International Journal of Agriculture Innovations and Research
Volume 6, Issue 3, ISSN (Online) 2319-1473
Manuscript Processing Details (dd/mm/yyyy):
Received: 15/11/2017 | Accepted on: 22/11/2017 | Published: 30/11/2017

Cloning of a Non - TIR NBS LRR type Resistance Gene Analogue (RGA) from Finger Millet

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Abstract **– Finger millet or ragi (*Eleusine coracana*) is a nutrient-rich crop suited to semi-arid climates. NBS-LRR proteins, coded by a large group of potential resistance (R) genes that have conserved structural domains, have specific roles in host-pathogen interactions. Degenerate oligonucleotide primers designed from the conserved domains of nucleotide binding site (NBS) were used in PCR to amplify resistance gene analogues (RGAs) from finger millet, a genomically under-exploited crop. The PCR reaction amplified 500 base pair long fragments that were cloned and sequenced. The sequences from five different clones revealed same sequence which was designated as EcRGA. The sequence when translated, was found to code for a continuous stretch of 170 amino acids without any stop codons in between. EcRGA displayed maximum similarity to RPS2 type RGA of rice. Phylogenetic analysis of the sequence indicated that it was closer to RPS2 type NBS-LRR protein class and among various RPS2 proteins from representative plant species, EcRGA was found to form a cluster with monocots, especially rice. EcRGA is a potential tool to isolate the complete gene and to be employed in disease management of the crop.**

Keywords **– Finger Millet, Resistance Gene Analogues, NBS-LRR, Phylogenetic Analysis.**

I. INTRODUCTION

During pathogen encounter, plants put forth several effective mechanisms to cope up with the challenge. Plants have a sophisticated immune system comprising of basal and adaptive immune mechanisms. Adaptive immune system is characterized by a genetic interaction between resistance genes (R) of the host and avirulence gene (*avr*) of the pathogen [1]. The R-gene product/avirulence factor complex is thought to trigger a defense response involving a series of events such as rapid oxidative bursts, cell wall strengthening, induction of defense gene expression and rapid cell death at the site of infection [2]. R-gene mediated immune response is more versatile in comparison to basal response as it is subject to diversification and selection in somatic cells of individuals [3].

Majority of R-genes in plants belong to the highly conserved family of proteins that possess two major domains-nucleotide binding site (NBS) and leucine rich repeats (LRR). This large group of proteins is involved in the detection of diverse pathogens including bacteria, viruses, fungi, nematodes and insects. The NBS domain is suggested to have a role of specific binding and hydrolysis of ATP. They have highly conserved and strictly ordered motifs, such as the P-loop, kinase-2 and Gly-Leu-Pro-Leu motifs [4]. Each LRR domain comprises a core of about 26 amino acids containing the Leu-xx-Leu-xx-Leu-x-Leu-xx-Cys/Asn-xx motif (x-any amino acid) which forms a beta sheet [5]. They are highly adaptable structural domains and primarily contributes towards protein-protein interaction. The NBS-LRR class of R genes are classified into two distinct subclasses based on the presence or absence of an N-terminal motif with homology to the cytoplasmic domains of the Drosophila Toll protein and the mammalian interleukin-1 receptor (TIR) [6]. They are the TIR and non-TIR subclasses of NBS-LRR R genes, respectively. Non-TIR class of NBS-LRR proteins are almost entirely absent in monocots.

The conserved motifs of the NBS domain are often used in the isolation of resistance gene analogues (RGAs) which may be part of functional R-genes, using PCR-based approaches with degenerate primers. Disease resistance genes from many of the plant species have been cloned and sequenced this way [7]-[9] and their role in disease resistance been proved [10]-[12]. This approach is highly useful in crops that do not have high-density genetic maps and hence cannot afford map based cloning of R-genes. Individual and genome-wide identification of RGAs will facilitate development of molecular markers towards R-gene mapping and cloning. This study is aimed at cloning RGAs from a genomically under-utilized crop, finger millet (*Eleusine coracana*), in order to enrich the genomic resources of the crop and also to identify functional disease resistance genes that can be made use in crop improvement programs.

II. MATERIAL AND METHODS

A. Designing of Degenerate Primers for R Gene

In order to design primers for NBS-LRR gene amplification from finger millet, a protein database search was carried out using *Hordeum vulgare* NBS protein sequence, CAD45031.1 as a query in NCBI BLASTP. Sequences from the first hundred matches (almost all monocotyledons) were downloaded and multiple sequence analysis was carried out using CLUSTALW. Two degenerate forward primers (RGF1: 5' GGNGGNGTNGGNAARACNAC 3' and RGF2: 5' GGTGGGGTTGGGAAGACAACG 3') and two degenerate reverse primers (RGR1: 5' ARNGCNARNGGNARNCC 3' and RGR2: 5' AGNGCNAGNGGNAGNCC 3') were designed from the conserved sequences and got custom synthesized (IDT).

B. PCR Amplification of RGAs, Cloning and Sequencing

DNA was extracted from IE2911 genotype of finger millet following CTAB method [13]. The quality of the DNA was checked on a 0.7% agarose gel and DNA was quantified using NanoDrop2000™ (Thermo Fischer Scientific). DNA was diluted to a final concentration of 100

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ng/µl and was used for PCR. PCR reaction mix consisted of 100 ng DNA, 20 pmoles each of forward and reverse primer, 1 unit of Taq polymerase and a final concentration of 100 µM of each dNTP in a total volume of 25 µl. PCR programme consisted of an initial denaturation of 5 minutes followed by 40 cycles of 30 s at 94°C, 30 s at 58°C and 30 s at 72°C and a final extension of 10 minutes at 72°C. Amplified DNA product was run on 0.8% agarose gel and was visualized with ethidium bromide staining (0.5 µg/ml concentration). The amplified DNA fragments were cloned in pJET1.2/blunt vector (Thermo Fischer Scientific). 150 ng of PCR product, 10 µl of 2X reaction buffer and 1 µl of DNA blunting enzyme were mixed to a final volume of 18 µl using de-ionised water (all components are from the kit) in an Eppendorf tube. The contents of the tube were mixed, centrifuged and incubated at 70°C for 5 minutes. To this mix added 1 µl of pJET1.2/blunt vector (50 ng/ µl) and 1 µl of T4 DNA ligase (5U/ µl). The contents were centrifuged briefly and incubated at room temperature for 5 minutes. 10 µl of the ligated mix was mixed with 100 µl of DH5α *E. coli* competent cells and incubated on ice for 45 minutes for transformation. Then the mixture was subjected to a heat-shock treatment at 42°C for 2 minutes, put on ice again, filled up with 1 ml LB medium and incubated at 37°C for an hour. The bacterial culture was then plated on Luria Bertini agarose plates containing 100 µg/ml of ampicillin. Plasmids were isolated from overnight grown bacterial culture using alkaline lysis method [14]. Plasmids were checked for presence of insert by restriction digestion with *Bgl*II. Positive clones were sequenced using Abi 3730XI (Sanger sequencing methodology).

C. Sequence Analysis and Construction of Phylogenetic Trees

Similarity search of the sequence was performed using NCBI BLAST (http://blast.ncbi.nlm.nih. gov/Blast.cgi) and multiple sequence alignment was done using ClustalW (http://www.genome.jp/tools/clustalw/). The nucleotide sequences obtained from Sanger's sequencing were translated to amino acid sequences using ExPASy translate tool (https://web.expasy.org/translate/). The conserved domains within the sequence was identified using NCBI Conserved Domain search (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). EcRPS2 sequence was compared with other major classes of NBS-LRR proteins from various plant species using MEGA7, a molecular evolutionary genetics analysis tool (http://www.megasoftware.net/) and dendrogram was constructed. The following sequences were used in the analysis; *Oryza sativa*1 (trIO48995I), *O. sativa*2 (trIA3BXA1I), *O. sativa*3 (trIQ69N78I), *Arabidopsis thaliana* RPS2 (U14158.1), *A. thaliana* RPM1 (spIQ39214I), Mla of *Hordeum vulgare* (ACZ65489.1), L6 of flax (trIQ40253I), N of *Nicotiana tobacco* (spIQ40392I) and RRS1 of *A. thaliana* (spIC4B7M6I). Another phylogenetic tree was constructed from RPS2 type NBS-LRR protein sequences of plants using the same programme. Accession numbers of RPS2 sequences used: *Brassica oleracea* (XP_013603678), *Brassica napus* (XP_013741401), *Raphanus sativus* (XP_018469792), *Camelina sativa* (XP_010433556), *Arabidopsis thaliana* (AAM90873), *Arabidopsis lyrata* (AAM90858), *Jatropha curcas* (XP_012076971), *Vitis vinifera* (XP_010656168), *Anthurium amnicola* (JAT59713), *Musa acuminata* (XP_009416673), *Zea mays* (XP_008674947), *Brachypodium distachyon* (XP_003564370), *Setaria italica* (XP_004984978), *Aegilops tauschii* (EMT19376), *Oryza sativa1* (XP_015611121), *Oryza sativa2* (XP_015611123). The sequences were aligned using CLUSTALW and phylogenetic trees were constructed following neighbor-joining method.

III. RESULTS AND DISCUSSION

A. Cloning and Sequence Analysis of E. Coracana RGA

Conserved amino acid sequences belonging to the conserved P-loop domain (GGVGKTTLA) and GLPLAL motif belonging to NBS domain were identified in the 100 protein sequences retrieved through NCBI BLASTP using *H. vulgare* NBS-LRR protein as the query sequence using the multiple sequence alignment tool, CLUSTALW. These sequences are widely used in rapid isolation of R-gene analogues (RGAs) from various plant species by PCR-based approach with degenerate primers. The degenerate primers were designed to yield an amplified product of approximately 500 bp (base pairs). PCR amplification of finger millet DNA prepared from IE2911 using the primer combinations, RGF1 + RGR1 and RGF1 + RGR2 gave multiple bands whereas RGF2 + RGR1 and RGF2 + RGR2 combinations produced a bright single band of ~0.5 kb. The PCR products were recovered from agarose gel, cloned into pJET1.2/blunt vector and sequences of five independent clones were determined. All clones turned out to be having the same nucleotide sequence after vector removal and the open reading frame was found to code for a 170 amino acids long protein. The nucleotide sequence obtained from Sanger's sequencing was submitted in NCBI Genbank (MG520627). Comparison of finger millet RGA to sequences available in NCBI database revealed that it was most similar to NBS-LRR proteins from other plant species especially the cereal, rice. The greatest similarity was shown with RPS2-like disease resistance protein (XP_015611123.1) of *Oryza sativa* japonica group (77%). RPS2 is a resistance gene first cloned from A*rabidopsis thaliana* that confers resistance against *Pseudomonas syringae* bacteria that express avirulence gene avrRpt2 [15]-[16]. Conserved domain search revealed that the protein contained an NB-ARC domain. The deduced amino acid sequence showed internal motifs characteristic of NBS domain namely P-loop, Kinase 2, Kinase 3a and GLPL (Fig. 1). Finger millet RGA has a tryptophan residue (W) in the end of kinase 2 motif which is indicative of the non-TIR sub-class of R-genes, the prominent class of R-genes in monocots (Fig. 1) [17].

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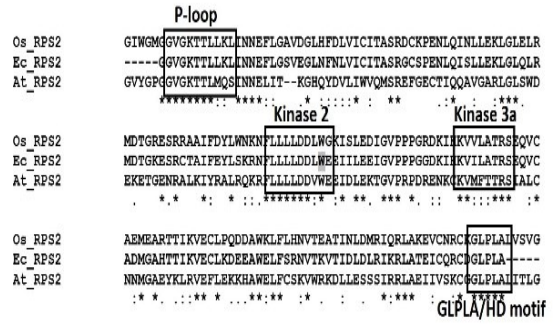

Fig. 1. CLUSTALW alignment of RPS2 sequences of *Arabidopsis* (ABN54588.1), *Oryza sativa* (XP_015611121) and finger millet showing the conserved motifs of nucleotide binding site (NBS). The tryptophan residue (W) highlighted within kinase 2 is conserved among non-TIR type NBS.

B. Phylogenetic Analysis

Phylogenetic analysis of finger millet RGA with various classes of plant RGAs indicated that it was similar to RPS2 type of R-gene (Fig. 2). Finger millet RGA formed a cluster with RPS2 type NBS-LRR proteins and among them it is closer to rice, a monocot like finger millet, RPS2 than to the RPS2 of *Arabidopsis*, a dicot. RPM1 and Mla proteins, which belong to CC-NBS-LRR group, formed a separate cluster next to the RPS2 type. The members of TIR-NBS-LRR class that were used in the analysis namely, L6, N and RRS1 formed a farther, separate cluster. RPS2 type R-genes that are reported in other plant species were used for phylogenetic analysis along with finger millet RGA. Finger millet RGA formed a cluster together with *O. sativa* RPS2 protein indicating the phylogenetic closeness of rice and finger millet, both being monocots (Fig. 3). The monocot species used in the study namely *S. italic, A. tauschii, B. distachyon, Z. mays, M. acuminata* and *A. amnicola* formed clusters that are closely spaced while dicotyledonous species together formed a farther cluster as expected.

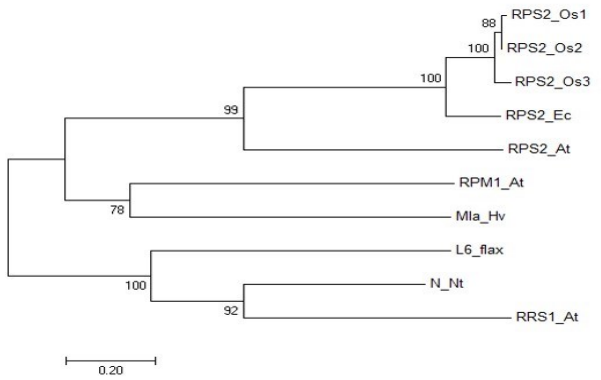

Fig. 2. Unrooted Neighbour-Joining phylogenetic tree of different classes of NBS-LRR type RGAs in plants with 500 bootstrap replicates. Accessions of the sequences used are: RPS2_os1 (trlO48995l), RPS2_os2 (trlA3BXA1l), RPS2_os3 (trlQ69N78l), RPS2_At (U14158.1), RPM1_At (splQ39214l), Mla_Hv (ACZ65489.1), L6_flax (trlQ40253l), N_Nt (splQ40392l) and RRS1_At (splC4B7M6l)

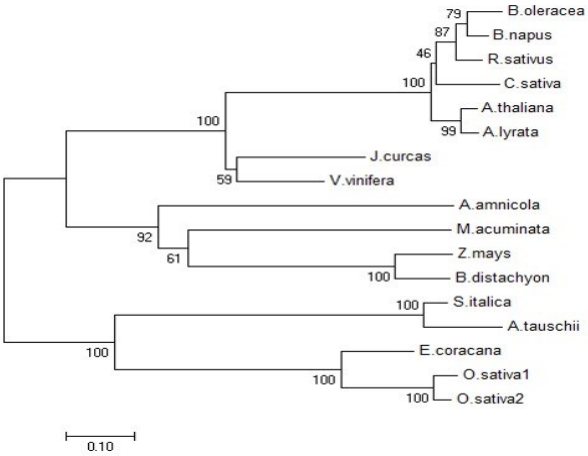

Fig. 3. Unrooted Neighbour-Joining phylogenetic tree of RPS2 type RGAs in plants. Numbers at node indicate the level of branch support with 1000 bootstrap replicates. Accession numbers of RPS2 sequences used: *Brassica oleracea* (XP_013603678), *Brassica napus* (XP_013741401), *Raphanus sativus* (XP_018469792), *Camelina sativa* (XP_010433556), *Arabidopsis thaliana* (AAM90873), *Arabidopsis lyrata* (AAM90858), *Jatropha curcas* (XP_012076971), *Vitis vinifera* (XP_010656168), *Anthurium amnicola* (JAT59713), *Musa acuminata* (XP_009416673), *Zea mays* (XP_008674947), *Brachypodium distachyon* (XP_003564370), *Setaria italica* (XP_004984978), *Aegilops tauschii* (EMT19376), *Oryza sativa*1 (XP_015611121), *Oryza sativa*2 (XP_015611123)

Map based cloning still remains one of the major strategies for cloning resistance genes. Identification of RGAs through PCR based method is an effective alternate way to identify R genes. Next generation sequencing technologies have enabled genome wide identification and characterization of RGAs from a number of plant species [18]-[21]. NBS profiling or RGA mapping is a powerful tool for development of markers linked to resistance loci in species where there is only limited genomic information, such as millets. Here, in this study, an RPS2 type RGA has been cloned from finger millet, a crop in which only a few genomic resources are available. This is the first RPS2 type RGA reported from finger millet. Cloning and expression analysis of RGAs not only facilitate the understanding of molecular mechanisms of interaction between host and pathogen but will also act as a valuable tool in resistance breeding in crop plants.

ACKNOWLEDGMENT

Author thanks Indian Council of Agricultural Research (ICAR) for the financial support for carrying out this research.

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