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Isolation and Characterization of NBS-LRR Resistance Gene in Banana (Musa *AAB cv. Nendran*)

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ABSTRACT

Banana which contributes significantly to food security, is plagued by a number of diseases due to its narrow genetic background and sterility. This results in a significant reduction in the production of banana. Identifying varieties of bananas with disease resistance genes can overcome this problem. One of the largest plant R-gene families encode proteins with nucleotide-binding site (NBS) and C-terminal lucine-rich repeat (LRR) domains. Conserved sequences of NBS-LRR gene may be used for the identification and isolation of plant defense genes in banana. In this study, a 517bp fragment of NBS-LRR type gene of *Musa AAB cv. Nendran* was amplified and sequenced (GenBank Accession no. JN033808). Nucleotide sequence comparison revealed 97-99% similarity with the NBS-LRR sequence of different *Musa* varieties. Identification of NBS-LRR type gene in the Nendran variety represents a source of disease resistance gene for transfer across different varieties. Further studies have to be conducted to identify NBS-LRR RGAs(resistance gene analogues) and to explore their diversity in *Musa AAB cv. Nendran*. Polymorphic RGAs may be developed as potential genetic markers for marker assisted selection of disease resistant banana and also for crop improvement through genetic transformation experiments.

Keywords : Musa AAB cv.Nendran, Disease resistance, R-genes, NBS-LRR

INTRODUCTION

Banana is one of the most important food crops in the world. India is the world's largest producer of banana. Most of the banana cultivars available today are susceptible to major diseases such as yellow sigatoka, panama disease, bunchy top disease etc. Currently, the most prevalent method used for combating these diseases is chemical control which may cause damage to the environment. An alternative to this is developing innate resistance in banana by various techniques. Because of the sterility of banana, conventional breeding methods are not suitable [6]. Hence, identification of disease resistance genes and transforming different varieties of bananas with these genes is the ideal solution for the disease ravaged banana cultivation.

Plants have defense mechanisms against pathogen infection that are produced prior to infection or during pathogen attack [7]. The presence of disease resistance(R) genes within plants has been

known since the early 1900s. Flor discovered the gene- for- gene hypothesis. It states that plant contains resistance (R) genes and pathogen contains avirulance (Avr) genes [12]. When a plant is infected by a pathogen, R gene recognize avr gene and initiate a defense response. This defense response is called hypersensitive response (HR). The HR leads to cell death at the site of pathogen attack which prevents disease progression and protect them from the pathogen. The absence of either of these genes leads to disease [5].

R protein activation leads to the initiation of plant defense reaction both locally (Local acquired resistance, LAR) and systemically (Systemic acquired resistance, SAR). LAR occur in the cells adjacent to infection. It results in the activation of defense genes in and surrounding the infected cell. This gene induces the salicylic acid biosynthesis, ethylene biosynthesis, strengthening of cell wall, lignification and production of various antimicrobial compounds [1]. It prevents the proliferation and further attack of pathogens. SAR is the signal transduction pathway which protects the non infected cells [10].

Five different classes of R genes have been identified. The most important class of R genes is Nucleotide binding site Lucine rich repeat (NBS-LRR) which belongs to class II [9]. This class of R gene contains an N terminal NBS domain and a C terminal LRR domain. NBS domain acts as an intramolecular signal transducer. It is also involved in pathogen recognition and signal transduction. LRR domain facilitate the recognition of pathogen attack by ligand binding which helps in ATP/ GTP binding and hydrolysis at the NBS domain. This provides energy for the activation of amino terminal domain to induce signal transduction [2].

Although a few NBS-LRR genes have been identified in different *Musa* varieties, this study has not been extended to indigenous varieties. The objective of this study was to amplify a specific NBS-LRR sequence from indigenous *Musa AAB cv Nendran.* The sequence analysis was done and evolutionary relationship among the various previously reported NBS-LRR sequences was studied.

MATERIALS AND METHODS

Plant material and DNA extraction

Musa AAB cv.Nendran leaves were used as the experimental sample which were collected from Kerala Agricultural university, Mannuthy,Thrissur. Total genomic DNA was isolated using a standard CTAB procedure and then analysed on agarose gel. The quantity and quality of the DNA was determined with a UV-spectrophotometer.

Primer Designing

For designing gene specific primers NBS-LRR gene sequence of *Musa acuminata* Var. Grand Nain (GenBank Accession Number: AJ534312) was selected as referrence. The primers were designed using the software Perl primer.

PCR amplification

PCR reaction was carried out in a 50µl reaction volume , containing 50ng of template DNA, 1.5mM $MgCl_2$, 0.2mM dNTPs, 10 picomoles of each primer, 1U of taq polymerase and 1X taq buffer. PCR was performed in an Eppendorf Mastercycler Personal using the following cyclic conditions: initial denaturation at 95ºC for 2 minutes, followed by 35 cycles each consisting of 94ºC for 30 seconds, 60ºC for 30 seconds and 72ºC for 30 seconds. A final extension at 72ºC for 10 minutes was also given. PCR product was analysed on a 2% agarose gel in 1X TAE buffer. The amplicon of expected size was gel eluted and purified using MinElute gel extraction kit (QIAGEN).

Cloning and Sequencing

The purified PCR product was ligated to a T/A cloning vector pTZ57R/T (Fermentas) and transformed into JM107 strain of E.coli using Ins TA clone PCR cloning Kit (Fermentas). The transformed recombinant

colonies were selected by blue white screening and the presence of the insert was confirmed by colony PCR. The plasmid DNA was isolated from overnight culture using GeneJET^{TM} Plasmid Miniprep Kit (Fermentas) and sequenced with insert specific primers at SciGenome, Cochin.

Sequence analysis

The sequence was analysed and edited using the software BioEdit [4]. It was submitted to GenBank. Homology search was performed using BLAST to identify similar sequences in the database. The sequence was compared and aligned with GenBank Sequences (GenBank Accession no. DQ119306, EU239821, AJ534312, EU855841, XM002448137, XM002448136, XM002458574 and AK330516) using ClustalX [13] multiple alignment program . The nucleotide sequences were translated and the corresponding amino acid sequences were aligned. Percentage of identity and similarity between nucleotide and amino acid sequences were determined by the ClustalW version 2 [8].

Phylogenetic Analysis

Phylogenetic analysis was performed using the 517bp partial NBS-LRR sequence obtained from the sample, *Musa AAB cv. Nendran* (GenBank Acc no. JN033808). Relationship among aligned nucleotide sequences of various NBS LRR gene sequences were determined using PHYLIP program. The evolutionary distance were calculated using PHYLIP software package (version 3.2) [3]. Phylogenetic tree was generated by Neighbour Joining method [11] and drawn by TREE EXPLORER program. Bootstrap analysis was done to determine confidence values on the clades within the tree.

RESULTS

PCR amplification and sequencing of targeted NBS-LRR fragment

Primers NBSF 5'-GAGCATGAGGAACCAAAGAG-3' and NBSR 5'-GGACAACGA ACTTCTTCCTCC-3' designed using Perl primer was used to amplify NBS-LRR gene from *Musa AAB cv. Nendran* (fig 1). The amplified product was gel eluted and cloned (fig 2). The recombinant clone sequenced using gene specific primers. This sequence was deposited in the GenBank database (Accession no. JN033808).

Sequence analysis

Nucleotide and aminoacid sequence comparison revealed that Nendran Variety showed 97-99% identity with the NBS-LRR sequence of different *Musa* varieties. The NBS sequence of Nendran Variety showed 99% identity with *Musa acuminata* NBS-LRR type disease resistance protein mRNA (GenBank Acc no. DQ119306), 98% identity with *Musa acuminata* subsp. malaccensis (GenBank Acc no. EU239821) and Musa ABB Group NBS resistance protein (RGA-K) gene (EU855841.1) and 97% identity with *Musa acuminata* AAA group Var. Grand Nain (AJ534312.1).

Phylogenetic analysis

Phylogenetic analysis was done using PHYLIP software to analyse the evolutionary pattern (fig 3). The statistical significance of tree was assessed by Bootstrapping. It was observed that NBS-LRR sequences of different varieties of *Musa* formed a single clade with a bootstrap value of 90 indicating common ancestry. This analysis demonstrated that NBS-LRR sequences of all the studied *Musa* varieties fall into a single phylo group. They clustered together to form a distinct clade. The sequences of *Sorghum bicolor* species shared common ancestry with Musa. The sequences of Sorghum were much more evolved than the different Musa species as evidenced from the phylogenetic tree.

DISCUSSION

Banana being the second most important fruit crop in India after mango, has immense socioeconomic importance. The indigenous commercial varieties of banana are plagued by diseases which affect its

yield. Isolation and identification of the disease resistance genes in these varieties may facilitate marker assisted selection as well as genetic improvement of susceptible varieties. In this study, we amplified NBS-LRR sequence from *Musa AAB* cv. *Nendran.* Gene specific primers were used to amplify NBS-LRR sequence of expected size. A 517bp partial gene was amplified and the amplified product was sequenced. The nucleotide sequence of *Musa AAB* cv. *Nendran* was translated and identity search of the nucleotide and aminoacid sequence was done using BLASTN and BLASTP algorithms respectively.

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. Multiple sequence alignment of *Musa AAB cv. Nendran* NBS-LRR gene and known R genes showed significant similarity among both nucleotide and aminoacid sequences. *Musa* genus itself showed a high level of similarity ranging from 97-99%. Phylogenetic tree based on the Neighbour Joining method using percentage identity of the reported NBS-LRR sequences identified their relatedness between different Musa sequences as well as with the reported sequence of other plants. It was observed that all the NBS-LRR sequence of Musa was included into a single clade. The grouping of Musa sequences into a single clade and remarkable sequence similarity is indicative of very recent evolutionary divergence. The present study confirms the presence of NBS-LRR type gene in the indigenous variety of *Musa*. Detailed study is needed to identify different RGAs in the wide array of indigenous *Musa* varieties. Information on more resistance gene sequences is necessary to explore the diversity and identify the ideal resistance gene analogues for the genetic improvement of Musa.

*Mus*a being a crop predominantly dependent on asexual vegetative propagation has a limited genetic variation due to their narrow genetic base. This has been a limiting factor in the development of natural resistance to different infections which plague the banana. The current strategy of agrochemical control of diseases is highly inappropriate as it causes grave environmental and socioeconomic problems. Development of alternate strategies is crucial for the survival of the banana plantations in this regard. Identification of disease resistance gene within the germplasm of indigenous *Musa* varieties will facilitate the genetic improvement of banana by marker assisted selection as well as genetic engineering.

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FIGURES

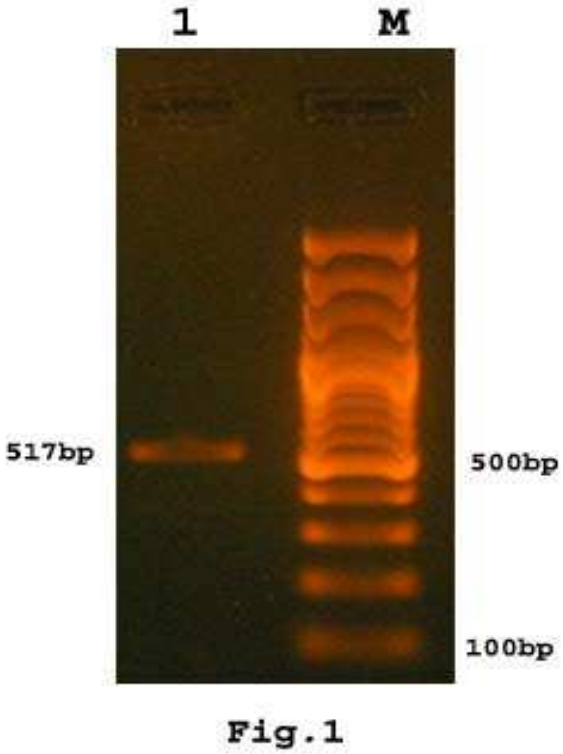

Fig.1

Figure 1 : PCR Ampilification of a 517 bp of NBS- LRR Gene
Lane M : 100 bp Ladder
Lane 1 : 517 bp NBS- LRR Gene

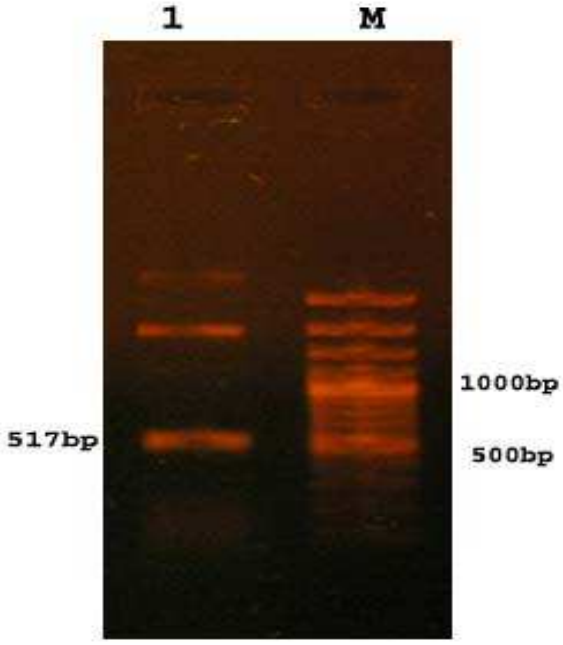

Fig.2

Figure 2 : Clone confirmation; pTZ57R/T-NBS-LRR clones were confirmed by PCR using NBS-LRR gene specific primers
Lane M: 100 bp Ladder
Lane 1: 517 bp NBS-LRR PCR product amplified from recombinant plasmid

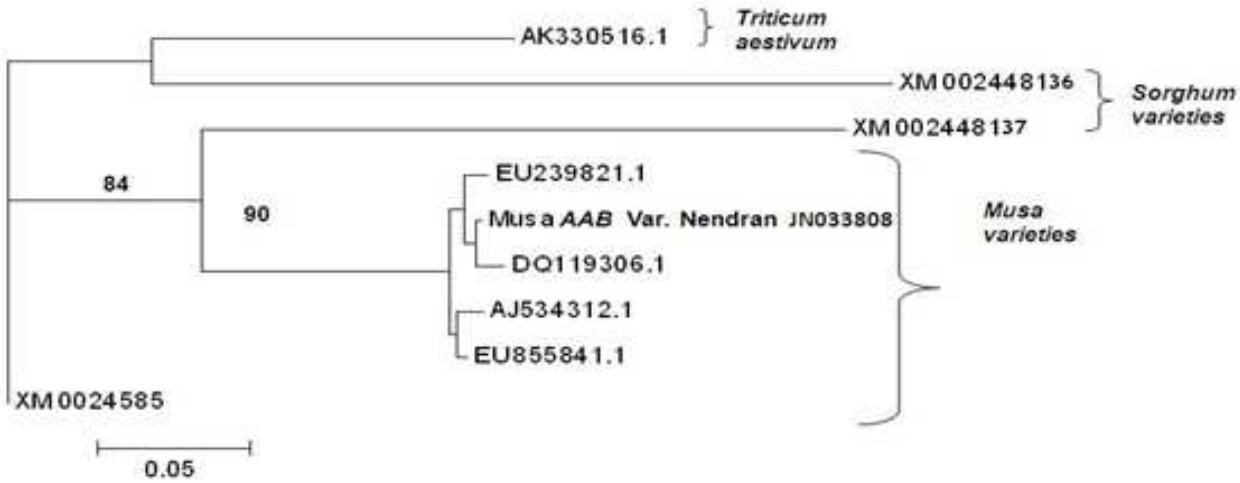

Figure 3 : Phylogenetic Tree of the Reported NBS-LRR Sequence with Similar Sequences. Bootsrap resampling values are indicated at the main branches. The analysis showed the clustering of all Musa varieties into a single clade with a bootstrap value 90.