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In-silico Characterization and Expression Analysis of NB-ARC Genes in Response to *Erwinia mallotivora* in *Carica papaya*

(Pencirian *In-silico* dan Analisis Pengekspresan Gen NB-ARC sebagai Gerak Balas kepada *Erwinia mallotivora* pada *Carica papaya*)

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ABSTRACT

Disease resistance in plants is commonly associated with resistance (R) genes that encode nucleotide binding site-leucine rich repeat (NBS-LRR) domains that are essential for pathogen recognition and defence signalling. In this study, we identified and analyzed the sequence of putative pathogen-responsive NB-ARC transcripts from Carica papaya *transcriptome database, carried out the structural and phylogenetic analysis, and determined the expression profile of the transcripts in* C. papaya *challenged with* Erwinia mallotivora*. The findings indicate CpNBS1, the only pathogen-responsive NB-ARC protein identified in this study belongs to the CC-NBS-LRR group. Semi-quantitative PCR showed CpNBS1 was differentially expressed in response to* E. mallotivora*. Structural analysis of the 4993-Eksotika and 4993-Viorica translated proteins showed striking differences in terms of the number of β-sheets and α-helixes as well their ligand-binding surface, suggesting the role of the LRR domain in determining the specificity of recognition of* E. mallotivora *effector. Collectively, this study provides new insights into the role of NBS-LRR genes in* C. papaya *and its implications for enhancing plant disease resistance through genetic engineering.*

Keywords: E. mallotivora; *nucleotide binding site-leucine rich repeat; resistance gene*

ABSTRAK

Kerintangan penyakit pada tumbuhan sering dikaitkan dengan gen kerintangan (R) yang mempunyai domain tapak pengikat nukleotida-ulangan kaya leusina (NBS-LRR) *yang berperanan untuk mengenal pasti patogen dan isyarat pertahanan. Dalam kajian ini, kami mengenal pasti dan menganalisis jujukan daripada pangkalan data transkriptom* Carica papaya*, menjalankan analisis struktur dan filogenetik serta memprofil pengekspresan transkrip* C. papaya *yang telah dicabar dengan* Erwinia mallotivora. *Keputusan kajian ini menunjukkan bahawa CpNBS1 adalah satu-satunya protein yang bergerak balas terhadap patogen dan berada dalam kumpulan* CC-NBS-LRR. *Analisis separa-kuantitatif PCR menunjukkan bahawa CpNBS1 telah diungkapkan secara berbeza sebagai gerak balas kepada* E. mallotivora. *Analisis struktur pula menunjukkan perbezaan yang nyata daripada segi bilangan kepingan beta dan heliks alfa serta permukaan ikatan ligan, yang mencadangkan peranan domain LRR dalam menentukan ketepatan pengecaman efektor* E. mallotivora*. Secara keseluruhannya, kajian ini mendedahkan pandangan baharu fungsi gen* NBS-LRR *dalam* C. papaya *dan kesannya kepada penambahbaikan kerintangan penyakit dalam tumbuhan melalui kejuruteraan genetik.*

Kata kunci: E. mallotivora*; gen kerintangan; tapak pengikat nukleotida-ulangan kaya leusina*

INTRODUCTION

Disease resistance in plants is commonly associated with resistance (R) genes that encode nucleotide binding site-leucine rich repeat (NBS-LRR) domains that are essential for pathogen recognition and defence signaling. Most NBS-LRR proteins comprise four distinct domains: a variable amino-terminal domain, NBS domain, LRR domain and variable carboxy-terminal domain that are joint

by a linker (Steele et al. 2019). Variation in the amino-terminal of NBS-LRR proteins defines the sub-families of these proteins either as a coiled-coil (CC) domain or toll-like interleukin (TIR) domain. This terminal serves as an interaction platform for downstream signaling (Bayless & Nishimura 2020). The central NBS domain, also known as NB-ARC domain consists of several highly conserved motifs characteristics of the Signal Transduction ATPases with Numerous Domains (STAND) such as P-loop, kinase-2 and Gly-Leu-Pro-Leu motifs. NB-ARC function as molecular switches in disease signaling pathways by catalyzing the hydrolysis of ATP (Zhou et al. 2019). The LRR domain is a highly adaptable structural domain that characteristically contain a series of β-sheets. This domain is involved in protein-protein interactions and is diverse to fit the binding specificities of various pathogens (Wang et al. 2020). Hence, the LRR region exhibits more variations than other regions of the gene.

The NBS-LRR proteins have very low steady state expression level. In the absence of pathogens, they remain in ADP-bound inactive state through intra-molecular interactions between their different domains or extra-molecular interaction with another host protein (Meunier & Broz 2017; Noman et al. 2019). These proteins are, however, induced in a timely manner when the pathogens come to interact with the plants. The interaction of plant NBS-LRR proteins and pathogen effectors has been extensively studied to understand the mechanism of plant and pathogen interaction. Generally, the NBS-LRR proteins directly or indirectly recognize effectors secreted by pathogens resulting in conformational changes of these proteins and exchange of ADP/ATP that causes activation of downstream signalling pathways leading to hypersensitive responses aimed to restrict the pathogen growth (Balint-Kurti 2019). Alternatively, the NBS-LRR proteins act as a guard by sensing and monitoring the status of other plant proteins targeted by pathogen effectors (Baggs et al. 2017). A classic example is the disease resistance protein *RPM1* in *Arabidopsis thaliana* where it detects the phosphorylation of RPM-1 Interacting Protein 4 (*RIN4*) triggered by the effectors AvrB and AvrRpm1 from *Pseudomonas syringae* pv. Glycinea and pv. Maculicola, respectively, and elicits the resistance response (Couto & Zipfel 2016).

There is considerable evidence indicating the involvement and contribution of the NBS-LRR proteins in resistance response in many pathosystems. Expression of TIR-NBS-LRR-encoded grapevine gene *VaRGA1* confers broad-spectrum resistance not only in grapevine but also in *Arabidopsis* and tobacco (Tian et al. 2020). Likewise, silencing of NBS-LRR gene SLNLC1 in a resistant *Solanum lycopersicum* cv. Motelle resulted in a susceptible phenotype (Cui et al. 2018).

Papaya (*Carica papaya* L.) is one of the important fruit crops in Malaysia planted for domestic and export markets. The main commercial variety grown in Malaysia is known as Eksotika which was developed by the Malaysian Agricultural Research and Development Institute (MARDI) three decades ago. The variety positioned the country as the second biggest contributor of world papaya (Sekeli et al. 2018). However, the production is severely affected by the outbreak of papaya dieback disease caused by *Erwinia mallotivora* (Mat Amin et al. 2011). The disease symptoms include greasy water-soaked lesions, spots on leaves and crowns defoliation and black spots of the papaya fruits. In an effort to keep the papaya industry afloat, a new hybrid variety called Viorica was developed by MARDI that is highly tolerant to dieback disease (Mohd Azhar et al. 2020). In order to elucidate the mechanism of tolerance in Viorica, a comparative transcriptomics was carried out between the Viorica and Eksotika in response to *E. mallotivora* (MARDI, unpublished data). Previously, genome data analysis has shown 54 NBS class R genes (Porter et al. 2009) and through the transcriptomic study, six NB-ARC transcripts were differentially expressed in response to the treatment. However, the involvement of papaya NB-ARC genes in response to dieback disease has never been validated. In the present study, bioinformatic analysis and expression profiling of NB-ARC candidate genes in two papaya varieties were carried out to obtain a clearer understanding of the involvement of the genes during interaction with *E. mallotivora*.

Materials and Methods

Plant material and inoculation

Carica papaya cv. Eksotika (susceptible, S) and Viorica (tolerant, T) seeds were germinated in a soil mixture consisting of topsoil, sand and poultry manure with a ratio of 3:2:1, respectively. After three weeks of germination, the seeds were transferred to 12” × 12” polybags and grown in a glasshouse under a controlled environment with a temperature range between 28 °C and 30 °C until it reached three months old. *E. mallotivora* strain BT-MARDI inoculum was prepared in Luria-Bertani media

broth for 40 h at 28 °C with agitation of 150 rpm. The main vein of fully expanded papaya leaves was inoculated using a sterile needle containing *E. mallotivora* culture at a concentration of 10^6 colony forming unit (CFU) (Supian et al. 2017). Control plants were treated similarly but with sterile water. The leaves were then harvested at 0, 24, and 48 h post-inoculation (hpi) before the onset of disease symptom.

IDENTIFICATION OF PUTATIVE PATHOGEN-RESPONSIVE NB-ARC TRANSCRIPTS FROM PAPAYA TRANSCRIPTOME DATABASE AND SEQUENCE ANALYSIS

The annotated *C. papaya* transcriptome database (available upon request to MARDI) were screened for NB-ARC transcripts that contained Pfam NB (NB-ARC) family (PF00931) domain with e-value cut off at 1.0, similar to the threshold set by Porter et al. (2009). Different transcript sequences that mapped to a single protein were removed. Candidate NB-ARC sequences with significant value of expression in Eksotika-S and/or Viorica-T (fold change more than 2.0) were selected. The transcript sequences were translated into proteins using ExPASy translate tool (https://web.expasy.org/translate/). Identification of open reading frame (ORF) in the translated protein sequences was done using an ORF finder (http://www.ncbi.nlm.nih.gov/projects/gorf/). The ORFs were aligned using the NCBI database BlastP to search for homologous sequences. Protein sequence hits with the highest similarity score were selected and further analyzed using the online software SMART (http://smart.embl-heidelberg/), NCBI CDD (https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi) and MOTIF (https://www.genome.jp/tools/motif/) to check for the domains. Annotation of predicted transmembrane regions was done using TMHMM Server v.2.0 (http://www.cbs.dtu.dk/services/TMHMM/) and coiled-coil motifs were predicted by the COILS program (Lupas et al. 1991) and HMMER (https://www.ebi.ac.uk/Tools/hmmer/).

PHYLOGENETIC TREE CONSTRUCTION

The predicted amino acid sequences of candidate NB-ARC proteins from *C. papaya* together with well-studied resistance proteins in other plant species were aligned using ClustalW in Mega X (Kumar et al. 2018). The alignment was used for the construction of a phylogenetic tree using Maximum Likelihood (ML) method in MEGA X with a bootstrap value of 500 to validate the tree topology.

ISOLATION OF TRANSCRIPT AND PROTEIN STRUCTURE ANALYSIS

Transcript 4993 of CpNBS1 was amplified from both Eksotika-S and Viorica-T and aligned using BioEdit program version 7.2.6 (Hall 1999). The position of LRR domains in the transcript was confirmed using NCBI CDD. The 3D structure and function of the proteins were predicted by an online server, I-TASSER (Zhang et al. 2016) using default parameters where all templates were included to maintain the quality of the I-TASSER modeling.

EXPRESSION PROFILING OF NB-ARC TRANSCRIPTS IN *C. papaya* CHALLENGED WITH *E. mallotivora*

Total RNA was extracted from the inoculated and control leaves of both Eksotika-S and Viorica-T by using RNeasy Plant Mini kit (Qiagen, Germany) according to the manufacturer's instructions. RNase-free DNase Set (Qiagen, Germany) was used to remove genomic DNA contamination. The integrity of RNA samples was examined through agarose gel electrophoresis and NanoDrop 1000 Spectrophotometer (Thermo Scientific, USA). Then, cDNA was prepared from 2 µg RNA using iScript Reverse Transcription Supermix (BioRad, USA). The expression level of NB-ARC transcripts was determined by semi-quantitative RT-PCR using the eukaryotic initiation factor 4A (*EIF*) gene (Genbank ID: FJ644949.1) as the internal reference. Primer pairs used to amplify all the transcripts were listed in Table 1. A total volume of 25 µL was prepared consisting of 1 µL cDNA, 2× MyTaq Red Mix™ DNA Polymerase (Bioline), 1 µM forward primer, 1 µM reverse primer and sterile water. PCR reaction was carried out as follows: pre-denaturation at 94 °C for 1 min, 27 cycles of denaturation at 94 °C for 30 s, annealing for 30 s, and extension at 72 °C for 30 s. Final elongation was carried out at 72 °C for 10 min.

RESULTS AND DISCUSSION

Plant disease resistance is well-known to be mediated largely by NBS-LRR proteins. The NBS-LRR domains residing in these proteins are essential for the recognition of pathogen effectors in order to activate defense signalling that eventually confer disease resistance in a plant (Lozano et al. 2015). In the present study, three ORFs with NB-ARC or AAA domain that showed the highest expression value were identified from the translated transcriptomic database of Eksotika-S and Viorica-T that have been challenged with *E. mallotivora*. The selected proteins were then named CpNBS1, CpNBS2

and CpNBS3 (Table 2). According to the BlastP analysis, all three sequences were highly homologous to protein from *C. papaya.* Interestingly, *CpNBS1* gene produced three truncated transcripts that matched the same protein in which two of them (transcript 4993 and 4756) are identical. No full-length CpNBS1 transcript was detected in the *E. mallotivora*-infected *C. papaya* transcriptome. The putative RGA3 protein (CpNBS1) also present high sequence identity with putative protein CpRGA1 and its isoforms X1, X2, and X3 (% query cover: 99, 98, 98, 92; % identity: 76, 83, 83, & 81, respectively). It is common for R gene transcripts to be produced in isoforms that contain only one or two domains from the full-length transcripts (Marone et al. 2013; Monteiro & Nishimura 2018). Such alternative transcripts were reported to remain stable and encode truncated proteins that contribute to successful immunity in certain cases of NBS-LRR expression control (He et al. 2018; Lai & Eulgem 2017). It is assumed that these truncated proteins gain their positive role by alleviating self-inhibition of the full-length proteins or by functioning as adaptors in the signal transduction pathway (Baggs et al. 2017). A previous study showed that a single domain of *Pi-ta*, a dominant blast-resistance gene in *O. sativa* called the TRX was sufficient to deliver the highest level of expression compared to full length or other truncated transcripts in a resistant rice variety (Costanzo & Jia 2009).

Genome analysis of *C. papaya* recorded the lowest number of NBS R genes (54), compared to *A. thaliana* (174), *O. sativa* (519) and *Vitis vinifera* (535) (Porter et al. 2009), which might explain the small number of *C. papaya* NBS genes that were responsive to *E. malllotivora* infection.

NBS-LRR proteins are assumed to be cytoplasmic since most of them lack signal peptide or transmembrane domain (Boyes et al. 1998; McHale et al. 2006). Based on the prediction using TMHMM server 2.0 and PSORT software, all the CpNBS proteins are lack of transmembrane region and were located in the cytoplasm. Analysis of conserved domains of the matched protein sequences from NCBI database showed the presence of NB-ARC or AAA domains (Figure 1). Only CpNBS1 contained NB-ARC domain while CpNBS2 and 3 contained AAA domain. According to the information available on the Pfam database (https://pfam.xfam.org/), both NB-ARC and AAA belong to the AAA family proteins under the P-loop_NTPase (CL0023) clan which often function as sensor and response factor. Hence, depending on the search criteria, protein sequences with either domain may or may not be included in the search result as potential disease resistance gene homolog (RGH). A similar case was reported where varying numbers of RGH from the same plant variety were identified in different studies using different search criteria (Sharma et al. 2017).

As the only pathogen-responsive NB-ARC proteins identified in this study, CpNBS1 belongs to the CC-NBS-LRR group of RGH based on the presence of coiled-coil domain at the N terminus and LRR domain at the C terminus (Figure 1). Based on the report by Porter et al. (2009), only six of the 54 NBS genes identified in *C. papaya* were predicted to encode CC motifs, and only four of those genes contain an LRR domain. One ORF in the study, 16.137 was predicted to encode two NBS domains, similar to CpNBS1. However, no sequence information provided by Porter et al. (2009) hindering direct sequence comparison between the two proteins. Transcripts 4993 and 4756 encode for the LRR domain while transcript 4221 does not specifically encode for any domain. The LRR domain with its slender conformation, is considered unique since it can maximizes surface area for protein-protein interaction and tolerate high levels of variability, making it an indispensable player in plant defence. NBS-LRR genes are known to undergo alternative splicing and the presence of alternative transcripts encoding receptors with an absent or truncated LRR domain is an interesting feature of certain members of the class (Padmanabhan et al. 2009). However, there has been no report of the presence of alternative transcript encoding LRR domain alone in response to pathogen infection as observed in this study. To date, ambiguities persist about the mechanistic role of LRR (Noman et al. 2019). Hence, the finding in this study could add a new link in signalling networks for plant innate immunity.

To examine the phylogenetic and evolutionary relationship of NB-ARC proteins from *C. papaya* and other plant species, a phylogenetic tree was constructed which include *A. thaliana*, *Solanum lycopersicum* (tomato), *O. sativa* (rice), *Nicotiana tabacum* (tobacco), *Zea mays* (corn), *Glycine max* (soybean), *Solanum tuberosum* (potato), *Malus domestica* (apple), and *Prunus persica* (peach). It was observed from the phylogenetic tree that CpNBS1 was closely related with CC-NBS-LRR protein I-2 (immunity to race 2) from *S. lycopersicum* and *N. tabacum* while CpNBS2 and CpNBS3 were grouped together with the majority of R genes from *Arabidopsis* (Figure 2). I-2 protein belongs to the Type I R-genes, which had frequent sequence exchanges among its homologues in the different genotypes of the same species (Wei et al.

2014). As a result, a particular plant species may harbor various sets of chimeric R-genes. However, extensive sequence analysis has to be conducted to make a plausible conclusion about CpNBS1.

To confirm the involvement of pathogen-responsive CpNBS transcripts to *E. mallotivora* infection, the transcripts were assayed for their expression patterns in a challenge experiment. The *C. papaya* Eksotika-S and Viorica-T exhibited different disease responses after exposure to dieback pathogen, *E. mallotivora*. Eksotika-S is highly susceptible whereas Viorica-T is tolerant (Mohd Azhar et al. 2020). Both Ekotika-S and Viorica-T were inoculated with *E. mallotivora* and leaf sample was collected at 0, 24, and 48 hpi before the onset of disease symptoms for semi-quantitative PCR. The NBS-LRR genes are finely regulated to ensure the correct dose of immune responses while limiting any detrimental effects on plant growth. Hence, they are basally expressed in plant tissues and only up-regulated upon pathogen detection to initiate defence responses (Lai & Eulgem 2017; Marone et al. 2013; Zhang et al. 2016).

Based on Figure 3, all transcripts were basally expressed in both tolerant and susceptible varieties of *C. papaya*. Transcript 4993 of *CpNBS1* was upregulated at 48 hpi in the susceptible plant in response to *E. mallotivora* whereas in the tolerant plant, it was downregulated. It seems like the reduced level of the transcript is favorable for *C. papaya* when under attack by *E. mallotivora*. Both transcript 3009 and 4999 seemed to respond to wounding based on the up-regulated and down-regulated expressions in the control sample of Eksotika-S and Viorica-T, respectively. It is not uncommon for wound-responsive gene to encode proteins involved in cellular responses to biotic stress since both factors share a number of components in their signalling pathways (Cheong et al. 2002). In response to *E. mallotivora*, the expression was slightly downregulated at 48 hpi in Eksotika-S but upregulated at 24 hpi in Viorica-T, which is opposite to the transcript 4993 of *CpNBS1*. In contrast, transcript 2792 of *CpNBS3* was stably expressed in both susceptible and tolerant *C. papaya*. Transcript of many R genes are known to accumulate transiently in a localized response to pathogen infection and plant defence response is a result of a complex interplay between many proteins and metabolites at different time points (Dang et al. 2019; Goyal et al. 2020; Lai & Eulgem 2017). At this stage, it is possible that the negative regulation of the transcript 4993 of *CpNBS1* and positive regulation of the transcript 3009 of *CpNBS2* genes give rise to the tolerant phenotype of Viorica to *E. mallotivora* infection. Functional characterization of the truncated transcripts in comparison to the full length would shed more knowledge on their exact role during *C. papaya*-*E. mallotivora* interaction.

Based on the sequence analysis and expression profiling, transcript 4993 of *CpNBS1* was selected for further analysis. The transcript sequences were amplified from both Eksotika-S and Viorica-T with the size of 1398 bp. Alignment of the two sequences showed five nucleotide variations at positions 404, 921, 958, 968, and 1041 where nucleotide A in 4993-Eksotika changed to G in 4993-Viorica at all these positions (Figure 4). These nucleotide variations led to differences at three amino acid positions in the translated sequences between the two varieties at position 135 (lysine to arginine), 320 (methionine to valine), and 323 (asparagine to serine) (Figure 5). Interestingly, amino acid variation at position 135 (lysine to arginine) lies in the conserved LRR domain. Even though both arginine and lysine are positively charged polar amino acids (Strickler et al. 2006), the protein function may be affected by the substitution of lysine to arginine owing to the geometric structure of arginine which provides more stability to the protein structure (Sokalingam et al. 2012).

To gain more information on the structure-function of the LRR domain in susceptible and tolerant *C. papaya*, a three-dimensional structure (3D) of the domain was predicted using comparative modelling approach. The structure of the translated protein sequences was predicted using the tertiary structure of LRR receptor-like serine/threonine- protein kinase FLS2 from *Arabidopsis* (PDB ID: 4mn8) as a template with 95% sequence coverage and 28% similarity. Secondary structures of protein like α-helix and β-sheets play an important role in the folding of a protein where α-helix is more favourable for the stability and organization of a protein (Deller et al. 2016; Stanger et al. 2001). Based on the 3D structures in Figure 6, 4993-Viorica is composed of 17 β-sheets and 15 α-helixes (Figure 6(A)) while 4993-Eksotika contains 19 β-sheets and 13 α-helixes (Figure 6(B)) suggesting that the former is more stable.

In addition, there is a remarkable difference in the amino acid residues involved in the ligand binding surface of 4993-Viorica with only seven amino acid residues (aspartate, serine, asparagine, cysteine, tyrosine, leucine, and glutamate at positions 150, 152, 174, 176, 198, 200, 222, respectively) (Figure 7(A)) in comparison to 15 amino acid residues (serine, threonine, glutamate, serine, aspartate, isoleucine, asparagine, histidine,

tyrosine, leucine, threonine, glutamate, valine, histidine, and arginine at position 73, 95, 96, 124, 150, 172, 174, 196, 198, 200, 220, 222, 225, 252, & 254, respectively) in 4993-Eksotika (Figure 7(B)). Similar proteins with different amino acid residues involved in ligand binding are expected to bind different types of ligand (Lee et al. 2017). This is because the amino acids at ligand binding surface will be arranged in a specific conformation that allows them to interact with a ligand that fit the conformation perfectly, similar to the lock-and-key concept in enzyme-substrate interactions. The observed difference between the LRR domain of 4993-Eksotika and 4993-Viorica suggests that the LRR domain may determine the specificity of recognition of *E. mallotivora* effector. In a recent study involving *Pvr4* disease-resistance gene of pepper, LRR domain swapping between the susceptible and resistant *Pvr4* allele showed that the LRR domains of the resistant allele are important for the recognition of avirulence protein NIb from Peper mottle virus (Kim et al. 2018). Both alleles contain an identical NB-ARC domain but a relatively different LRR domain.

TABLE 1. Primers used in this study

Transcript ID/Gene name	Forward	Reverse	Annealing temperature (°C)
RT-PCR			
Ref_Papaya_ Transcript_16838_4993	ACATTTGAATGCTTGGTGGTGG	AGTGGAGGCAACTGCTCAAT	58
Ref_Papaya_ Transcript_39327_3009	CCGGATACCATGGAGAAGCT	CGTAGCCAACCTCATCCAGT	56
Ref_Papaya_ Transcript_38330_2792	GTTCTGGGTTTCTTCCAAAACCA	AGGCTTCCTCCCCAAATCTA	57
EIF	GGGAAGACGCCA GGTGTA	GCTTGGATTTGGCAGAGAAG	58
Transcript isolation			
Ref_Papaya_ Transcript_16838_4993	CCCGGGATGGAAAAAGGTGAGTTGTACTTT	CTCGAGTCACCAAACTTTAAAATGCCAG	55

TABLE 2. List of pathogen-responsive LRR proteins identified in current study

Transcript ID	Gene name	ORF length	Blast results (% query cover, E value, identities, accession no., description, [species])
Ref_Papaya_ Transcript_16838_4993	*CpNBS1*	474	100, 0.0, 100, XP_0121904876.1, putative disease resistance protein RGA3 [*Carica papaya*]
Ref_Papaya_ Transcript_16838_4756		474	100, 0.0, XP_0121904876.1, putative disease resistance protein RGA3 [*Carica papaya*]
Ref_Papaya_ Transcript_16835_4221		297	95, 0.0, 100, XP_0121904876.1, putative disease resistance protein RGA3 [*Carica papaya*]
Ref_Papaya_ Transcript_39327_3009	*CpNBS2*	537	100, 0.0, 99.07, XP_021897305.1, cell division cycle protein 48 homolog [*Carica papaya*]
Ref_Papaya_ Transcript_38330_2792	*CpNBS3*	685	100, 0.0, 100, XP_021897864.1, uncharacterized protein ycf45 [*Carica papaya*]

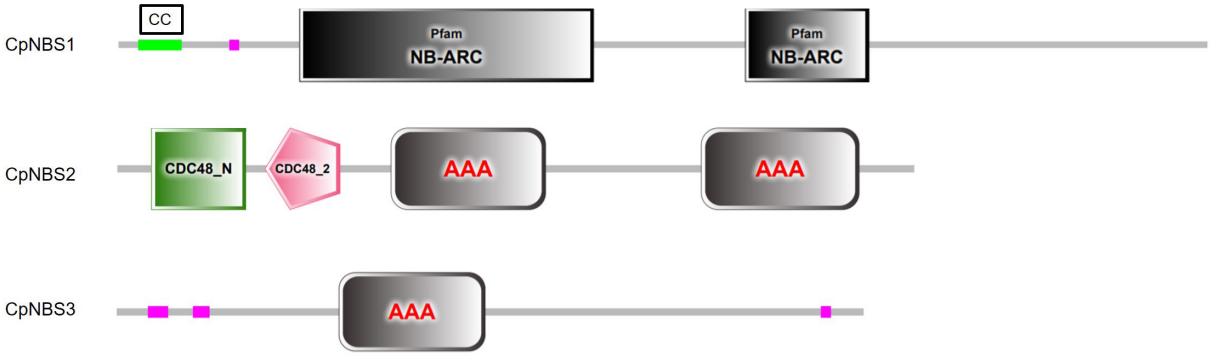

FIGURE 1. Domain structure of CpNBS1, CpNBS2 and CpNBS3 proteins analysed using SMART. Only confidently predicted domains, repeats, motifs and features are shown. Purple box indicates a low complexity region

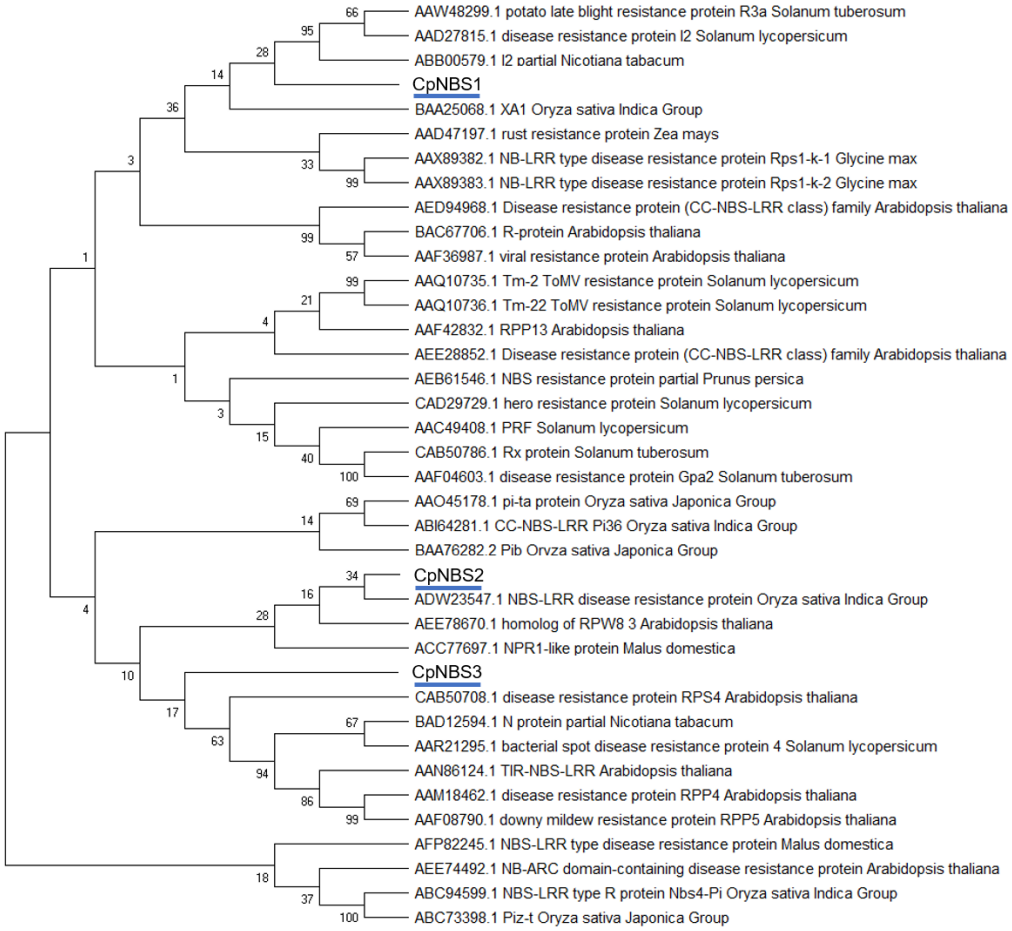

FIGURE 2. Phylogenetic tree showing relationships of LRR proteins between *C. papaya* and well-studied resistance proteins in other plant species. The LRR proteins from *C. papaya* identified in this study were denoted with a blue underline. The unrooted phylogenetic tree was constructed with the maximum likelihood (ML) tree. Tree reliability was assessed using 500 bootstrap replicates. Bootstrap percentages are indicated at branches

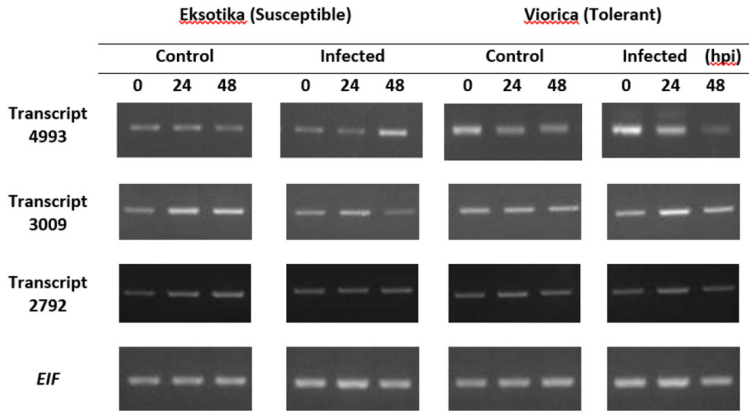

FIGURE 3. Modulation of NBS-LRR transcripts expression by *E. mallotivora* in *C. papaya* cv. Viorica and Eksotika. The semi-quantitative expression was recorded at 0, 24, and 48 h post-inoculation (hpi) with *E. mallotivora*. Eukaryotic initiation factor 4α (*EIF*) was used as the internal reference gene

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                10        20        30        40        50        60        70        80        90       100
Eksotika  ATGGAAAAAGGTGAGTTGTACTTTAATAATTTGGCTATGCGCTCCTTTTTCCAAGACTTCAGAAAACATAAAACCAATTTAGCATGCAAGATGCATGATG
Viorica   ATGGAAAAAGGTGAGTTGTACTTTAATAATTTGGCTATGCGCTCCTTTTTCCAAGACTTCAGAAAACATAAAACCAATTTAGCATGCAAGATGCATGATG

               110       120       130       140       150       160       170       180       190       200
Eksotika  TAGTGCATGATTTTGCTAAACATTTGACAAAAAATGAATTTTTTTTTAGTAAACTCTGCTAATAATGAAGCGTGTGATAATGAATTATATGAAAAAGTGCG
Viorica   TAGTGCATGATTTTGCTAAACATTTGACAAAAAATGAATTTTTTTTTAGTAAACTCTGCTAATAATGAAGCGTGTGATAATGAATTATATGAAAAAGTGCG

               210       220       230       240       250       260       270       280       290       300
Eksotika  TCATTCAATGATACTTTCTCGGTCAGATTTACCATTTCCTGTCTCTGTTTACAAGTTAAAGAAGGTACGAAGCCTCTTGCTTACGGAGCTTATGCGGAGA
Viorica   TCATTCAATGATACTTTCTCGGTCAGATTTACCATTTCCTGTCTCTGTTTACAAGTTAAAGAAGGTACGAAGCCTCTTGCTTACGGAGCTTATGCGGAGA

               310       320       330       340       350       360       370       380       390       400
Eksotika  AACTCTGAGACCGAAAAACATCTAGTTGATTTATGTGGTCAATTGACATGCCTTAGGTCATTGGATTTGAGTGTGCATATTAACTGTAGTAATTCACTGA
Viorica   AACTCTGAGACCGAAAAACATCTAGTTGATTTATGTGGTCAATTGACATGCCTTAGGTCATTGGATTTGAGTGTGCATATTAACTGTAGTAATTCACTGA

               410       420       430       440       450       460       470       480       490       500
Eksotika  GTAAACTTCCGAAAGGGATAGAACAACTGATACATTTGAGACTTCTTGACTTGTCTTCGAATACAGCCCTAAGAGTATTACCTGAATCTTTGTGTCATTT
Viorica   GTAGACTTCCGAAAGGGATAGAACAACTGATACATTTGAGACTTCTTGACTTGTCTTCGAATACAGCCCTAAGAGTATTACCTGAATCTTTGTGTCATTT

               510       520       530       540       550       560       570       580       590       600
Eksotika  ATATAATCTTCAAATCCTGAATATTTGTTGTTGTCTTTTTCTTAGAGGACTTCCTCATGGGATGGAAAAACTAATCAACTTGAGGCATTTATATAACTTG
Viorica   ATATAATCTTCAAATCCTGAATATTTGTTGTTGTCTTTTTCTTAGAGGACTTCCTCATGGGATGGAAAAACTAATCAACTTGAGGCATTTATATAACTTG

               610       620       630       640       650       660       670       680       690       700
Eksotika  GGAACTTATAAGCTAACCTTTATTCCCAAAGGGTTGGGGAGATTAACTTCTCTTAGCACATTTGAATGCTTGGTGGTGGGAAGTAATAATAATGATGGTG
Viorica   GGAACTTATAAGCTAACCTTTATTCCCAAAGGGTTGGGGAGATTAACTTCTCTTAGCACATTTGAATGCTTGGTGGTGGGAAGTAATAATAATGATGGTG

               710       720       730       740       750       760       770       780       790       800
Eksotika  AAGCTATGACTCTAGGGGATCTGCAACAACTAAACAACCTTAAGGGAACTCTCCACATTAGAGGACTAGGAAATGTGAAAGATGTCCTGGAGGCTACAAA
Viorica   AAGCTATGACTCTAGGGGATCTGCAACAACTAAACAACCTTAAGGGAACTCTCCACATTAGAGGACTAGGAAATGTGAAAGATGTCCTGGAGGCTACAAA

               810       820       830       840       850       860       870       880       890       900
Eksotika  TGCTCAGCTTCTAAATAAGAAACATCTTCGTGACTTGCAGCTTAGATTTGATGGACTGATAACAGACGCAGAGAGAATTGATGATCTTCTTCTTGAAGCC
Viorica   TGCTCAGCTTCTAAATAAGAAACATCTTCGTGACTTGCAGCTTAGATTTGATGGACTGATAACAGACGCAGAGAGAATTGATGATCTTCTTCTTGAAGCC

               910       920       930       940       950       960       970       980       990      1000
Eksotika  CTGCAACCACCTTCGGACTTAGAAATTTTAGAAATTACTGATTATATAGGCCCTACTATGTGTCCAAATTGGTTAATGCAACTGCCAAGGCTAAGGAAGC
Viorica   CTGCAACCACCTTCGGACTTGGAAATTTTAGAAATTACTGATTATATAGGCCCTACTGTGTGTCCAAGTTGGTTAATGCAACTGCCAAGGCTAAGGAAGC

              1010      1020      1030      1040      1050      1060      1070      1080      1090      1100
Eksotika  TTACACTTGGGTCGTTCATAAATATTGAGCAGTTGCCTCCACTTGGGATCCTGCCGGGTCTCCAGGTGCTCAAGATTAGAAACATGCCTAAAGTGAAAAA
Viorica   TTACACTTGGGTCGTTCATAAATATTGAGCAGTTGCCTCCGCTTGGGATCCTGCCGGGTCTCCAGGTGCTCAAGATTAGAAACATGCCTAAAGTGAAAAA

              1110      1120      1130      1140      1150      1160      1170      1180      1190      1200
Eksotika  ACTGGGCCTTGAGTTTCTAGGAATAGGCACTCTTCCATTTTCATCTTCTGTTGCTGCCTTCCCTCAACTCCAAAAGCTTCATTTTAGTAACATGAAAAAC
Viorica   ACTGGGCCTTGAGTTTCTAGGAATAGGCACTCTTCCATTTTCATCTTCTGTTGCTGCCTTCCCTCAACTCCAAAAGCTTCATTTTAGTAACATGAAAAAC

              1210      1220      1230      1240      1250      1260      1270      1280      1290      1300
Eksotika  TGGGAAGAGTGGGATTTTGAAATCACAACAGGAACTGTTCATCATAGTAATAGTAGTACCAGTAGCTGCATATTTATGGTAATGCCACGCCTCAGTTATT
Viorica   TGGGAAGAGTGGGATTTTGAAATCACAACAGGAACTGTTCATCATAGTAATAGTAGTACCAGTAGCTGCATATTTATGGTAATGCCACGCCTCAGTTATT

              1310      1320      1330      1340      1350      1360      1370      1380      1390
Eksotika  TGCTCATTCAGAACTGTGACAAGTTGAAGGAATTAAAACAGCCTTACGAGGATTTAAGAAAAGACGGTCTTATACGCTGGCATTTTAAAGTTTGGTGA
Viorica   TGCTCATTCAGAACTGTGACAAGTTGAAGGAATTAAAACAGCCTTACGAGGATTTAAGAAAAGACGGTCTTATACGCTGGCATTTTAAAGTTTGGTGA
```

FIGURE 4. Nucleotide sequence alignment of transcript 4993 isolated from *C. papaya* cv. Eksotika and Viorica. Nucleotide variations at positions 404, 921, 958, 968, and 1041 are indicated by grey-shaded letters

```
                10        20        30        40        50        60        70        80        90        100
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Eksotika        MEKGELYFNNLAMRSFFQDFRKHKTNLACKMHDVVHDFAKHLTKNEFFLVNSANNEACDNELYEKVRHSMILSRSDLPFPVSVYKLKKVRSLLLTELMRR
Viorica         MEKGELYFNNLAMRSFFQDFRKHKTNLACKMHDVVHDFAKHLTKNEFFLVNSANNEACDNELYEKVRHSMILSRSDLPFPVSVYKLKKVRSLLLTELMRR

                110       120       130       140       150       160       170       180       190       200
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Eksotika        NSETEKHLVDLCGQLTCLRSLDLSVHINCSNSLSKLPKGIEQLIHLRLLDLSSNTALRVLPESLCHLYNLQILNICCCLFLRGLPHGMEKLINLRHLYNL
Viorica         NSETEKHLVDLCGQLTCLRSLDLSVHINCSNSLSRLPKGIEQLIHLRLLDLSSNTALRVLPESLCHLYNLQILNICCCLFLRGLPHGMEKLINLRHLYNL
                                                  *
                210       220       230       240       250       260       270       280       290       300
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Eksotika        GTYKLTFIPKGLGRLTSLSTFECLVVGSNNNDGEAMTLGDLQQLNNLKGTLHIRGLGNVKDVLEATNAQLLNKKHLRDLQLRFDGLITDAERIDDLLLEA
Viorica         GTYKLTFIPKGLGRLTSLSTFECLVVGSNNNDGEAMTLGDLQQLNNLKGTLHIRGLGNVKDVLEATNAQLLNKKHLRDLQLRFDGLITDAERIDDLLLEA

                310       320       330       340       350       360       370       380       390       400
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Eksotika        LQPPSDLEILEITDYIGPTMCPNWLMQLPRLRKLTLGSFINIEQLPPLGILPGLQVLKIRNMPKVKKLGLEFLGIGTLPFSSSVAAFPQLQKLHFSNMKN
Viorica         LQPPSDLEILEITDYIGPTVCPSWLMQLPRLRKLTLGSFINIEQLPPLGILPGLQVLKIRNMPKVKKLGLEFLGIGTLPFSSSVAAFPQLQKLHFSNMKN
                                  * *
                410       420       430       440       450       460
                ....|....|....|....|....|....|....|....|....|....|....|
Eksotika        WEEWDFEITTGTVHHSNSSTSSCIFMVMPRLSYLLIQNCDKLKELKQPYEDLRKDGLIRWHFKVW
Viorica         WEEWDFEITTGTVHHSNSSTSSCIFMVMPRLSYLLIQNCDKLKELKQPYEDLRKDGLIRWHFKVW
```

FIGURE 5. Amino acid sequence alignment of 4993 translated sequence from *C. papaya* cv. Eksotika and Viorica. Conserved LRR domains are underlined in red. Amino acid variations at positions 135, 320, and 323 are indicated by an asterisk and coloured letters

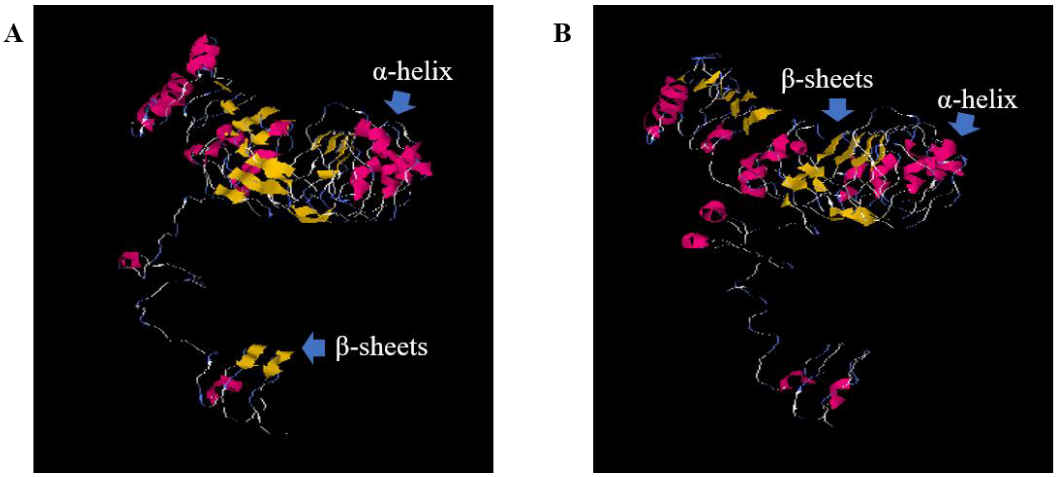

FIGURE 6. The molecular modelling of 4993 translated sequence using I-TASSER software. A) Structural 3D model of 4993 translated sequence from *C. papaya* cv. Viorica with a TM score of 0.67+-0.13 and Cs of −1.14 and B) Structural 3D model of 4993 translated sequence from *C. papaya* cv. Eksotika with a TM score of 0.68+-0.12and Cs of −0.25. Secondary structure features, β-sheets (yellow) and α-helix (pink) are indicated by a blue arrow

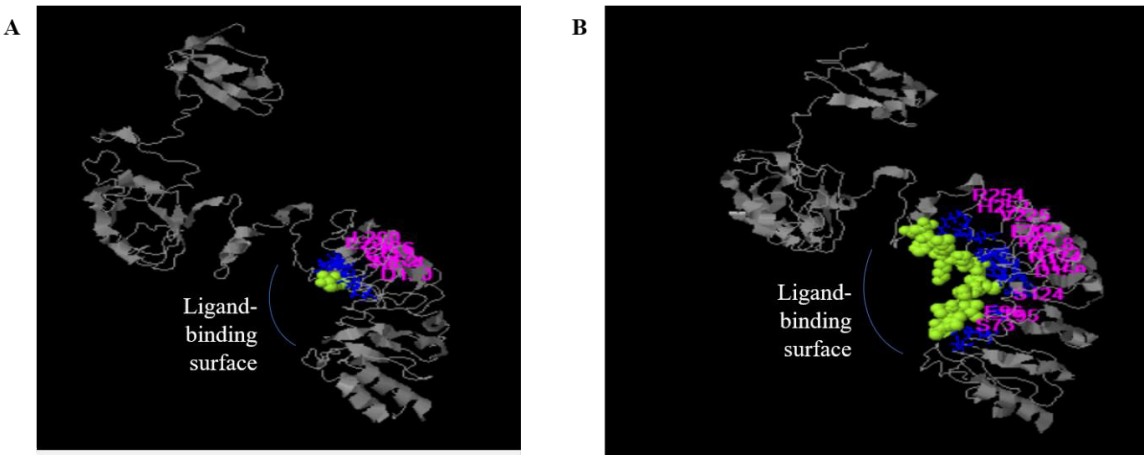

FIGURE 7. Predicted amino acid residues involved in the ligand binding surface of 4993 translated sequence. A) Ligand binding surface of 4993 translated sequence from *C. papaya* cv. Viorica and B) Ligand binding surface of 4993 translated sequence from *C. papaya* cv. Eksotika. The amino acid residues involved are denoted in purple

CONCLUSION

In the present study, LRR-encoding transcripts (4993, 4756, and 4221) were identified in *C. papaya* translated transcriptome database of Eksotika (susceptible) and Viorica (tolerant) in response to bacterial pathogen *E. mallotivora*. The matching *C. papaya* proteins were named CpNBS1, CpNBS2, and CpNBS3, respectively. Despite truncated, two of the identified transcripts, 4993 and 4756 were identical and encode a complete LRR domain. CpNBS1 which is annotated as resistance gene analog 3 (RGA3) is of particular interest since it belongs to the CC-NBS-LRR class of the R gene. Semi-quantitative PCR showed that transcripts 4993 and 4576 were differentially expressed in response to *E. mallotivora*. Structural analysis of the 4993-Eksotika and 4993-Viorica translated proteins showed striking differences in the number of β-sheets and α-helixes as well their ligand-binding surface, suggesting the role of the LRR domain in determining the specificity of recognition of *E. mallotivora* effector. Overall, this study provides new insight into the role of NBS-LRR gene in papaya that could have implications in enhancing of plant disease resistance through genetic engineering.

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