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Molecular characterization and phylogenetic analysis of *NBS-LRR* genes in wild relatives of eggplant (*Solanum melongena* L.)

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ABSTRACT

Eggplantor brinjal (*Solanum melongena* L.), is highly susceptible to various soil-borne diseases. The extensive use of chemical fungicides to combat these diseases can be minimized by identification of resistance gene analogs (RGAs) in wild species of cultivated plants.In the present study, degenerate PCR primers for the conserved regions ofnucleotide binding site-leucine rich repeat (NBS-LRR) were used to amplify RGAs from wild relatives of eggplant (Black nightshade (*Solanum nigrum),* Indian nightshade (*Solanumviolaceum)*and *Solanu mincanum)* which showed resistance to the bacterial wilt pathogen, *Ralstonia solanacearum*in the preliminary investigation. The amino acid sequence of the amplicons when compared to each other and to the amino acid sequences of known RGAs deposited in Gen Bank revealed significant sequence similarity. The phylogenetic analysis indicated that they belonged to the toll interleukin-1 receptors (TIR)-NBS-LRR type *R*-genes. Multiple sequence alignment with other known *R* genes showed significant homology with P-loop, Kinase 2 and GLPL domains of NBS-LRR class genes. There has been no report on *R* genes from these wild eggplants and hence the diversity analysis of these novel RGAs can lead to the identification of other novel *R* genes within the germplasm of different brinjal plants as well as other species of *Solanum.*

Key words: Eggplant, Nucleotide binding site-.Leucine rich repeat, Resistance Gene Analogs.

INTRODUCTION

India is blessed by rich biodiversity and hence offers immense potential for studying the wild relatives ofcultivatedcrops. These wild plants offer a diversity of traits including pathogen resistance for crop improvement. Studies should be focussed on major genes and defined characters present in these plants which may be absent in the cultivated gene pool. Eggplant (brinjalor aubergine, *Solanum melongena* L.), an important solanaceous fruit crop is widely grown in Asia, Africa and the subtropics, including India and Central America. However this poor man's vegetable is susceptible to numerous soil-borne diseases leading to enormous loss in yield and quality. They have partial resistance to most of the soil-borne pathogens, but often at insufficient levels(Daunay *et al*., 1991). In order to combat the pathogen attack, farmers mostly depend on chemical fungicides which are expensive, cause environmental problems and health risks. Hence, the ideal strategy to combat these pathogens is to produce disease resistance in cultivated egg plants.

Cloning resistant genes, subsequent transformation and sequencing genomic DNA fragments amplified by degenerative PCR primers for conserved domains is one of the recent solutions for obtaining disease resistant plant varieties.Researchers have isolated disease resistant (*R*) genes from genomes of several plant species like *Arabidopsis thaliana, A lyrata*, rice cultivars, *Brachypodium*, woody species grape, poplar, cotton, apple, legume species, potato (*Solanum tuberosum*), tomato (*S. lycopersicum*) and pepper (*Capsicum annuum*) (Wei *et al*., 2016).

Most of the *R* genes belong to the nucleotide binding site- leucine rich repeats (NBS-LRR) class. Bacterial wilt, caused by *Ralstonia solanacearum*, is one of the most destructive diseases of eggplant especially in the tropical regions (Chandrashekara *et al*., 2012). There have been earlier reports of RGAs or *R* genes from other eggplants namely, *Solanummelongena, S aethiopicum* gr. Gilo, *S linnaeanum, Sintegrifolium, Ssisymbriifolium,* and*S khasianum*(Zhuang*et al*., 2012). But to the best of our knowledge, no research work on *R* genes was carried out in the three wild eggplants namely,Black nightshade (*Solanumnigrum),* Indian nightshade (*Solanum violaceum)* and *Solanum incanum*(a species of night shade) which showed resistance to the bacterial wilt pathogen,*R solanacearum* in the preliminary investigation.Therefore, the main objective of this work was to characterize the diversity of NBS-LRR class of genes in these wild plants and to study their evolution with regard to the reported homologous *R* genes in other plant species. This could pave way for the

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utilization of wild species to improve the disease resistance in cultivated eggplant.

MATERIALS AND METHODS

DNA extraction and PCR amplification by degenerative primers: The leaf samples of *S nigrum, S violaceum* and *S incanum* were collected from Aromatic and Medicinal Research Centre, Odakkali, Ernakulam, Kerala, India. DNA was extracted from the fresh leaf tissues by Dneasy® Plant Mini Kit Based Method. Degenerate primers, forward 5'GGNGGNRTNGGNAAGACGAC3' (Noir *et al*., 2001) and reverse 5'GAGGGCTAAAGGAAGGCC3' (Deng *et al*., 2000) were used to amplify the regions between P-loop and GLPL, conserved domains of plant *R* genes. The primers were predicted to amplify approximately 500 bp DNA fragments. The PCR reaction was performed in a total volume of 20µl, containing 50 ng of template DNA, 1 X PCR buffer with $MgCl_2$ 0.25 mMdNTPs, 0.20 mM of each primer, and 1U Taq DNA polymerase. PCR amplification was performed in Eppendorf thermal cycler with initial denaturation at 94°C for 4 min,followed by 35 cyclesof 94°C for 30 s, 55°C for 30 s and 72°C for 60 s with final extension of 72°C for 10 min. Amplified PCR products were electrophoresed in 1.5% w/v agarose gels stained with ethidium bromide. PCR fragments were excised under UV trans-illuminator and purified using NucleoSpin® Gel and PCR Clean-Up Kit.

Cloning and sequencing of PCR products: Eluted purified PCR fragments of expected size were cloned into the vector pTZ57R/T using InsT/AcloneTM PCR Product Cloning Kit and transformed into competent *Escherichia coli* strain DH5α by following manufacturer's instructions. The transformed colonies were screened and confirmed for the presence of insert by colony PCR. Plasmid DNA was isolated by NucleoSpin® Plasmid / Plasmid (NoLid) kit. Sequencing of the purified plasmid was done at SciGenom Labs Pvt Ltd, Kochi.

Phylogenetic analysis of cloned sequences: The vector sequences and primer sequences were removed with GeneDoc 2.7 software and the region between Ploopand GLPL was taken for further analysis.The nucleotide sequences were translated to predicted amino acid sequence (Bikandi *et al*., 2004) and BLASTp algorithm with default settings was used for performing homology searchthrough National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov), to identify *R*genesas well as homologous sequences. The conserved motif structures of predicted RGAs were analyzed using MEME software (Multiple Expectation Maximization for Motif Elicitation) (Timothy and Charles, 1994). A phylogenetic tree was constructed using MEGA (Molecular Evolutionary Genetic Analysis) Version 6.0 (Tamura *et al*. 2013) to evaluate the set of NBS sequence analogs of the present study with other plants resistance genes. Cluster analysis was carried out using CLUSTALW(Thompson *et al*. 1997) based on the neighbor-joining tree (Jones Taylor-Thornton (JTT) model) with 1000 bootstrap replications.

RESULTS AND DISCUSSION

Solanum nigrum, Solanum violaceum and *Solanum incanum* are wild relatives of eggplant mostly prevalent in tropics showing resistance to bacterial wilt. Since there have been no earlier reports on the characterization of *R* genes in these plants, this study was undertaken. *S nigrum* constitute a minor food crop, with the shoots and berries not only being used as vegetables and fruits, but also for various medicinal uses. *S violaceum and S incanum* are also used as vegetables and for various medicinal treatments.

Identification and characterization of NBS-LRR fragment: The discovery of conserved motifs among the NBS-LRRclass resistance genes opens the avenue for the use of PCR based strategy in isolating and cloning *R* gene family members and analogs using degenerate or specific primers.PCR amplification of nucleotide binding site-encoding genomic DNA regions of the wild eggplants in this study generated fragments of expected size Figure 1. These fragments were extracted from 1.5% agarose gel,purified and then cloned into pTZ57R/T vector. Fifteen recombinant clones from resistant type/cultivated species were selected for sequencing.Of these, six sequences had no match with *R* genes and had potential stop codons. The remaining nine sequences which had uninterrupted ORF were considered as RGAs. Homology matrix was done for the nine sequences and three sequences which have <50 % homology with other sequences and highest identity to different NBS-LRR RGAs analyzed through BLAST were shortlisted. These were considered for further analysis and named as *Sincanum*(Smi), *S nigrum* (Sn), *S violaceum* (Sv). Protein sequences of the three nucleotide sequences were used for identity search with BLASTp algorithm which indicated that all these sequences had a high level of similarity to RGAs and *R*-genes of other

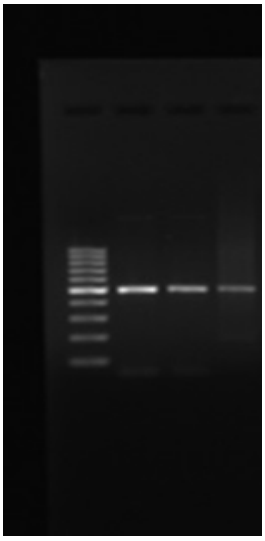

Fig 1: PCR amplification of DNA samples of *Solanum nigrum, Solanum violaceum* and *Solanum incanum* using degenerative primers

plant species deposited in GenBank (Table 1).Nucleotide blast helped to identify the sequence diversity among the different varieties whereas protein level search and comparison helped to identify the conserved regions of the polypeptide chain. MEME motif identification software was used todetect conserved and relative motifs in RGAs and *R* genes of highly homologous species (Figure 2). Five major, conserved motifs (Kinase 2, RNBS-C, P-loop, GLPL and RNBS A-non TIR) were identified along the whole length of eggplant RGAs (Fig. 3) and this represented similarities of eggplantRGAs with the structural character of NBS domain of NBS-LRR *R*-genes. It was observed that the cloned sequences were highly similar to *R* genes or the RGAs identified in other plant species, especially solanaceous plants. Thus, these genes, especially those from the wild species, may encode resistance gene products of unknown specificity.

Multiple amino acid sequence alignment of TIR and non-TIR-NBS-LRR RGAs in eggplant: Multiple sequence alignment of RGAs isolated in this study with previously isolated RGAs by Reddy *et al*. (2015) and other known *R*genes of different plants,including *Solanumbul bocastanum, Lagenaria siceraria, Solanum melongena, Solanum tuberosum, Capsicumannum, Solanumly copersicum, Solanum tuberosum, Luffa aegyptiaca* also revealed various conserved motifs of NBS, including the highlyconserved P-loop (GKTT), GLPL, RNBS-A, Kinase-2, RNBS-B and RNBS-C (Figure 4). This explains that the RGAs isolated in this study may be considered as NBS-LRR class of resistance genes. The three isolated RGA analogs also had an aspartic acid (D) or aspartate (N), suggesting their resemblance to TIR type NBS-LRR class of *R*-genes.

Table 1: Similarity search between eggplant RGAs and GenBank accessions carried out using the BLASTp algorithm.

Eggplant	Plant species	Description	Identity	e value
Sm	*Lagenaria siceraria*	NBS- LRR resistant protein(AEV46136.1)	67	3e-43
	Solanum melongena	NBS –LRR protein (AHL69098.1)	88	5e-70
	Solanum tuberosum	PREDICTED : TMV resistance protein N-like XP006359566.1)	85	4e-55
Sn	*Solanum bulbocastanum*	Putative resistance protein (AFD18779.1)	75	3e-75
	Capsicum annum	Resistance protein RGAO6 (AEV76889.1)	69	6e-66
	Solanum lycopersicum	Resistance protein homolog (AAD08712.1)	69	4e-68
Sv	*Solanum tuberosum*	PREDICTED: LOW QUALITY protein: uncharacterized protein (LOC102590743) (XP015159498.1)	83	2e-75
	Luffa aegyptiaca	NBS- LRR resistance protein (AEV46141.1)	69	1e-75

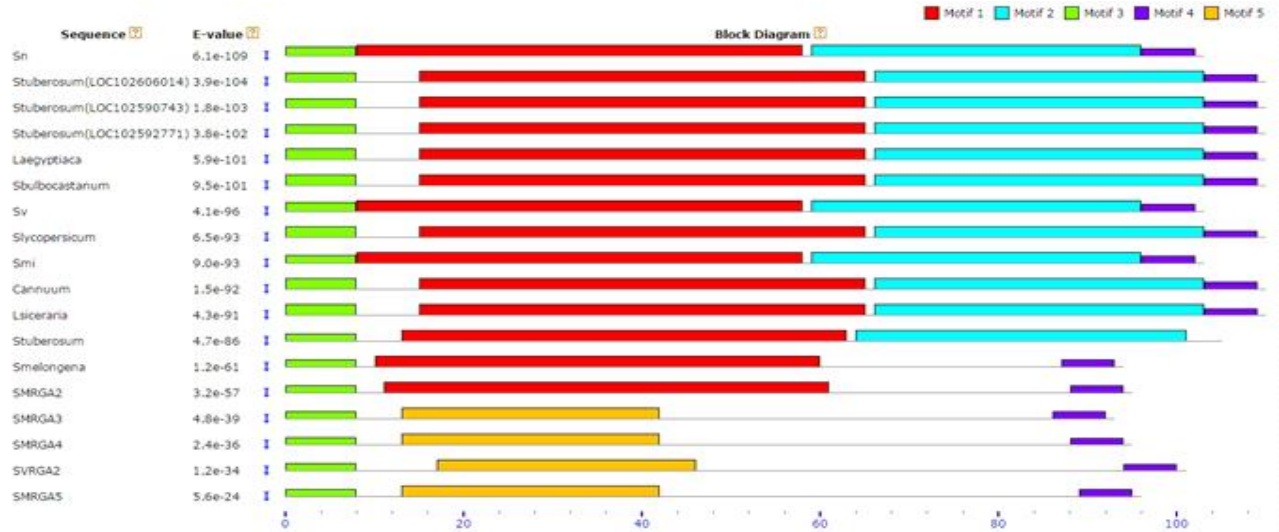

Fig 2: Conserved amino acid motifs identified within the eggplant RGAs NBS domains through MEME software analysis. The black line represents the length of different eggplant RGAs and coloured boxes represent the motifs along the length of each eggplant RGAs.

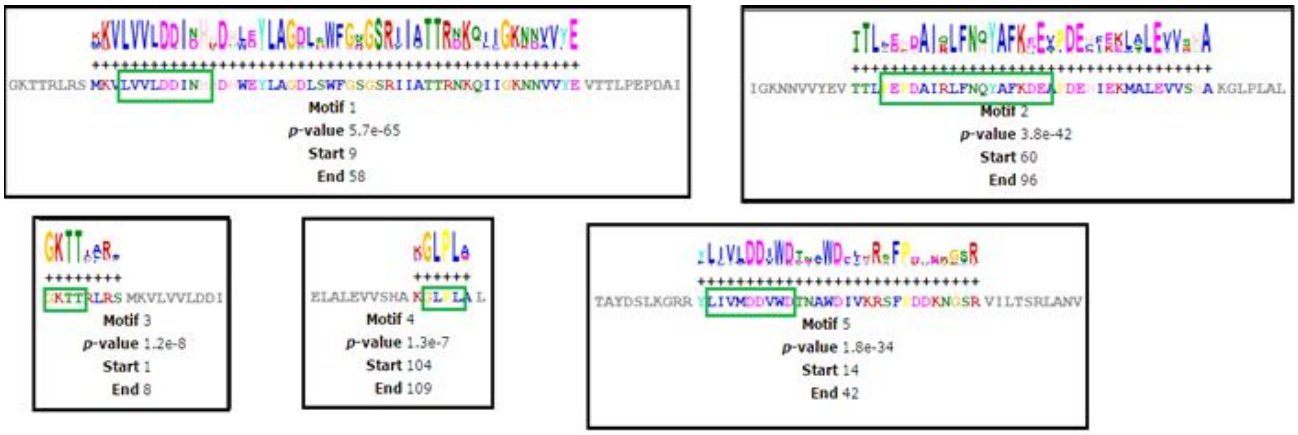

Fig 3: Sequence signature of five major conserved motifs in eggplant RGAs NBS region along with their e-values (motif 1: Kinase 2, motif 2: RNBS-C motif 3: P-loop, motif 4: GLPL, motif 5: and RNBS A-non TIR)

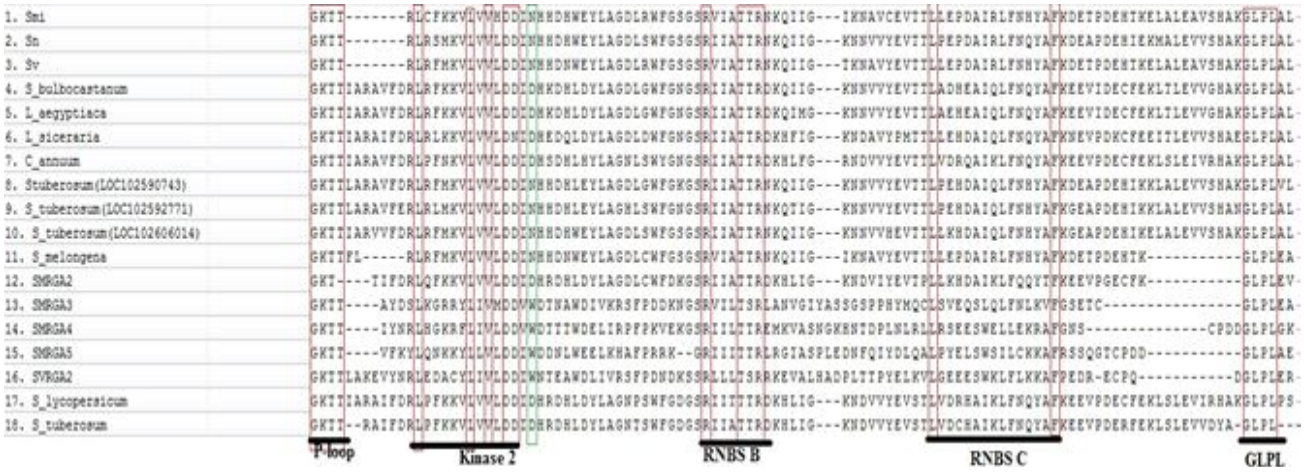

Fig 4: Multiple sequence alignment of eggplant RGAs of current study along with other plant R-genes/RGAs. Majorconserved motifs (P-loop, Kinase 2, RNBS-B, RNBS-C and GLPL) are highlighted in above alignment. Gaps to optimizemultiple sequence alignment are indicated by (.). The constructionof multiple sequence alignment was performed by using the MEGA6.0 software.RNBS-A motif is represented as green box.

Phylogenetic analysis of eggplant RGAs: MEGA 6.0 (Tamura *et al*. 2013) software was used to analyze evolutionary relationship among eggplant RGAs of present study with already characterized known resistance genes from different plants. The phylogeny was constructed using neighbor joining method along Jones Taylor-Thornton (JTT) model with 1000 bootstrap replications. The region between P-loop and GLPL was identified for analysis and it is present in both TIR and non-TIR-NBS-LRR of resistance genes. Sequences were classified into two majortypes - non-TIR and TIR sequences (Fig.5). Non-Toll-interleukin receptor type genes SMRGA3, SVRGA2, SMRGA4 and SMRGA5(Reddy *et al*., 2015) were grouped together in a separate class.The wild species of eggplant in this study along with other closely related plant species belonged to the TIR typedue to the presence of highly conserved motif aspartic acid (D) and aspartate (N) at the end of Kinase 2 domain. Multiple sequence alignment with other known *R* genes in different plants showed significant homology with P-loop, Kinase 2 and GLPL domains of NBS-LRR class

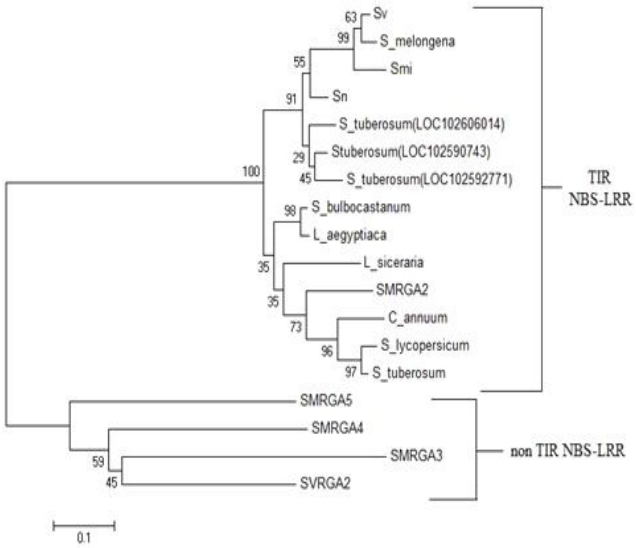

Fig 5: Phylogenetic tree of the deduced amino acid sequences of eggplant RGAs and other plant *R* genes, based on the neighbor joining method. The numbers on the branches indicate bootstrap values (1,000 iterations).

genes. A class of protein conferring resistance to several strains was found in TIR NBS-LRR class of *R* gene products of *R solanacearum* (Deslandes *et al*., 2002). This reinforces the possibility that the newly cloned eggplant RGAs can also function as *R. solanacearum* resistant genes.

CONCLUSION

Plant RGAs are a large group of potential *R*-genes that have conserved domains and structural features which have specific roles in host-pathogen interactions. Studies on *R*-genes and RGAs identification and characterizationwill assistmarker development, cloning of plant resistance genes and resistance breeding (Sekhwal *et al*., 2015). However the mechanism and physiology of the action of NBS–LRR protein has not been fully understood. Information on more *R*-gene sequences is necessary to delineate more structural domains, which is the basis for the search of RGAs in any crop plant (Totad *et al*. 2005).The diversity among the novel RGAs characterized in this study may lead to identification of different *R*- genes within the germplasm of different brinjal plants as well as other species of *Solanum*. Two more potential *R* genes from wild eggplants (*S torvum* and *S surattense*are under investigation, which will provide a better understanding of the role of potential *R* genes and facilitate the utilization of wild species for eggplant breeding.Novel primer sets can also be designed for analysis of brinjal wild relatives to target novel genomic resources for the genetic improvement of this crop.

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