MOLECULAR CHARACTERIZATION AND PHYLOGENETIC ANALYSIS OF *NBS-LRR* GENES IN CULTIVATED AND WILD VARIETIES OF BRINJAL (*Solanummelongena*L)

[1]Sona S Dev*, [2]Sneha Francis and [3]Vineetha K.U
[1]Assistant Professor, [2]PG Student, [3]PG Student
Department of Biotechnology, St.Peter's College, Kolenchery, Ernakulam, Kerala, India

Abstract - Brinjal or egg plant is an agronomically and economically important solanaceous crop. It is rich in nutrients and is very much beneficial to human health. Brinjal is prone to infection by fungal, bacterial and viral pathogens as well as insect pests leading to significant economic loss. Some of the diseases that affect brinjal are fruit rot, *Verticillium* wilt, bacterial wilt etc. In such conditions understanding the molecular basis of disease, pest and herbicide resistance in plants proves to be helpful. The identification of resistance gene analogs holds great promise to minimize the extensive use of fungicides and to develop disease resistant plants. In the present study, degenerate primers based on conserved regions of NBS-LRR were used to amplify and clone Resistant Gene Analogs (*RGAs*) from cultivated (*Solanummelongena*) / wild species (*Solanumsurattense, Solanumtorvum*) of brinjal. Sequencing of the cloned gene were carried out. Comparison of their predicted amino acid sequences with each other and other amino acid sequences of known *R*-genes revealed high level of identity with NBS-LRR family of *RGAs* deposited in GenBank. A phylogenetic tree constructed incorporating the sequences of the closest type of RGAs, using MEGA 6.0 software revealed genetic divergence among the three isolated*RGAs*. *S melongena* and *S.torvum* were closely related, while *S surattense*was in another sub-cluster. The *NBS* analogs that we isolated can be used as guideline to eventually isolate numerous *R*-genes in brinjal.

Key words - *NBS-LRR, Solanum*sp, Resistance Gene Analogs (RGAs)

I. INTRODUCTION

Brinjal or eggplant (*Solanummelongena L.*) is an important solanaceous crop of sub-tropics and tropics. It has originated in the Indian sub-continent and China (Thompson and Kelly, 1957; Purewal, 1957; Martin and Rhodes, 1979). The name brinjal is popular in Indian subcontinents and is derived from Arabic and Sanskrit whereas the name eggplant has been derived from the shape of the fruit of some varieties, which are white and resemble in shape to chicken eggs. It is also called aubergine (French word) in Europe. Brinjals have partial resistance to most of the soil-borne pathogens like fusarium wilt, bacterial wilt and verticillium wilt, but often at insufficient levels. In order to combat the pathogen attack, farmers mostly depend on chemical fungicides. Fungicides are too expensive and since it causes environmental problems and health risks, the other remedy against pathogen is to produce disease resistant plants or to improve the level of disease resistance in plants.

The isolation and characterization of these *R* genes provide information about complex mechanisms of resistance. The disease resistance in brinjal is mainly seen in the wild species than in the cultivated species. Information regarding *NBS-LRR* class *RGA*s in egg plant is very scanty and work presented here explains isolation and characterization of resistance genes analogs in cultivated (*Solanummelongena*) and wild egg plants (*Solanumsurattense, Solanumtorvum*). This would be helpful in understanding and developing cultivated varieties resistant to soil borne pathogens.

The aim of this study was to isolate resistant gene analogs (*RGA*s) from different species of egg plant through amplification of the nucleotide binding site – leucine rich repeat (*NBS-LRR*), molecular characterization and phylogenetic analysis using suitable bioinformatics tools.

II MATERIALS AND METHODS

3.1 Specimen collection and sample preparation

Three species of brinjal (*Solanummelongena (Sm), Solanumsurattense (Ss)* and *Solanumtorvum (St)*) were collected from the houses near and around Kolenchery and from Aromatic & Medicinal Plants Research Station, Odakkali in Ernakulam District, Kerala, India.

Solanummelongena *Solanumsurattense* *Solanumtorvum*

Fig 1: Cultivated and wild varieties of *Solanumsp* used in this study

Isolation of high molecular weight plant genomic DNA is an essential requirement for most genome characterization, mapping and isolation of genes for genetic engineering. DNeasy® Plant Mini Kit was used for DNA isolation from leaves of the above mentioned species of brinjal. The quality of the isolated DNA was judged by Agarose Gel Electrophoresis.

3.2 PCR amplification using specific primers

PCR was carried out for samples *Solanummelongena (Sm), Solanumsurattense (Ss), Solanumtorvum (St)* using a set of primers - 5´GGNGGNRTNGGNAAGACGAC3´ (Ploop – F1) and 5´GAGGGCTAAAGGAAGGCC3´ (GLPL-R1) in Thermal Cycler (Master cycler Gradient-Eppendorf™. The 20µl PCR mix comprised of template DNA (100ng), primers (each 10 µM), Taq buffer (5X), dNTPs (2.5mM), Taq polymerase (5Units/µl) and distilled deionized water. PCR programme consisted of initial denaturation step at 94°C for 4 min followed by 35 cycles of denaturation at 94°C for 30 sec, primer annealing at 55°C for 30 sec and primer extension at 72°C for 1 min; this was followed by a final primer extension at 72°C for 7 min. Amplified product was analysed on 1.5% agarose gel along with 100 bp DNA ladder. The band was excised from the gel with sterile blade under UV-transilluminator and eluted from the gel using Nucleospin® Gel and PCR Clean-Up kit as per the manufacturer's instructions.

3.3 Cloning and sequencing of PCR products

The purified PCR fragments of all the three samples mentioned in the previous section were cloned into plasmid vector pTZ57R/T using InsTAclone PCR Product Cloning Kit (Fermentas Life Sciences) 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.

3.4 Phylogenetic analysis of cloned sequences

The vector sequences and primer sequences were removed with GeneDoc 2.7 software and the region between P loop and GLPL was taken for further analysis. Sequenced data were edited using BioEdit Sequence Alignment Editor (Version 7.2.0), aligned and analysed for finding the closest homolog using National Center for Biotechnology Information (NCBI) GenBank database. A phylogenetic tree was constructed using MEGA (Molecular Evolutionary Genetic Analysis) Version 6.0 (Tamura et al. 2013) with the set of NBS sequence analogs of present study and other closely related plant resistance genes. Cluster analysis was carried out using CLUSTALW (Thompson *et al.* 1997) based on the Neighbour-joining tree (Jones Taylor-Thornton (JTT) model) with 1000 bootstrap replications.

III. RESULTS AND DISCUSSION

Solanumsurattense and *Solanumtorvum*are two wild eggplants while *Solanummelongena*is a cultivated variety of brinjal, mostly prevalent in tropics. *S surattense* commonly known as Kanteli, yellow-berried nightshade, Kantakari, Nidigdhika is a perennial herb and is considered as one of the most useful traditional medicine in India. *S tovrum* commonly known as 'Turkey berry' is a small erect spiny shrub of about 4m tall evergreen and is native of Africa and West Indies (Adjanohoun*et al*., 1996). Its edible fruits, commonly available in the market are used as a vegetable and used for medicinal or ritual purposes.

By using degenerate primers designed in accordance with the sequence of the subdomains P-loop and GLPL that are conserved in the NBS region of *R genes* from other plants, three unique NBS–LRR class RGAs were isolated from the above mentioned eggplants and sequenced. PCR products of expected size (500 bp) were obtained and then purified (Fig 2). DNA fragments of 500-600 bp were considered to be specific for RGA amplicons as earlier attempts with 300 and 900 bp did not produce any significant homology to known *R*-genes (Joshi *et al.*, 2010).

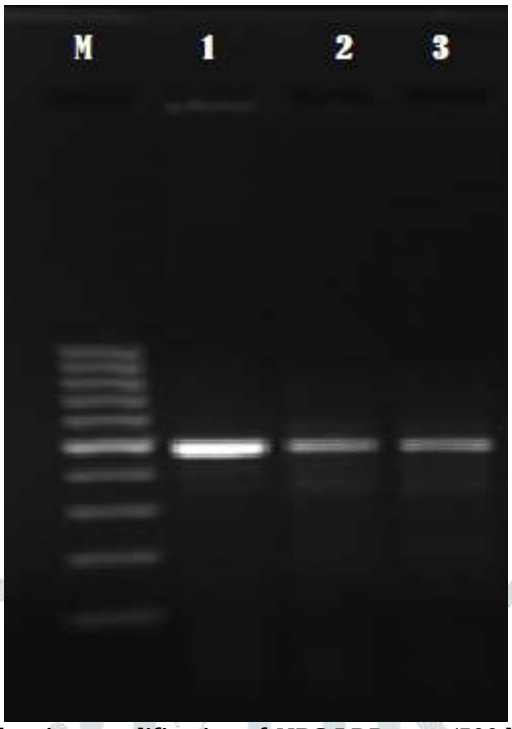

Fig 2: Agarose gel showing amplification of *NBS LRR* gene (500 bp) using primer set
Lane M = DNA marker 100 bp, lane 1: *Solanummelongena* (Sm), Lane 2: *Solanumsurattense* (Ss) and Lane 3: *Solanumtorvum* (St)

The purified PCR fragments of all the three samples mentioned in the previous section were cloned into plasmid vector and transformed into competent *Escherichia coli* strain DH5α to produce blue/white colonies when plated on LB agar plates having Xgal, IPTG and Ampicillin. White coloured colonies obtained on transforming with the PCR amplified RGAs obtained from *S.melongena, S.surattense, S.torvum*were subjected to colony PCR.The plasmids isolated from confirmed colonies were loaded in 1.5% agarose gel and were sequenced. Similar method was followed earlier by Bahramnejjad(2014).

The results obtained on sequencing the plasmids isolated from the three brinjal species were trimmed to remove the vector contamination using GeneDoc 2.7 software. The nucleotide sequence was translated and identity search of the nucleotide and amino acid sequence was done using BLASTN and BLASTP algorithms respectively. This search of the NCBI database indicated that the sequences of these three *Solanum* species showed a high level of identity with several plant *R* genes 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. It was observed that the cloned sequences have substantial variation within nucleotide sequences as well as protein sequences. They were highly similar to *R genes* or the RGAs identified in other plant species, especially *Solanaceae* plants. Thus, these genes, especially those from wild species, may encode resistance gene products of unknown specificity.

In silico analysis of NBS analogue of *Solanummelongena, Solanumsurattense*and *Solanumtorvum*gave the following results. *Solanummelongena* showed 82% identity with *Solanumtuberosum, Solanumpennellii* TMV resistance protein N-like misc RNA .*Solanumsurattense* showed 89% identity with *Solanumtuberosum*TMV resistance protein N-like, 88% with *Lycopersiconesculentum* isolate Q147 nucleotide binding region of resistance-like gene, 87% identity with *Solanumtuberosum* TMV resistance protein N-like *Solanumtorvum* showed 88% identity with *Solanumtuberosum* 87% identity with *Lycopersiconesculentum* isolate Q147 nucleotide binding region of resistance-like gene, partial sequence and 86% identity with *Solanumpennellii* chromosome ch01, complete genome

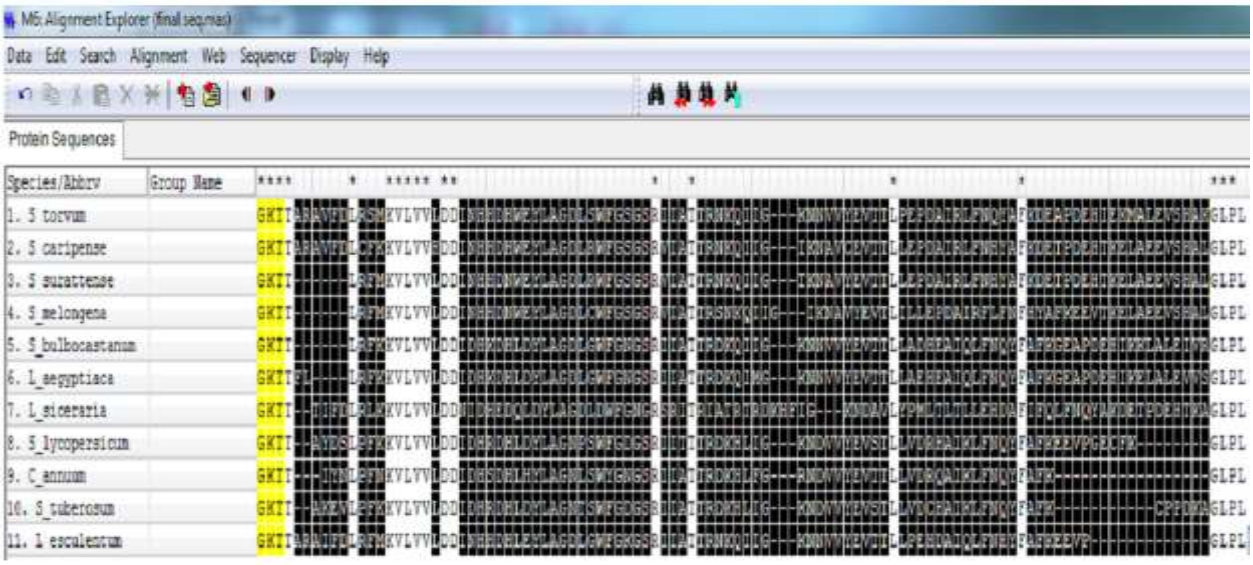

Fig 3:Multiple sequence alignment of eggplant RGAs of current study along with other plant R-genes/RGAs showing major conserved motifs (GKTT/P-loop and GLPL). Gaps to optimize multiple sequence alignment are indicated by (.). The construction of multiple sequence alignment was performed by using the MEGA6.0 software.

Multiple sequence alignment of RGAs isolated in this study with previously isolated RGAs by Reddy et al., (2015) and other known *R* genes for different plants, including *Solanumbulbocastanum, Lagenariasiceraria, Solanummelongena, Solanumtuberosum, Capsicum annum, Solanumlycopersicum, Solanumtuberosum, Luffaaegyptiaca*revealed various conserved motifs of NBS, including the highly conserved P-loop (GKTT) and GLPL (Fig. 3). This explains that the RGAs isolated in this study may be considered as NBS-LRR class of resistance genes..

To estimate the genetic divergence between the three *Solanum* species and the species selected based on the sequence similarity, a neighbor-joining phylogenetic tree was constructed (Fig 4). Sequences were classified into two major clusters; One cluster comprising of *S. bulbocastanum, Luffaaegyptiaca, L. esculentum, C. annuum, S. tuberosum, S. lycopersicum*and *L. siceraria* the other consisting of *S. surattense* (as one sub cluster), *S. caripense* (as one sub cluster) S. *melongena* and *S. torvum* (another sub cluster).Among the three isolated RGAs, *S. melongena* and *S.torvum* were very close together, while *S. surattense*was in another sub-cluster. These diversities were of evolutionary time factor. A similar method was followed in phylogenetic tree construction of resistant gene sequences in citrus (Deng et al., 2000). Phylogenetic analysis identified the relatedness between different brinjal sequences as well as with the reported sequence of other plants. The present study confirms the presence of NBS LRR type gene in the wild and cultivated varieties of brinjal. Different genetic mechanisms have been proposed for the evolution of *R* genes, for example recombination, unequal crossing-over, gene conversion, and point mutations (Michelmore et al., 1998).

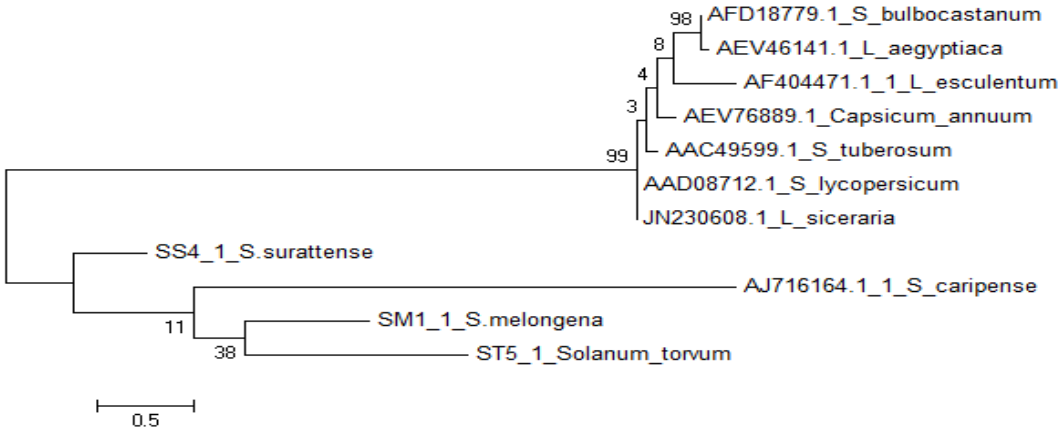

Fig 4: Phylogenetic tree of the reported NBS LRR sequence with similar sequences. Bootstrap re sampling values are indicated at the main branches

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. The results provided a valuable genomic resources and insight for functional and evolutionary studies of *NBS LRR* genes in *Solanum* species. By using phylogenetic reconstruction, we can analyse the phylogenetic distribution of the nucleotide binding site–leucine rich repeat (*NBS-LRR*) architecture to demonstrate that using the new genomic technologies genetic determinants which provides resistance to diseases in plants can be easily identified. 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). Further investigation is therefore needed to identify different RGA's within the germplasm of different brinjal species so as to facilitate the genetic improvement by marker assisted selection as well as genetic engineering.

REFERENCES

[1] Adjanohoun, J.E., Aboubakar, N., Dramane, K., Ebot, M.E., Ekpere, J.A., Enoworock, E.G., Focho, D., Gbile, Z.O., Kamanyi, A., Kamsu, K. J., Keita, A., Mbenkum, T., Mbi, C.N., Mbiele, A.C., Mbome, J.C., Muberu, N.K., Nancy, W.L., Kongmeneck, B., Satabie, B., Sowora, A., Tamze, V., Wirmum, C.K.1996. Organization of African Unity; Scientific, Technical and Research Commission, editor. *Traditional Medicine and Pharmacopoeia*: Contribution to ethno botanical and floristic studies in Cameroon.

[2] Bahramnejad, B. 2014. Identification and characterization of a NBS–LRR class resistance gene analog in *Pistaciaatlantica* subsp. Kurdica. MolBiol Res Commun, 3(3): 175–185.

[3] Deng, Z., Huang, S., Ling, P., Chen, C., Yu, C., Weber, C., Moore, G.A., Gmitter, Jr. F.G. 2000. Cloning and characterization of NBS-LRR class resistance-gene candidate sequences in citrus. TheorAppl Genet,101:814–822.https://doi.org/10.1007/s001220051548

[4] Ferrier-Cana, E., Geoffroy, V., Macadre´, C., Creusot, F., ImbertBellore´, P., S´vingac, M., and Langing, T. 2003. Characterization of expressed *NBS–LRR* resistance gene candidates from common bean. Theor. Appl. Genet., 106: 251–261. https://doi.org/10.1007/s00122-002-1032-z.

[5] Huettel, B., Santra, D., Muehlbauer, F.J., and Kahl, G. 2002. Resistance gene analogues of chickpea (*Cicerarietinum* L): isolation, genetic mapping and association with a *Fusarium* resistance gene cluster. Theor. Appl. Genet., 105: 479–490.doi:10.1007/s00122-002-0933-1

[6] Joshi, R.K., Mohanty, S., Subudhi, E., Nayak, S. 2010. Isolation and characterization of *NBS-LRR*- resistance gene candidates in turmeric (*Curcuma longa* cv. surama). Genet Mol Res, 9(3):1796-806. doi: 10.4238/vol9-3gmr910.

[7] Martin, F.W and A.M. Rhodes. 1979. Subspecific grouping of eggplant cultivars. Euphytica, 28: 367-383.https://doi.org/10.1007/BF00056595.

[8] Michelmore R W, Shen K A, Meyers B C, Islam- Faridi M N, Chin D, Stelly D M (1998), Resistance gene candidates identified by PCR with degenerate oligonucleotide primers map to clusters of resistance genes in lettuce. Mol Plant Microbe Interaction**,** 11:815-823.

[9] Purewal, S.S., 1957. Vegetable cultivation in North India. ICAR Farm Bulletin No. 36, Indian Council of Agricultural Research (ICAR), New Delhi, India.

[10] Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. MolBiolEvol, 30(12):2725-9. doi: 10.1093/molbev/mst197.

[11]Thompson, C.H. and C.W. Kelly. 1957. Vegetable Crops. McGraw-Hill Book Co. Inc., New York, Pages: 501.

[12] Thompson, J.D., Gibson, T.J., Higgins DG. 2002. Multiple sequence alignment using ClustalW and ClustalX. *CurrProtoc Bioinformatics.* ; Chapter 2: Unit 2.3. doi: 10.1002/0471250953.bi0203s00.

[13] Totad A S, Fakrudin B, Kuruvinahetti M S, 2005, Isolation and characterization of resistance gene analogs (RGAs) from Sorghum (*Sorghum bicolor* L. Moench). Euphytica,143: 179-188.

[14] Yaish, M.W.F., Sa´ez de Miera, L.E., and Pe´rez de la Vega, M. 2004. Isolation of a family of resistance gene analogue sequences of the nucleotide binding site (NBS) type from *Lens* species. Genome, 47: 650–659. doi:10.1139/g04-027.