection, we add a paragraph explaining that PCR based cloning strategy in combination with primer walking was applied to isolate the complete full length of putative distal promoter NPR1 gene region (5,950 bp). The terminology "99% homology" was changed to "99% identity". We add that the BLAST analysis didn't show any significant homology with another promotor sequence available in the NCBI database, indicating a limitation of promoter sequence availability. We further expand on this and add a reference to Lee *et al.*, 2006.

We expand on our validation process and add a new reference (Nova *et al.*, 2019).

We have updated Figure 3 with 1 TCA motif and 3 CGTA motifs in the cis-acting diagram and the word "nine" is changed to "eleven".

We added a new paragraph explaining the findings following analysis with PlantCare and PlantPAN 3.0.

In the **Conclusion:** we add TCA and CGTA to the list of *cis*-acting elements and mention that the role of *cis*-acting elements in gene expressions needs to be confirmed.

Any further responses from the reviewers can be found at the end of the article

Introduction

The non-expressor of pathogenesis related gene 1 (*NPR1*) protein is the main regulator in the systemic acquired resistance response of many plants. Overexpression by modifying the distal promoter in the W-box element of the *OsNPR1* gene in rice could increase its resistance against *Xanthomonas oryzae* pv. Oryzae up to 4.3 times (Hwang & Hwang, 2010). Similar results were reported by Zhong *et al.* (2015), who modified the RAV1 element on the GhNPR1 which increased *Gladiolus hybridus* resistance against *Curvularia gladioli* by up to 18.6 times compared to control. Based on those studies, we expect more prospects of the *NPR1* gene promoter in the improvement of plant resistance against many pathogens.

Here we report characteristics of the distal promoter segment of the *NPR1* gene isolated from chili pepper (*Capsicum annuum*) genotype *Berangkai*, a local genotype potentially produces more yields compared to other genotypes cultivated in West Sumatera.

Methods

Genomic DNA isolation

Healthy young leaves collected from chili pepper genotype Berangkai was used as the plant material in this study. This genotype is known to produce more yield, but susceptible to geminivirus infection. Resistant genotype however, is not available in our collection so far, so comparison of both two genotypes is not possible to be performed. The plant was grown in a greenhouse and maintained for 8 weeks before being used for DNA isolation. Genomic DNA isolation was performed using protocol as described by Jamsari & Pedri (2013).

Fragment isolation and PCR conditions

PCR-based cloning combined with a primer walking approach was applied for the isolation complete segment of distal promoter of the *NPR1* gene. All PCR reactions in this study were performed using the KOD-Plus-Neo kit provided by Toyobo-Japan. Chromosome 7 of *Capsicum annuum* cv. *Zunla-1* (Qin *et al.*, 2014), accession number: NC_029983.1, was used as the reference sequence. Primer combinations were designed to cover the interval segment spanning from base 112.600.897 to 112.606.847 on the reference sequence (Table 1). All primers used in this study are listed in Table 1. First-step isolation was started from both termini and continued with successive isolation until both termini formed a complete contig of the target segment. The initial PCR condition was started with 95°C for 3 minutes for pre-denaturation and amplified in two different loops using 14 and 24 cycles. The first 14 cycles were performed using a touch down steps started by denaturation at 95°C for 30 seconds. Annealing was initiated with 70°C for 30 seconds and gradually decreased 1°C for every cycle before finally elongated in 72°C for 2 minutes. The next 24 cycles started with denaturation at 95°C for 30 seconds with annealing at 55°C for 30 seconds and elongation of at 70°C for 2 minutes and elongated for 72°C for 2 minutes. The final extension was maintained at 72°C for 5 minutes. PCR reaction was performed in 50 µl of final volume. PCR composition was set according to manufacturer's recommendation, containing 5 µl 10x Buffer KOD-Plus-Neo, 5 µl of 2 mM dNTPs, 3 µl of 25 mM $MgSO_4$, 1.5 µl of 10 ng/µl of each primer, 1 µl of KOD-Plus-Neo (Toyobo, Japan) and 1 µl of 10 ng/µl of DNA template. The volume was filled with 32 µl of nuclease-free water.

Bioinformatic tools and sequence analysis

The PCR product generated from each walking step was processed to sequencing reaction using its both forward and reverse primers. All sequencing processes were performed by 1st BASE-Singapore. A number of bioinformatic tools were applied during analysis of the sequence. Trimming, editing and building of the sequence contig were performed using BioEdit v7.2.5 (Hall, 1999). Homology search of the sequence with all available sequences deposited in the NCBI database was run using the BLASTn tool (Altschul *et al.*, 1990). The *cis*-acting elements were identified using PLACE, developed by Higo *et al.* (1999), PlantCARE (Lescot *et al.*, 2002) and PlantPAN.

Results and discussion

Characteristics of the *NPR1* distal promoter

We successfully isolated the distal promoter of the *NPR1* gene from our local chili pepper genotype Berangkai, exhibiting 5,950 bp in size and designated *PD_CbNPR1*. The fragment was

Table 1. Primers ID, sequence, length of expected product and verified sequence obtained in this study.

Primer ID	Sequence 5' to 3'	Expected PCR product (bp)	Verified sequence (bp)
F1-F	TGA TCG CAC TCA CCG AAC	3,597	970
F1-R	CCG TAC CTT GTT AAC CCC ATC		993
F2-F	CAT GGG GTT AAC AAG GTA CC	3,351	Skipped for sequencing
F2-R	CCC AAG GCG TAA CTA TTG AAC		
F1.1-F	CGG ACC CAC ACC AGG TTA TAT	970	871
F1.1-R	CCC CCT CCT TAG CTT CTC TTC		
F1.2-F	CCC TTG GTC CTT AGT CAG TGA	1,204	1,115
F1.2-R	CCA TGC GCT CAT ATG GTG A		
F2.1-F	CCC GTC AAG AGG TTT CAC	1,915	1,801
F2.1-R	CCC ATC AAC TAC AGA TCA GAG C		
F2.2-F	CCG GAC ATA GAC TAA GGG ATC	1,811	1,724
F2.2-R	CCC AAG GCG TAA CTA TTG AAC		

isolated from two steps round of walking via PCR-based cloning. In the first step, the whole distal promoter region was expected to be covered using two primer combinations (F1 F/R and F2 F/R). However, after verification of the sequence data using BioEdit, the upstream primer combination (F1 F/R) successfully produced a contig spanning only 1,963 bp, while the downstream primer combination (F2 F/R) was skipped for the sequencing process. In the second round of primer walking, four new primer combinations (F1.1-F/R, F1.2-F/R, F2.1-F/R, and F2.2-F/R) (Table 1) were designed in order to extend sequence coverage. Combining all verified sequences obtained from the first and the second round of primer walking successfully produced a contig with a size of 5,950 bp (Figure 1).

PCR based cloning strategy in combination with primer walking was applied to isolate the complete full length of putative distal promoter *NPR1* gene region (5,950 bp). This approach is considered to be the most appropriate since the sequencing read capacity used in this study is limited for about only 500 bp on average. Even though the KOD-Plus-Neo could amplify up to 24 kb according to manufacturer's claim, but the full-length fragment (5,950 bp) still can not be sequenced in one step read due to limited reading capacity of the sequencing machine. Validation of every single nucleotide data was confirmed by at least two overlapping validated segment.

Homology analysis between *PD_CbNPR1* and its reference sequence via BLAST search showed 99% identity. The data was verified by only one single nucleotide polymorphism (SNP) shown as a substitution event at the position -6,335 from the ATG start codon (Figure 2). Furthermore, BLAST analysis showed no significant homology with another promotor sequence available in the NCBI database, indicating a limitation of promoter sequence availability. The only promoter sequence showed homology is the promoter region of *Capsicum annuum* pathogenesis related protein-1 (PR-1) gene (DQ201633.1) published by Lee *et al.* (2006). However, the comparable nucleotide of both sequences spanned only 180 bp.

In order to validate our claim, we constructed a contiguous segment with our isolated core promoter (MK310185.1) and the *NPR1* gene sequence isolated from a similar genotype *Berangkai* (Nova *et al.*, 2019). BLAST analysis using the *NPR1-Berangkai* cDNA sequence exhibited 43 significant identity hits with other cDNA sequences of *NPR1* gene for instance, *Capsicum annuum* (NM_001325099.1), *Capsicum chinense* (AM900559.1), *Solanum lycopersicum* (KX198701.1, NM_001247629.2), *Nicotiana* sp. (DQ837218.1, AF480488.1) *Carica papaya* (XM_022041103.1, AY550242.1) and some others. Tree analysis showed that our *NPR1-Berangkai* cDNA sequence clustered to similar clade with AM900559.1 and NM_001325099.1 and other three solanaceae (*S. lycopersicum*-KX198701.1, NM_001247629.2; *S. tuberosum*-XM_006357647.2; *S. pennellii*-XM_015227358.2 and *S. torvum*-KJ995663.1). All those data obviously indicated that our segment landed in the right chromosome segment.

Cis-acting regulatory elements

PLACE analysis successfully indicated 9 *cis*-acting elements with multi repetition (Figure 3). All 9 *cis*-acting elements contained the W-box (15), WLE1 (W-box like elements) (8), RAV1AAT (20), TATA-box (22), CAAT-box (26), GARE (1), GT1 (20), Enhancer (4) and Silencer (1). The PlantCare analysis successfully showed 2 other *cis*-acting element motifs namely 1 TCA motif and 3 CGTA motis which could not be shown by PLACE (Figure 3).

The W-Box element binds the WRKY protein, acting as a transcription factor during expression of *NPR1* (Yu *et al.*, 2010). Mutation in this element in *Arabidopsis thaliana* delayed the *NPR1* expression induced by salicylic acid (SA). PLACE analysis

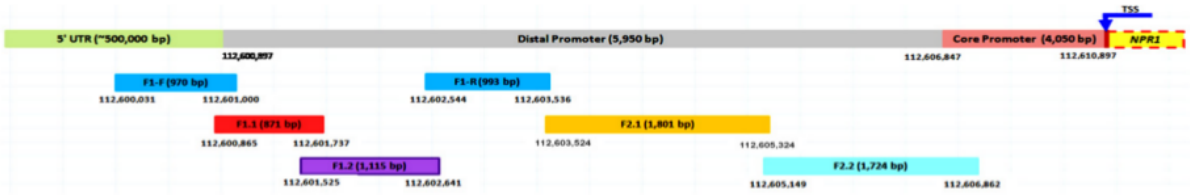

Figure 1. Primer walking steps along distal promoter sequence based on the *Capsicum annuum* cv. Zunla-1 genome. Base position of start and end segment are shown by number on each terminus. Length of verified sequence of each segment is shown by number in bracket on each segment. Transcription start site of the *NPR* gene located between core promoter and *NPR1*'s ORF is shown by arrowhead.

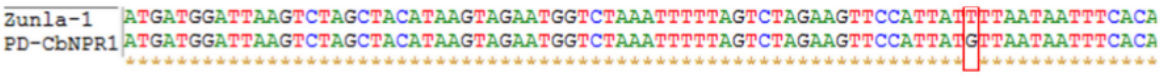

Figure 2. Partial multi-alignment showing one single SNP (T/G) at position -6,335 from ATG (red box).

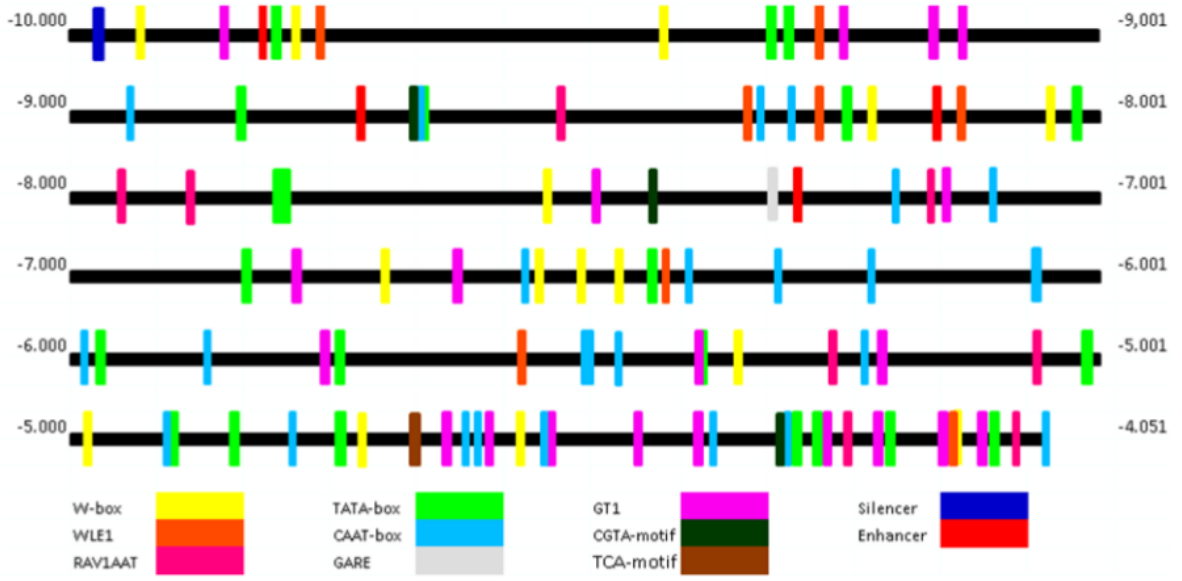

Figure 3. Position of eleven *cis*-acting elements along *PD_CbNPR1* from *Capsicum annuum* genotype Berangkai. Colors indicate each element as labeled below the figure.

indicated that *PD-CbNPR1* is characterized by three consensus sequences: TTGAC, TGACY, and TGACT. The TGACY pattern could also be found in the promoter of *ERF3* isolated from tobacco (Nishiuchi *et al.*, 2004), while TGACT could be found in the promoter of the *Iso1* barley gene (Sun *et al.*, 2003). The WLE1 (W-box like elements) with TGACA pattern has an analog function with the W-box identified in the promoter of *OsNPR1* (Hwang & Hwang, 2010).

Another *cis*-acting element found in *PD_CbNPR1* is RAV1AAT, which acts as binding site for protein RAV1 (Hwang & Hwang, 2010). The RAV1 protein is a transcription factor associated with some pathogenesis-related genes (Sohn *et al.*, 2006). Two consensus patterns of *RAV1AAT* elements found in *PD_CbNPR1* are CAACA and TGTTG. The CAACA pattern was also found by Kagaya *et al.* (1999) in *Arabidopsis thaliana*, while the TGTTG pattern is similar to the *OsNPR1* promoter

(Hwang & Hwang, 2010). Notably, the ASF1 element is absent in the *PD_CbNPR1*. The ASF1 element has been previously reported by Hwang & Hwang (2010) and Zhong *et al.* (2015) as a *cis*-acting for common promoter associated with pathogen infection and SA induction.

PLACE analysis identified 22 putative TATA-boxes in our *PD_CbNPR1*, which could be classified into TATA box 3, TATA box 4, TATA box 5 and TATA box OspaI. During transcription, the TATA-box binds with RNA polymerase II and many transcription factors like TFIID, TFIIA, TFIIF, TFIIE, and TFIIH to build the TATA-box complex.

Another interesting element found in *PD_CbNPR1* is the CAAT-box (26 repeats) which is associated with the regulation of many genes involved in pathogen infection (Imran *et al.*, 2016). The CAAT-box consensus sequence found in *PD_CbNPR1* is characterized with CAAT which is similar that have been found in promoter of the *legA* gene from *Pisum sativum* (Shirsat *et al.*, 1989).

We also found a gibberellin-responsive element (GARE) motif, which has been previously reported by Ogawa *et al.* (2003) as the binding site with some transcription factors induced by gibberellic acid. The consensus sequence of thoses motifs is TAACAAR. Another *cis*-acting element induced by abiotic factors such as salt, light and pathogenesis is GT1 motif (Biłas *et al.*, 2016; Park *et al.*, 2004). The identified GT1 element in *PD_CbNPR1* is characterized by a consensus GRWAAW motif and present in 20 repeats. They could be classified into two forms, GT1CONSENSUS and GT1GMSCAM4, as described previously by Terzaghi & Cashmore (1995). The last GT1 class, GT1GMSCAM4, is initially identified by Park *et al.* (2004) with consensus pattern GAAAAA.

PLACE analysis also successfully identified four CCAAT-box motifs, which is commonly reported as an enhancer of transcription (Thonpho *et al.*, 2013). This element is able to up-regulate the transcription and replication rate of eukaryotic genes by binding many types of transcription factors (Biłas *et al.*, 2016). da Silva *et al.* (2013) previously reported that a greater number of CCAAT-boxes could improve promoter capability in increasing of replication rate by binding efficiently with transcription factors. In contrast with the enhancer, we found only one motif of GAGAAATT which is also known as silencer (Lai *et al.* (2009). This element is reported to be able to down-regulate the kinesin-like protein 1 (*AtKP1*) gene in *Arabidopsis thaliana* by up to 80.9%.

Analysis with PlantCare (Lescot *et al.*, 2002) resulting 2 new additional elements, which are designated TCA element and CGTA-motif (Figure 3), while analysis with PlantPAN 3.0 did not show any new different element compared to PLACE analysis. The TCA element has a function as a *cis*-acting element which is involved in salicylic acid responsiveness. Moreover we also found the CGTA motif playing a role in regulating me-JA responsiveness. In our sequence only 1 single TCA element and 3 CGTA motifs could be detected.

Conclusion

We successfully isolated the distal promoter of the *NPR1* gene from chili pepper genotype Berangkai (designated *PD_CbNPR1*) spanning 5,950 bp. The *PD_CbNPR1* contain 11 *cis*-acting elements: W-box, WLE1, RAV1AAT, TATA-box, CAAT-box, GARE, GT1, TCA, CGTA, enhancer and silencer elements. Engineering of the *cis*-acting elements may have future prospects, particularly in improving chili pepper resistance against biotic and abiotic stresses by up- and down-regulating *NPR1* gene expression. However, their role in the gene expression has to be confirmed empirically. For that reason, we are currently using the *cis*-acting elements found in this study for replicase (*Rep*) gene expression in the bacterial system.

Data availability

The sequence of the distal promoter of the *NPR1* gene sequenced in this study has been deposited in GenBank under accession number MK281381; https://identifiers.org/ena.embl:MK281381.

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