Int.J.Curr.Microbiol.App.Sci (2019) *8*(1): 2773-2781

International Journal of Current Microbiology and Applied Sciences
***ISSN: 2319-7706* Volume 8 Number 01 (2019)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2019.801.292

Isolation and *in silico* Characterization of Disease Resistance Gene Analogues (RGAs) from Minor Millets

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Keywords

Setaria, Eleusine, NBS, LRR, Resistant gene, *in silico*

Article Info

Accepted:
17 December 2018
Available Online:
10 January 2019

A B S T R A C T

Minor millets are under-exploited plant species often dubbed as 'Crops for the future' as they are best adapted to less fertile area and can survive limited water situations and relatively free from pests and diseases. Degenerate oligo-PCR strategy was employed to isolate resistant gene analogues from minor millets by amplifying samples using NBS-LRR gene specific primers. Genomic DNA was isolated from the young leaves of minor millets *viz.*, Foxtail millet (*Setaria italica*) var. CO 5 and Finger millet (*Eleusine corocana*) var. CO (Ra) 14 by following CTAB method with minor modifications. PCR amplification of the genomic DNA with degenerate primers, revealed presence of 500bp product in the minor millet samples typical to the size of other reported R-gene analogues. The amplicons TENAIKKM1 sequenced was of size 527 bases and RagiKKM1 was of size 509 bases, the sequences with high similarity to NBS-LRR family are deposited in GenBank, NCBI. *In silico* characterization of the sequences were done using bioinformatics tools by similarity search, pattern recognition and diversity analysis, which resulted in classifying the genes as those belonging to NBS-LRR class as that of other grass family NBS-LRR kind of proteins. Further, *in silico* characterization ensured that they belong to Non-TIR family of R genes as evident by the conserved residues in the kinase 1 and kinase2 domains. The diversity among the novel RGAs characterized in this study may lead to mining of *R- genes* in other under-exploited minor millets.

Introduction

Many plant defense responses are initiated by resistance genes, providing a mechanism by which the plant can recognize a pathogen and execute a defense against it. In recent years many different disease-resistance genes (R-genes) have been cloned from plants. R-genes contain a leucine-rich repeat region (LRR), a strong candidate for pathogen recognition specificity. Some of the LRR R-genes contain domains possibly involved in signal

transduction such as nucleotide binding sites (NBS), leucine zippers, or Toll–Interleukin-1 Cytoplasmic receptor (TIR) domains. Still others contain protein kinase domains and conserved domains of unknown function (Meyers *et al.,* 1999).

Genes from the NBS-LRR classes were reported to confer resistance to bacteria, fungi, virus, nematodes and aphids (Timmerman-Vaughan *et al.,* 2000, Dogimont *et al.,* 2014). In the R-genes, NBS region is thought to be important for ATP binding and overall functionality of the resistant protein coded by it (Saraste *et al.,* 1990). The NBS and NBS-LRR class of *R* genes have been cloned and well characterized from *Arabidopsis thaliana*, flax, tobacco, tomato, rice,peanut, Kiwifruit and other under-exploited plant species (Bent *et al.,* 1994; Mindrinos *et al.,* 1994; Whitham *et al.,* 1994; Grant *et al.,* 1995; Lawrence *et al.,* 1995; Milligan *et al.,* 1998; Yoshimura *et al.,* 1998; Tirumalaiandi *et al.,* 2008, Maiti *et al.,* 2011, Lozano *et al.,* 2015, Li *et al.,* 2016, Chong *et al.,* 2017). CC-NBS-LRR class of gene from *Haynaldia villosa* was cloned and characterized (Xing *et al.,* 2018).

Discovering specific genes from the gene pool can be achieved by various strategies, among them degenerate-oligo Polymerase chain reaction (PCR) is more sensitive and easily employed in isolating conserved gene sequences and would therefore be more effective in isolating potential NBS/LRR disease resistance genes (Shen *et al.,* 1998). Using this strategy, it has been possible to amplify analogous sequences from many plant species (Kanazin *et al.,* 1996; Leister *et al.,* 1996; Yu *et al.,* 1996). Leister *et al.,* (1996) used PCR and oligonucleotide primers designed from conserved domains of *N* (tobacco) and *RPS2* (*Arabidopsis*) to amplify products with NBS homology to known resistance genes. Minor millets, also described as nutritious millets have received far less research and development attention than other crops with regard to crop improvement and utilization. On positive note, these crops possess inherent mechanism to resist biotic stresses and are less prone to major diseases. In the present study, attempts were made to isolate R genes from minor millets viz., finger millet (*Eleusine coracana*) and foxtail or Italian millet (*Setaria italica*) and characterize them using publicly available bioinformatics tools.

Materials and Methods

Seeds of minor millets, Foxtail millet (*Setaria italica*) var. CO 5 and Finger millet (*Eleusine corocana*) var. CO (Ra) 14 were obtained from the Department of Millets, Tamil Nadu Agricultural University, Coimbatore. Seeds were raised in pots and leaf samples were collected from the plants for total DNA isolation.

Genomic DNA isolation

Genomic DNA was isolated from the young leaves of finger millet (*Eleusine coracana*) and foxtail millet (*Setaria italica*) by adopting CTAB procedure (Doyle and Doyle, 1990) with necessary modifications. The leaves were subjected to pretreatment with absolute alcohol for 30 min., air dried and ground using liquid nitrogen in a prechilled Pestle and Mortar. Cells were lysed with DNA extraction buffer using liquid nitrogen. After cell lysis, the genomic DNA was separated using 6 volumes of Chloroform: isoamylalcohol (24:1) and precipitated using ice cold iso-propanol and the DNA was further purified by washing with 70% ethanol, the samples were electrophoresed in 1% Agarose gel electrophoresis for checking the quality.

Primer Designing

Oligonucleotide primers with optimized primer parameters were designed using Primer3 (Untergasser *et al.,* 2012) based on

the conserved domains of *N* (tobacco) and *RPS2* (*Arabidopsis*) genes and was to be used for amplification of products with NBS homology to known resistance genes.

R-gene isolation by degenerate oligo-PCR

PCR amplifications were carried out in a 50 µl reaction containing 100 ng genomic DNA, 0.2 µ*M* of each primer The primer sequences are ACK1F-5'-GGTGGGGTTGGGAAGACAAC G-3' and ACK1R-5'-CAACGCTAGTGG CAATCC-3' (designed from consensus sequences of NBS region), 0.25 m*M* each of the four dNTPs (Fermentas Life Sciences, Canada), 2 mM MgCl2, 1 x *Taq* buffer (GeNei, India) and 2.5 U *Taq* DNA polymerase (GeNei, India). PCR was performed in GeneAmp PCR6700 thermal cycler (Applied Biosystems Inc., USA) with initial denaturation at 92°C for 5 min, then cycling for 30 times 92°C for 1 min, 53°C for 1 min., 72°C for 1 min, then final extension at 72°C for 5 min. Amplified PCR products were electrophoresed in 1.5% w/v agarose gels (Xcelris Genomics, India) stained with ethidium bromide. PCR fragments were excised under UV transilluminator (Biorad, USA) and purified using GenElute^{TM} Gel Extraction Kit (Sigma Aldrich, USA). To further confirm, the PCR products were reamplified using same PCR conditions. Amplicons of 500bp were gel eluted and subjected to sequencing in an automated ABI 3100 Genetic Analyser based on Sangers's sequencing method. The sequences are deposited in GenBank, NCBI, USA with accession numbers KM454474 and KM454475.

In silico characterization and diversity analysis

The nucleotide sequences of the cloned fragments were translated in to amino acid sequences using ExPASy (Expert Protein Analysis System) Translate Tool, a proteomics server of Bioinformatics (www.expasy.org). The deduced amino acid sequences were subjected to motif analyses using the online version of CLUSTALW multiple alignment program (Thompson *et al.,* 1997) of European Bioinformatics Institute (www.ebi.ac.uk). The amino acid sequences of the RGAs were compared with protein sequences deposited in the GenBank using BLASTP algorithm (Altschul *et al.,* 1997). Pair wise comparison of RGA sequences with known *R*-gene sequences at NBS region was made. In addition to these, amino acid sequences of representative classes of RGAs isolated from the model legumes *Glycine max* (Kanazin *et al.,* 1996), N *gene of* tobacco, *RPS2, RPS5 and RPP8 of A. thaliana, L6 of Linum and XA1 of* rice were retrieved from the GenBank and were trimmed to start and end at the P-loop and GLPL motifs to facilitate accurate alignments with the sequences in the present study. Postsctipt pattern observed using ESPript 2.2 (Gouet *et al.,* 1999). The deduced amino acid sequences of the RGAs were compared with protein sequences using WU-BLAST. The alignment file output was fed to phylogenetic tree prediction tool- TREE TOP (Yushmanov and Chumakov, 1988) with boot strapping in a BLOSUM62 matrix. Best possible open reading frames were identified and pattern recognition was done using PROSITE tool in the same ExPASy server. Conserved domain (CD) search was performed in NCBI-CD database (Marchler-Bauer *et al*., 2007) for classifying the protein to the respective protein super family.

Results and Discussion

PCR amplification of Resistance Gene Analogues

Use of PCR based strategies has been successful and has opened up an avenue for isolation and cloning of R genes from plants

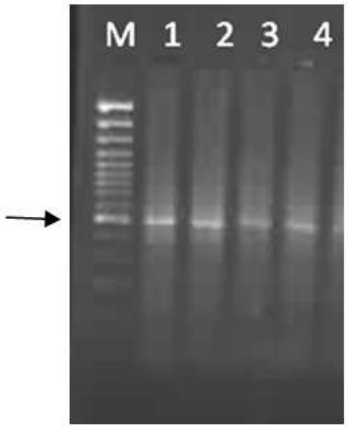
based on conserved motifs among the NBS-LRR class resistance genes. This oligo Primed-PCR approach provides an alternative to the classical methods of transposan tagging and map-based cloning strategies (Seah *et al.*, 1998). Earlier reports indicates co-amplification of non-specific fragments apart from the expected amplicon (500-600 bp) in crops like soybean (Yu *et al.*, 1996), rice (Mago *et al.*, 1999) and sorghum (Totad *et al.*, 2005). In the present study we could successfully amplify the targeted RGA fragment (expected size from the distance between the sequence motifs in *N* gene of tobacco and *RPS2* of *Arabidopsis thaliana*) from the genomic DNA of *Eleusine coracana* and *Setaria italica* due to high stringency PCR conditions with degenerate primers (Fig. 1). The amplicon from *Setaria italica* was designated as TenaiKKM1 and of *Eleusine coracana* as RagiKKM1. The sequences are deposited in GenBank of National Center for Biotechnology Information, USA and accessioned as KM454474 (TenaiKKM1) and KM454475 (RagiKKM1).

Sequence analysis for conserved motifs

The deduced amino acid sequences of RGAs in the present investigation were compared with other known *R* genes *viz.*, *N* gene of tobacco, *RPS2, RPS5* and *RPP8* of *A.thaliana*, *XA1* of rice and *L6* of flax in NBS region using CLUSTALW multiple alignment program (Fig. 2). The various motifs of NBS, *i.e.*, kinase-1a, kinase-2 and kinase-3a were conserved in *Eleusine coracana (RAGIKKM1)* and *Setaria italica (TENAIKKM1)* RGAs. The hydrophobic region represented by the GLPL domain was also observed in all the four RGA sequences. The Best ORF in the 5'3' reading frame of TENAIKKM was with 173 aa and for RAGIKKM1 was with 175aa which contained GGFGKTT - P Loop, LIVLDD - Kinase 2 Loop and GLPL – motif. Conserved domain search identified NB-ARC (nucleotide-binding adaptor shared by APAF-1, R proteins, and CED-4) domain) and classified the protein to Pfam 00931 family. Pattern recognition done using PROSITE tool detected a major Protein kinase C phosphorylation site in both the cases. Sequence similarity search using WU-BLAST indicated good homology of the TENAIKKM1 sequences with the NBS-LRR class of proteins belonging to other cereals and grasses *viz., Zea mays, Sorghum bicolor, Saccharum sp., Oryza sativa, Hordeum vulgare etc.*, Similar results were obtained with the RAGIKKM1 sequences that's showed >55% homology with for the reported RGA sequences from *Sorghum bicolor, Brachypodium distachianum, Triticum, Hordeum vulgare, Secale strictum* and *Oryza sativa.* High sequence homology of the RGAs with NBS motifs of N, RPS2, RPS5, RPP8, L6 and XA1 clearly establishes that the RGAs cloned in the present investigation might belong to NBS-LRR class of *R-genes*.

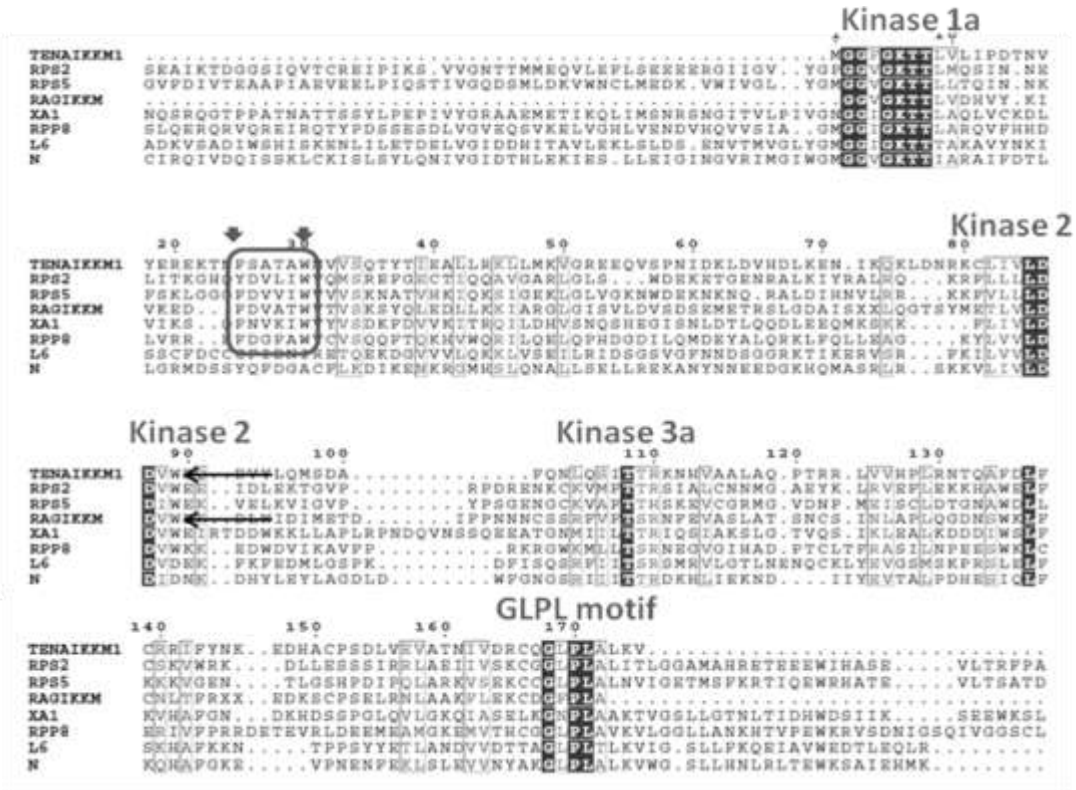
Among R genes, the NBS-LRR class of genes are usually grouped into two subfamilies (Meyers *et al.*, 1999; Pan *et al.*, 2000) in which the subfamily I contains the TIR element (Toll-Interleukin-1 Receptor like domain) mostly present only in dicots, while subfamily II lacks the TIR domain found in both the monocots and dicots. The partial sequence of the NBS portion is usually sufficient to assign a given gene to either subfamily I or II. The last residue of the kinase-2 domain can be used to predict with 95% accuracy whether an RGA would belongs to the TIR-NBS or the non-TIR-NBS family; conservation of tryptophan (W) at this location is tightly linked to non-TIR *R*-genes (*RPS2, RPS5* and *RPP8* of *A. thaliana*), whereas conservation of aspartic acid (D) or its uncharged derivative aspartate (N) is characteristic of TIR class of *R*-genes (*N* and *L6*) (Meyers *et al.*, 1999; Pan *et al.*, 2000; Jeong *et al.*, 2001; Penuela *et al.*, 2002).

Fig.1 PCR amplification of targeted R genes with degenerate primers



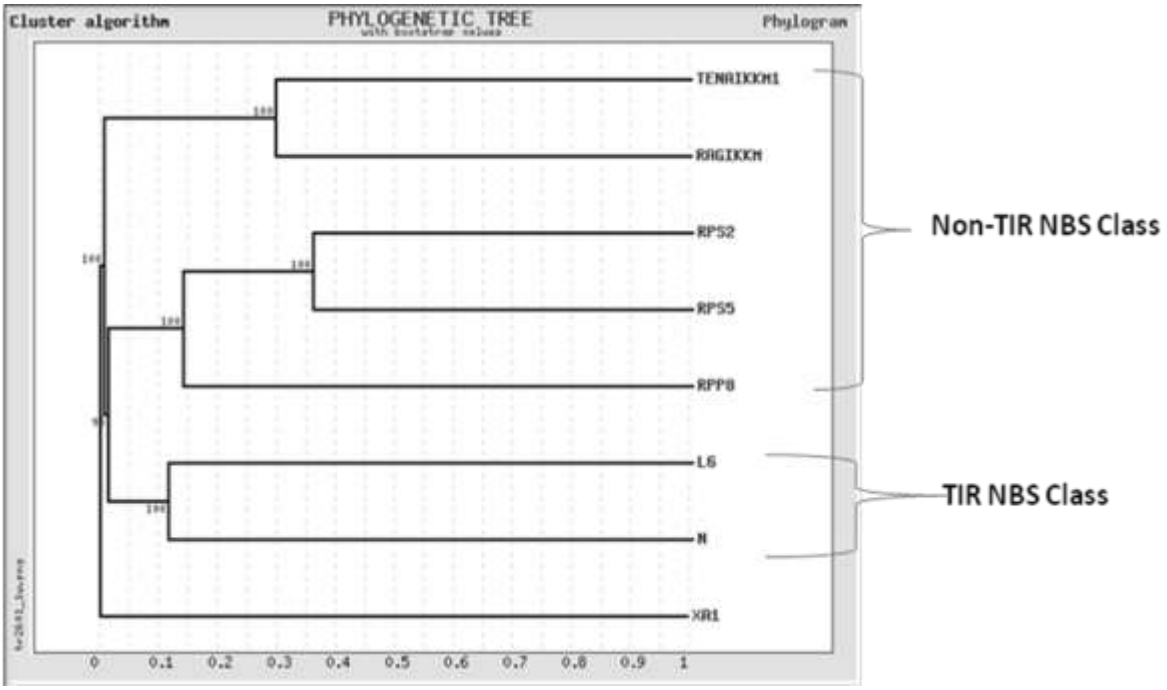
M-1 Kb DNA ladder, 1,2-foxtail millet RGA ; 3,4-finger millet RGA. Arrow indicates 500bp amplicons, size expected for NBS coding regions of R-Genes

Fig.2 Multiple sequence alignment of the novel RGAs with representative NBS-LRR class of R-genes



N gene of tobacco, *RPS2, RPS5 and RPP8 of A. thaliana, L6 of Linum and XA1 of* rice in NBS region using CLUSTALW2 and Postscript pattern observed using ESPript 2.2

Fig.3 Average distance tree for identified RGAs with known R-genes using TREETOP tool



Values are boot strapped. R genes, N (acc. no.U15605), RPS2 (acc. no. U14158), RPS5 (acc. no. AF074916), RPP8 (acc. no. AF089710), XA1 (acc. no. AB002266) and L6 (acc. no. U27081)

In this study, the TENAIKKM1 and RagiKKM1 genes have Tryptophan conserved in the kinase2 loop, establishing the fact that it's a NON-TIR Class of R gene. In TIRNBS-LRR group, a characteristic consensus motif FXXXXF and a highly conserved glycine is present between kinase1a and kinase-2 domains, whereas the non- TIR-NBS-LRR group contains the consensus sequence FXXXXW (Pan *et al.*, 2000). The presence of consensus motif FXXXXW between kinase 1a and kinase-2 domains of TENAIKKM1 (KM454474) and RAGIKKM1 (KM454475) suggests RGA sequences further ensures their position in non-TIR-NBS-LRR subfamily.

Diversity analysis based on phylogeny

Phylogenetic analysis of RGA sequences with already characterized *R-genes* was done by constructing average distance tree using BLOSUM62 in the alignment editor. TIR-NBS-LRR genes evolved after the divergence of monocotyledonous and dicotyledonous crop species and clustering of RGAs occurs due to their origin from common evolutionary mechanisms. The RGAs of *Eleusine coracana* and *Setaria italic* were clustered together with well- characterized non-TIR-NBS-LRR genes *RPS2, RPS5* and *RPP8* of *A. thaliana*, while the TIR-NBS-LRR genes *N* and *L* were grouped as a separate cluster. The RGAs isolated from *Eleusine coracana* and *Setaria italica* were clustered together in a single sub-clade and exhibit their sequence similarity (Fig. 3).

Resistance gene analogues generally are putative disease resistance genes identified on the basis of their structure. Hence studies on R-genes and RGAs are still explorative in nature. Further information on R-gene sequences are necessary to delineate more

structural domains, which would be the basis for the finding of novel RGAs in any crop plant (Totad *et al.,* 2005). The diversity among the novel RGAs characterized in this study may open up scope and further lead to mining of R-genes in other under-exploited minor millets. To know their exact function they need to be characterized and linked with the genes actually conferring resistance phenotype. Mapping of RGAs generates perfect markers that are linked to known resistance genes. This can be employed to follow resistance genes in segregating populations, to possibly link them and then ultimately use as candidates for gene isolation.

Acknowledgements

The authors gratefully acknowledge the crews of NCBI, USA; EMBL-EBI, UK and ExPASy proteomics server of SIB, Switzerland for making the computational biology data/tools publicly available for use in analysis.

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How to cite this article:

Subramanian Rajesh, Sivaraj Preetha, Gurusamy Anand, Balasubramanian Selvi and Navaneethakrishnan Shunmugavalli. 2019. Isolation and *in silico* Characterization of Disease Resistance Gene Analogues (RGAs) from Minor Millets. *Int.J.Curr.Microbiol.App.Sci.* 8(01): 2773-2781. doi: https://doi.org/10.20546/ijcmas.2019.801.292