Available online at www.ijpab.com

DOI: http://dx.doi.org/10.18782/2320-7051.6644

ISSN: 2320 – 7051

Int. J. Pure App. Biosci. **6 (3):** 137-145 (2018)

Research Article

International Journal of Pure & Applied Bioscience

OPEN ACCESS

Characterization, Structural Modeling and Docking Study of CnCNLR1, A CC-NBS-LRR Protein from Coconut

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Received: 22.05.2018 | Revised: 19.06.2018 | Accepted: 26.06.2018

ABSTRACT

Coconut, a versatile crop of the family Arecaceae, is highly susceptible to bacterial and fungal pathogens. The identification of resistance gene analogues (RGAs) holds great promise for development of disease resistant coconut cultivars. Plant NBS-LRR genes, a class of RGAs, trigger multifaceted defense responses by recognizing several conserved microbial/pathogen associated molecular patterns. In our previous studies, different classes of RGAs were identified from coconut root (wilt) resistant leaf transcriptome and also by comparative genomics approach. CnCNLR1, a coiled-coil nucleotide-binding-site-leucine-rich-repeat (CC-NBS-LRR) R-gene, was found up-regulated in healthy palms in comparison to root (wilt) disease affected palms. In the present study, we have predicted the primary, secondary and 3D (three dimensional) structure of CnCNLR1 based on threading. In addition, docking interaction of CnCNLR1 with the ligand β-(1, 3)-D-glucan was also predicted. These predictions suggested that two amino acid residues viz.; Tyr250 and Gly247 *act as catalytic residues and are involved in hydrogen bonding with the ligand. Elucidation of the molecular structure will help to provide a deeper understanding of the structural basis of ligand binding to CnCNLR1 protein, thus paving ways for development of novel strategies for disease control in coconut palm.*

Key words: *Coconut, Disease resistance, CC-NBS-LRR, β-(1,3)-D-glucan, Threading, Docking*

INTRODUCTION

Plants have been endowed with an elaborate and complex system during evolution to resist themselves against invasion of phytopathogens. The classic 'gene-for-gene' concept of plant disease resistance involves two processes: perception of the pathogen attack, followed by responses to limit disease spread[1]. Perception of pathogens, determined by disease resistance genes (*R* genes), involves receptors possessing a high degree of specificity. Effective defense is characterized by a model wherein the products of *R* genes act as receptor for the direct or indirect products of pathogen avirulence *(Avr)* genes[2]. Plants, therefore, can trigger highly effective inducible defense responses, comprised of hypersensitive response[3], tissue reinforcement and production of phytoalexins at the site of infection[4].

Cite this article: Rachana, K.E., Gangaraj, K.P. and Rajesh, M.K., Characterization, structural modeling and docking study of CnCNLR1, a CC-NBS-LRR protein from coconut, *Int. J. Pure App. Biosci.* **6(3):** 137-145 (2018). doi: http://dx.doi.org/10.18782/2320-7051.6644

A number of disease resistance *(R)* genes or resistance gene analogues (RGAs), which confer resistance to diverse pathogens, have been cloned and isolated from several plant species by map-based cloning or transposon tagging[5]. Amino acid sequence resemblances among these R proteins have clearly revealed a certain degree of structural similarity and conservation of specific domains. These conserved domains in R proteins participate in pathogen recognition, protein-protein interactions and activate signal transduction cascades[6,7]. The *R* genes have been categorized into eight classes based on the structural similarities of their predicted protein products[8]. Most of the *R* genes contain a nucleotide binding site (NBS) firmly attached to a C- terminal leucine rich repeat (LRR) of flexible length. Such genes are called NBS-LRR genes and represent the largest *R* gene class in plant genome[9]. The presence of NBS domain are the characteristic of various ATP or GTP binding proteins as they are highly essential for the catalytic activity, functioning directly in ATP and GTP binding[10] and generally comprise of P-loop, kinase-2α, kinase-3α and GLPL motifs[11]. NBS-LRR class *R* genes are further subdivided into TIR-NBS-LRR and CC-NBS- LRR groups, depending on the presence or absence of an N-terminal domain. TIR-NBS-LRR genes are characterized by a N-terminal TIR (Toll/interleukin-1 receptor) domain[12], whereas CC-NBS- LRR genes contain a leucine zipper coiled-coil domain instead of an N-terminal TIR domain[13].

Coconut (*Cocos nucifera* L.; family Arecaceae) is an important and useful palm possessing high economic significance. Coconut yield is in decline in many countries with pathogens (bacterial, fungal and phytoplasma) causing major yield losses. Due to several constraints associated with the utilization of chemicals against disease control, development of disease resistant cultivars could be the most likely solution for sustaining yields. In our previous studies, different classes of RGAs were predicted in coconut viz., from coconut root (wilt) resistant leaf transcriptome[14] and through comparative genomic approach[15]. *CnCNLR1*, a coiled-coil nucleotide-binding-site-leucine-rich-repeat (CC-NBS-LRR) *R*-gene, was found to be up-regulated in healthy palms compared to palms affected by root (wilt) disease.

It is essential to determine the three dimensional (3D) model of CnCNLR1 to assess its structural integrity and biological relevance. A modeling based protein structure prediction of CnCNLR1 could be an efficient strategy taking into account the time consuming and cumbersome nature of X-ray diffraction or nuclear magnetic resonance spectroscopy (NMR)[16]. Several reports have suggested that various *R* genes and pathogenesis related proteins could play major roles in detection as well as controlling the spread of phytopathogens, mainly as a part of plant defense signaling machinery[17]. B-(1,3)-D-glucan, the major cell wall constituents of phytopathogenic oomycetes, often acts as elicitors of defence reactions and serve as pathogen associated molecular pattern (PAMP)[18]. Recognition of these PAMPs by *R* genes are crucial for the activation of defense reactions in plants. The present study was carried out to comprehensively characterize CnCNLR1 using computational tools, predict its 3D structure based on threading and to predict its interaction with β-(1, 3)-D-glucan through docking studies.

MATERIAL AND METHODS

Data set and domain analysis

The complete coding sequence of CnCNLR1 disease resistance protein has been deposited by us in NCBI (Accession no. MH500085). The major domains present in CnCNLR1 protein were confirmed through Conserved Domain Search Service (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and Pfam database (https://pfam.xfam.org/) search.

Primary sequence analysis

The various chemical and physical parameters of predicted protein sequence of CnCNLR1was computed by online server ProtParm (http://web.expasy.org/protparam/).

The computed parameters include the molecular weight, theoretical pI, amino acid composition, atomic composition, extinction coefficient, instability index, aliphatic index and grand average of hydropathicity (GRAVY).

Prediction of secondary structure

Secondary structure prediction was done by using by GOR (ver. 1.1)[19], SOPMA[20] and PSIPRED[21].

3-Dimensional modeling of CnCNLR1

The protein structure of CnCNLR1 was predicted through threading method[22] since homology-based modeling could not be undertaken due to lack of homologues structures in PDB. SPARKS-X (http://sparks-lab.org/yueyang/server/SPARKS-X/) and MUSTER (https://zhanglab.ccmb.med.umich.edu/MUSTER/) were utilized to undertake threading. The predicted structures were visualized and analyzed by using RasMol[23]. Rampage server was then used to validate the predicted structures (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php). The best structure was then taken for further refinement using Mod-Refiner (http://zhanglab.ccmb.med.umich.edu/ModRefiner/), an algorithm for atomic-level, high-resolution protein structure refinement. The refined structure was visualized in PyMOL (https://pymol.org/2/) and Ramachandran plot was obtained through the protein preparation wizard module in Schrödinger package.

Docking studies

Docking studies were performed in order to determine the binding affinity of the ligand β-(1,3)-D-glucan on to the CnCNLR1 protein. The chemical structure of the ligand β-(1,3)-D-glucan was obtained from chemical Book (https://www.chemicalbook.com/ProductChemicalPropertiesCB2683841_EN.htm) and the 9012-72-0.mol file were opened in PyMOL and converted as 9012-72-0.pdb file. The refined structure of CnCNLR1 protein, obtained in the previous step, was used for docking with β-(1,3)-D-glucan. Molecular docking simulations were carried out using AutoDock Vina software (http://vina.scripps.edu/index.html) utilizing Lamarckian Genetic Algorithm. Grids were generated by using grid option in AutoDock Tools-1.5.6 (grid= x:144; y:116; z116) and grid centre was described as -90.82, 80.61 and -102.84 along with X, Y and Z-axis. The best conformation was then identified and the ligand-protein complexes were analyzed using Maestro environment (Schrödinger package). The number of hydrogen bonds and bond lengths were also calculated.

RESULTS AND DISCUSSION

Primary sequence analysis

The primary sequence analysis, using ProtParm, revealed that the predicted molecular weight of CnCNLR1 was found to be 137.07 kDa. The total number of negatively charged residues (Asp + Glu) and positively charged residues (Arg + Lys) was found to be 158 and 146 respectively. Instability index of the protein, which gives an indication of the protein stability, was found to be 47.92, thus classifying the protein as an unstable one. Aliphatic index of the protein, which is defined as the relative volume occupied by aliphatic side chains, was found to be 105.01; this provides details on the thermal stability of effector protein. Grand average of hydropathy (GRAVY) of of CnCNLR1 was -0.166 and the theoretical pI was 6.35. The extinction coefficient 142680, Abs 0.1% (=1 g/l) was 1.041, assuming all pairs of Cys residues form cystines and extinction coefficient 141180 Abs 0.1% (=1 g/l) was 1.030, assuming all Cys residues are reduced). Atomic composition of the protein sequence was seen to comprise of 6154 carbon atoms, 9813 hydrogen atoms, 1661 nitrogen atoms, 1765 oxygen atoms and 55 sulfur atoms.

Domain analysis

CnCNLR1 protein from coconut was found to comprise of 1192 predicted amino acids, possessing CC domain (residues 29-151), NB-ARC domain (residues 229 to 492) and LRR domain (residues 606-1080) (Table 1). Similar plant R-protein have been reported to comprise of a N-terminal effector domain, behind which there is a STAND (signal transduction

ATPases with numerous domains) family NTPase domain, and a series of LRRs constituting the sensor domain[24]. The effector domain of CnCNLR1 was seen to comprise of the coiled coil (CC) domain. The LRR repeats of the sensor domain chiefly function as an vital phytopathogen sensing machinery[24]. The conserved motif 'EDIVD', which has been reported to be present in CC domains of all CC-NBS-LRR proteins, was found in CnCNLR1 too (data not shown). Mutations in this motif has been shown to cause perturbations in the intramolecular interactions between NBS and LRR domains and cause diminished resistance response to pathogen attack[25]

Table 1: Details of predicted domains in CnCNLR1 protein

Sl. No.	Domain name	Description	Interval
1	RX-CC_like	Coiled-coil domain of the potato virux X resistance protein and similar proteins	29-151
2	NB-ARC super family	NB-ARC domain	229-492
3	LRR	Leucine-rich repeat (LRR) protein [Transcription]	606-1080

Analysis of secondary structure

The comparative results of the secondary structure of CnCNLR1, as predicted by SOPMA, GOR and PSIPRED, as provided in Table 2. CnCNLR1 was seen primarily to comprise of alpha helices, random coils and extended strands.

Table 2: Secondary structure of CnCNLR1, as predicted by SOPMA, GOR and PSIPRED

Secondary structure	SOPMA	GOR	PSIPRED
Alpha helix (Hh)	689 (57.80%)	579 (48.57%)	768 (64.4%)
3_{10} helix (Gg)	0 (0.00%)	0 (0.00%)	0 (0.00%)
Pi helix (Ii)	0 (0.00%)	0 (0.00%)	0 (0.00%)
Beta bridge (Bb)	0 (0.00%)	0 (0.00%)	0 (0.00%)
Extended strand (Ee)	72 (6.04%)	170 (14.26%)	145 (12.2%)
Beta turn (Tt)	0 (0.00%)	0 (0.00%)	0 (0.00%)
Bend region (Ss)	0 (0.00%)	0 (0.00%)	0 (0.00%)
Random coil (Cc)	431 (36.16%)	443 (37.16%)	279 (23.4%)
Ambiguous states (?)	0 (0.00%)	0 (0.00%)	0 (0.00%)
Other states	0 (0.00%)	0 (0.00%)	0 (0.00%)

3-Dimensional modeling of CnCNLR1

The 3D structure of CnCNLR1 was predicted utilizing threading procedure with SPARKS-X and MUSTER programmes. The confidence score (Z score) of the template (CnCNLR1) and queries using both programmes, as can be seen from Tables 3 and 4, was more than 7.5; therefore matching profiles can be considered significant. Model2 in SPARKS-X showed more significance among the predicted structures as per the validation results, in comparison to other models, and was selected for further analysis. The refined structure is shown in Figure 1 and its Ramachandran plot in Figure 2.

Table 3: Structural summary and Ramachandran plot validation details of predicted structures of CnCNLR1 using SPARKS-X server

Model	Template	Z score	Structural details				Ramachandran plot validation		
			Group	Helices	Strands	Turns	Favoured region	Allowed region	Outlier region
Model1	2a0zA	10.20	1192	39	32	142	1080 (90.8%)	86 (7.2%)	24 (2.0%)
Model2	4z0cA	10.78	1192	41	41	156	1092 (91.8%)	80 (6.7%)	18 (1.5%)
Model3	3w3gA	9.49	1192	46	34	148	1067 (89.7%)	91 (7.6%)	32 (2.7%)
Model4	2a5yB	13.37	1192	40	34	150	1069 (89.8%)	85 (7.1%)	36 (3.0%)
Model5	4kxfK	9.69	1192	45	37	137	1069 (89.8%)	87 (7.3%)	34 (2.9%)

Table 4: Structural summary and Ramachandran plot validation details of predicted structures of CnCNLR1 using MUSTER server

Model	Template	Z score	Structural details				Ramachandran plot validation		
			Group	Helices	Strands	Turns	Favoured region	Allowed region	Outlier region
Model1	2a0zA	9.066	872	0	6	111	763 (87.7%)	80 (9.2%)	27 (3.1%)
Model2	4z0cA	8.877	872	6	24	114	756 (86.9%)	88 (10.1%)	26 (3.0%)
Model3	3w3gA	8.488	872	16	38	130	754 (86.7%)	84 (9.7%)	32 (3.7%)
Model4	2z64B	8.525	872	8	39	111	764 (87.8%)	78 (9.0%)	28 (3.2%)
Model5	5gmfA	8.450	872	18	36	132	744(85.5%)	91(10.5%)	35(4.0%)

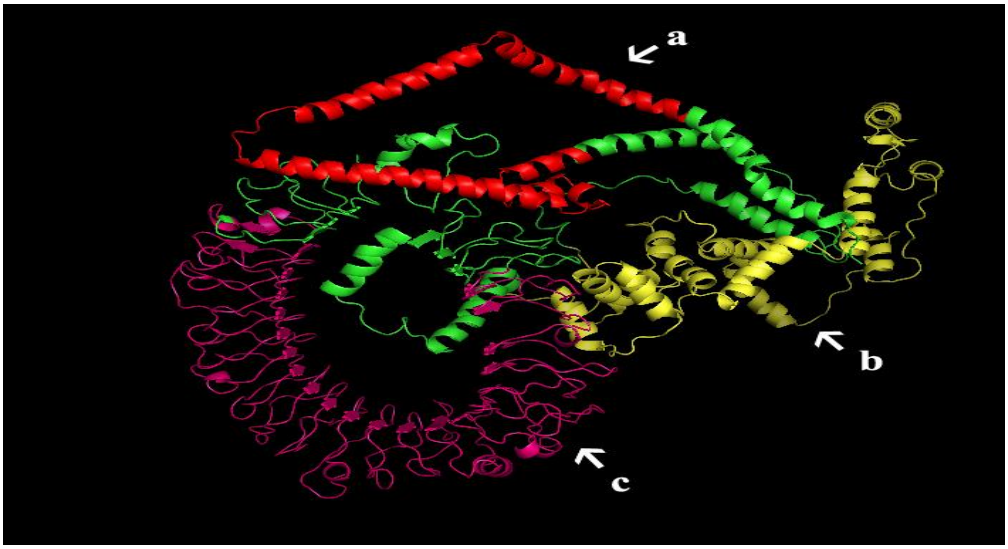

Fig. 1: Predicted 3D structure of CnCNLR1 protein. a) coiled coiled domain, b) NB-ARC domain, c) LRR domain

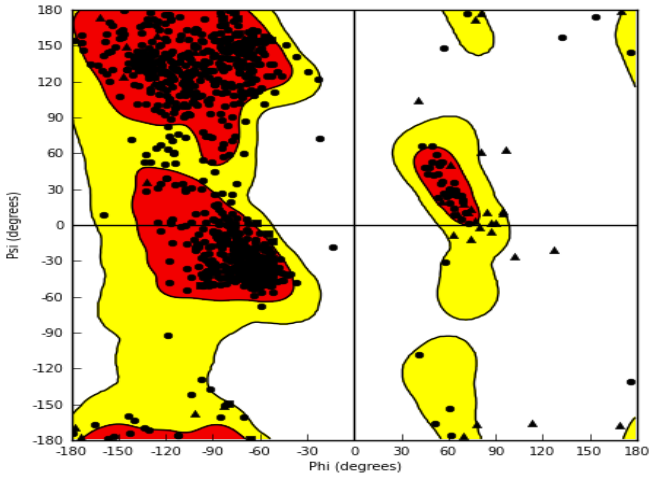

Fig. 2: Ramachandran plot for the refined 3D structure of CnCNLR1 protein

The NBS domain has been recognized in plant NBS-LRR proteins on the basis of its close resemblance to the homologous sequences in the animal APAF-1 and CED-4 proteins[26]. The NBS domain is characterized by NTPase activity- it has been reported that this domain assays a vital role as a molecular switch activating signal transduction which occurs due to changes in its conformation as a result of reversible nucleotide binding, leading to activation/deactivation of the whole receptor[27]. The coiled-coil (CC) domain has been reported to be composed of two or more α-helices, which are, interestingly, twisted super-helically around each other in both parallel- and anti-parallel orientations[27].

R proteins possessing CC domain, and which can bind to target proteins of pathogen effectors, have been well characterized. One such example is the CC domain of *Arabidopsis thaliana* RPS5 protein which has been shown to interact with PBS1, a target of *Pseudomonas syringae* effector AvrPhB[28]. In a recent study, rice *Panicle blast 1* gene, which confers a durable non-race-specific blast resistance and encodes CC-NB-LRR protein, has been shown to specifically interact with a WRKY transcription factor (belonging to the rice salicylic acid signalling pathway). This interaction, which is mediated through its CC domain, is vital for conferring blast resistance[29]. It is envisaged that CnCNLR1 might also be involved in recognition and binding to target proteins of pathogen effectors, given the similar domains and structure shared with other plant CC-NBS-LRR proteins.

Tertiary structure of a single LRR domain is typically a horse shoe-shaped superhelix, as seen in Fig. 1, with individual repeats forming other coils of the superhelix. It is presumed that LRR domains constitute an arena for protein-protein interactions[27]. The inner surface of horseshoe-shaped structure of LRR is usually comprised of parallel β-strands, which is composed of hydrophobic, aliphatic residues. The inner surface could be presumed to be a site of precise interaction with other proteins and with respect to plant R proteins, this could be the specific site for recognition of elicitors secreted by phytopathogens[27]. The outer part of the domain, which is generally usually made up of α -helices, is linked by β-strands and β-turns[30].

Docking studies

The interaction of β-(1, 3)-D-glucan molecule with CnCNLR1 protein was evaluated through molecular docking analysis. The best binding affinity was found to be -6.8 Kcal/mol with two hydrogen bonds: (i) hydrogen bond between oxygen in Tyr250 and hydrogen in ligand at a distance of 2.14 A^o and (ii) hydrogen bond between nitrogen in Gly247 and hydrogen in ligand molecule at a distance of 2.11 A^o (Figure 3). The ligand interaction diagram of β-(1, 3)-D-glucan molecule - CnCNLR1 protein complex is given in Figure 4.

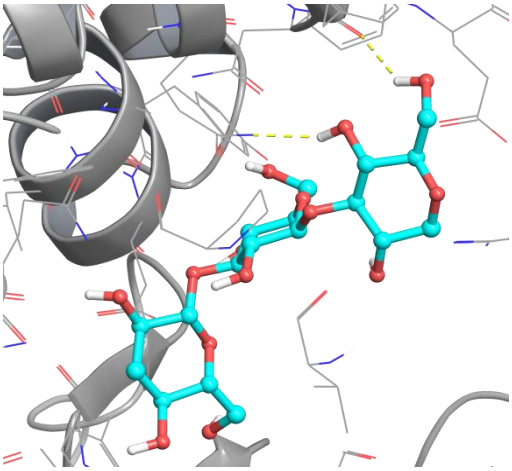

Fig. 3: Interaction complex formed between CnCNLR1 protein and β-(1, 3)-D-glucan

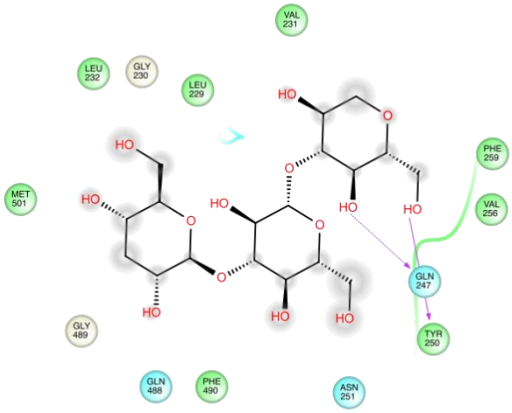

Fig. 4: Ligand interaction diagram of CnCNLR1and β-(1, 3)-D-glucan comp

CONCLUSION

Plant disease resistance genes (*R*-genes) are in the forefront of plant defence mechanism as vital mechanisms towards detection of the pathogen effectors and subsequent activation of the plant defense response. In the present study, we have characterized a CC-NBS-LRR *R*-gene CnCNLR1 in coconut utilizing computational tools and discussed the possible function of the *R*-gene encoded protein in regulating defense mechanism in coconut. Structural analysis revealed the presence conserved domains viz., coiled coil, NB-ARC and LRR domains, found typically in plant CC-NBS-LRR proteins. Molecular docking analysis predicted high affinity between CnCNLR1 and (1, 3)-β-D-glucan, a major component of the oomycete cell wall with favorable hydrogen bonding of two negatively charged amino acid residues Tyr250 and Gly 247 within the docked complex. We hope that comprehensive characterization of R proteins in coconut will pave the way towards elucidation of disease resistance mechanism in coconut and lead to design of novel disease control strategies.

Acknowledgements

The authors would like to thank Indian Council of Agricultural Research (ICAR) and Department of Biotechnology, Government of India for funding (Distributed Information sub-Centre).

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