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Full Length Research Paper

Cloning and characterization of NBS-LRR resistance gene analogues of *Musa* spp. and their expression profiling studies against *Pratylenchus coffeae*

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Resistance gene analogues (RGAs) were isolated from two banana cultivars viz., Karthobiumtham and Rose using degernate primers designed from the conserved motifs of different plant resistance genes. A total of 40 sequences were hit with various R genes, of which 20 sequences were having uninterrupted open reading frame (ORFs). Based on the conserved domains like P loop, internal kinase 2, kinase 3a and hydrophobic domain motifs of the deduced amino acid sequences were grouped as NBS-LRR class of resistant genes. The phylogentic analysis of RGAs showed that all the *Musa* RGAs are grouped under non-TIR branch and grouped into six distinct *Musa* RGA cluster. To investigate the expression profile of the RGAs, specific primers were designed for one representative RGA from each RGA cluster and it was found that C1 and C5 were induced upon root lesion nematode infection in the resistant (cv. Karthobiumtham) and not in susceptible (cv.Nendran) cultivar. C6 was expressed only in resistant cultivar not in susceptible one. But there was no change in the expression of C2 and C3 in both resistant and susceptible cultivars. These results indicate that in depth study on C1, and C5 RGAs will be helpful for further improvement of *P. coffeae* resistance in banana.

Key words: Banana, *P. coffeae*, resistance gene analogues, expression level.

INTRODUCTION

Pest and disease resistance genes (R-genes) comprise a large and diverse group of related sequences in plant genomes. Based on common molecular features, R-genes can be broadly divided into at least five broad structural classes (Hammond-Kosack et al., 1997). Among these, the second gene family (Class II), represented by majority of the functionally described R-genes (72%), encodes cytoplasmic receptor like proteins that contain a leucine rich repeat (LRR) domain and a nucleotide binding site (NBS). Genes from the NBS-LRR class condition resistance to bacterial, fungal and viral pathogens, aphid and nematode pests, have been cloned from a number of plants including *Arabidopsis thaliana* (Bent et al., 1994; Mindirons et al., 1994; Grant et al., 1995), flax (Lawrence et al., 1995), tobacco (Whitham et al., 1994), tomato (Milligan et al., 1998) and rice (Yoshimura et al., 1998). Young (2000) stated that NBS-LRR and other R genes are organized in large, extended clusters in the genome.

The presence of conserved domains in resistance genes gave an opportunity to clone numerous additional resistance genes from diverse species by polymerase chain reaction (PCR) with degenerate oligo-nucleotide primers to the conserved motifs (Joyeux et al., 1999; Deng et al., 2000; Donald et al., 2002; Lacock et al., 2003; Totad et al., 2005). Aarts et al. (1998) reported that

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1 to 2% of the total coding capacity of the *Arabidopsis* genome is contributed by the NBS-LRR sequences, and genetic mapping studies of resistance gene analogues (RGAs) provided evidence that they co-segregated with resistance markers. The RGA fragments were also used as molecular markers for tagging the disease resistance loci in *A. thaliana* (Aarts et al., 1998), wheat (Chen et al., 1998), cocoa (Lanaud et al., 2004), etc.

Therefore, isolation of RGAs could be an effective strategy to identify genomic regions linked to disease resistance (Yu et al., 1996). These RGAs can be transformed into molecular markers for use in marker assisted selection or even lead to the cloning of the full length functional R genes (Kuhn et al., 2003; Quint et al., 2003).

Banana (*Musa* spp.) is one of the most important horticultural crops produced in tropical and subtropical countries of the world. The ruling cultivars of banana are highly susceptible to over 50 fungal pathogens, as well as a number of bacterial pathogens, nematodes (*P. coffeae*, *Radopholus similis*, *Meloidogyne incognita*, *Pratylenchus coffeae* and *Helicotylenchus multicinctus)*, viruses and insect pests.

Greatest threats to global banana production are currently caused by the fungal pathogens *Mycosphaerella* complexes, (*M. fijiensis*, *M. mucicola,* etc.) causal organism of leaf disease and *Fusarium oxysporum* f. sp. *cubense* race 1 to 4, which causes *Fusarium* wilt (Ploetz, 1993) and next to this root lesion, nematode (*P.coffeae*). Bridge et al. (1997) observed 62% of yield reduction due to *P. coffeae* in plantain. Although some of the land races and wild species are found to be resistant to many pest and diseases, introgression of resistance into edible cultivars through conventional breeding is a difficult task owing to problem associated with the polyploidy and low fertility (Roux et al., 2004).

These drawbacks of conventional breeding can be conquered through the use of plant resistance genes (R-genes) by molecular breeding approaches. A better understanding of the molecular and genetic basis of genes conferring resistance would enhance the effectiveness of *Musa* improvement programme. Nevertheless, the identification and characterization of RGAs provides new tools that will allow studies on the evolution of the diverse regions of R-genes like sequences and their implications for plant -pathogen interactions. Peraza-Echeverria et al. (2008) isolated and cloned a full length R gene (RGC2) which is resistance to FOC race 4.

This showed potential source of R genes in *Musa,* which encouraged isolating the nematode resistant R genes. Hence this present study was carried out 1) to isolate the RGAs; 2) to evaluate their diversity and phylogenetic relationships to the known R-gene from other plant species and 3) to examine the transcript expression profiles of different *Musa* RGAs in *P. coffeae* resistant and susceptible cultivar.

MATERIALS AND METHODS

DNA isolation

Cigar leaves of two resistant cultivars (Karthobiumtham AAB and Rose AA) were collected from National Research Centre for Banana, Trichy, India. Total genomic DNA was isolated by using the modified CTAB protocol as described by Sambrook and Russel (2001).

Primers and PCR conditions

Twelve (12) degenerate primers were designed from the conserved motif of the R-genes of different species and listed in Table 1. PCRs were carried out in a total volume of 25 µl containing 20 ng template DNA, 0.25 µM of each primer, 1.5 mM MgCl$_2$ and 0.5 U of *Tag polymerase* (Sigma). Cycling conditions consisted of a 3 min initial denaturation at 94°C followed by 40 amplification cycles consisting of 94°C for 1 min, 45 to 60°C (Table 1) for 45 s and 72°C for 1 min and a final extension of 72°C for 7 min. PCR products were separated by electrophoresis on 1% agarose gel and stained with ethidium bromide for visualization.

Cloning and analysis of PCR products

Bands of the expected size of amplification were excised from gel and purified using the PCR elution kit (Qiagen). The obtained DNA was cloned using the pGEM-T Easy vector system following the manufacturer's instructions (Promega, Madison, Wis) and transformed into *Escherichia coli* DH5α. Recombinant plasmid DNA was extracted using alkaline lysis, and digested with *Eco*RI to verify the presence of the expected insert. For discrimination between the different RGA sequences, clones were characterized by restriction analyses with *Hin*fI and *Rsa*I and classified according to their restriction pattern. After visual inspection, at least one clone of each class was selected for sequencing.

Sequence analysis

The sequences obtained were first exposed to the VecScreen algorithm (http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html) in order to remove the contaminating vector sequences. BLASTN, algorithms (Altschul et al., 1997) were used to compare the insert sequences to sequences available at the Entrez nucleotide and protein databases (http://www.ncbi.nih.gov/BLAST). The CLUSTALW algorithm (Thompson et al., 1997) was used for the multiple sequence alignments and the BL2SEQ algorithm (Altschul et al., 1997) was used for comparing two aminoacid sequences to each other. Phylogenetic analyses were conducted using the MEGA software (version 3) (Kumar et al., 2004). Robustness of clustering was checked by bootstrapping 1000 replicates. Searches for ORF were done using ORF finder at the NCBI server.

Inoculation of nematode and sample collection for RNA isolation

Suckers of nematode resistant (Karthobiumtham) and susceptible (Nendran) cultivars were planted in individual earthen pots containing sterilized mixture of soil, sand and farm yard manure (2:1:1) and kept under green house condition. Nematode infected root samples of Nendran were collected from *P. coffeae* sick plot and root lesion nematode was extracted and used as nematode inoculums. One month after planting individual plastic cup, hole on the side was placed by removing the soil near the plants in pots and

Table 1. Degenerate primers used to amplify RGAs from resistant cultivars of Musa.

S/N	Primer	Oligonucleiotide sequence (5'-3')	Anealing temperature (°C)	Amplified	Expected size (bp)	Conserved domain	Conserved domain
1	P6-F P6-R	GGACCTGGTGGGGTTGGGAAGACAA CAACGCTAGTGGCAATCC	60	+	500	P- loop GLPL	Ohmori et al. (1998)
2	S2 P6-R	GGIGGIGTIGGIAAIACIACI CAACGCTAGTGGCAATCC	45	+	500	P- loop GLPL	Kanazin et al. (1996)
3	F11 R11	GGDGTDGGNAARACWAC AGIGCHAGNGGNAGNCC	46	-	500	P- loop GLPL	Chen et al. (2007)
4	P12-F P12-R	TAGGGCCTCTTGCATCGT TATAAAAAGTGCCGGACT	50	+	500	LRR LRR	Kanazin et al. (1996)
5	LRR-F LRR-R	CCGTTGGACAGGAAGGAG CCCATAGACCGGACTGTT	48	+	450	LRR LRR	Nair and Thomas (2007)
6	NBS- F NBS-R	GGAATGGGNGGNGTNGGNAARAC YCTAGTTGTRAYDATDAYYYTRC	50	+	350	P-loop RNBS B	Yu et al. (1996)
7	S2 AS3	GGIGGIGTIGGIAAIACIACI IAAIGCIAGIGGIAGICC	45	+	500	P- loop GLPL	Mago et al. (1999)

those cups were filled with potting mixture. Single root was selected in each plant and inserted through the hole into the plastic cup. Each cup was inoculated with 3000 active root lesion nematodes. The root samples were collected at 0, 2, 3, 4, 5, 6 and 7 days after inoculation from nematode inoculated cup in three biological replications. Roots were washed with DEPC water and frozen by using liquid nitrogen and kept in -80°C for later analysis.

Root RNA isolation and cDNA Synthesis

Total RNA was extracted from the *P. coffeae* infected as well as uninfected roots of cv. Karthobiumtham and cv. Nendran using the modified protocol of Clendennen and May (1997).   The total RNA was treated with DNAase for

removing the genomic DNA contamination from RNA. The purity of RNA was verified by optical density (OD) absorption ratio OD260 nm/OD280 nm between 1.80 and 2.06 (mean = 2.0). RNA quality was analyzed using the RNA6000 Nano LabChip® Kit (Agilent Technologies GmbH, B¨oblingen, Germany) and the Agilent 2100 bioanalyser (Agilent Technologies) for electrophoretic separation. RNA quantities acquired by the Agilent 2100 bioanalyser were relatively regarded the same as measured spectrophotometrically. Constant amount of total RNA (1 µg) were used for isolating the poly A + mRNA. Purification of poly A + mRNA were performed using a Qiagen Oligotex mRNA kit for isolating mRNA from total RNA (Cat. # 70042) following the manufacturer's protocol given. First strand cDNA was synthesized from mRNA by reverse transcription with oligo-(dT) primers according to

the manufacturer's protocol (Promega corp.)

Expression analysis by semi-quantitative RT-PCR

To analyse the transcript level of the RGAs in nematode inoculated root tissues, cDNAs isolated from roots of *P. coffeae* resistant cv. Karthobiumtham and susceptible cv (Table 2). Nendran harvested at different days after nematode inoculation were used. Three biological replicates were used for expression analysis and two technical replicates were analysed for each biological replicate. The specific primers which are designed from each RGA family were used in the amplification of root cDNA both cultivars (Table 3). The PCR program initially started with a 94°C denaturation for 5 min, followed by 20 to 30 cycles of 94°C/1 min, 52-58°C /1 min, 72°C/1 min.

Table 2. Number of RGAs obtained in cv. Karthobiumtham and cv. Rose with different degenerate primer combinations.

Primer combination	Karthobiumtham			cv. Rose		
	Number of clones sequenced	Number of RGAs	Number of RGAs without stop codon	Number of clones sequenced	Number of RGAs	Number of RGAs without stop codon
P6F/ P6R (I)	12	11	3	2	2	2
P12 F/ P12R (II)	6	0	0	2	1	0
S2/P6R (III)	10	7	4	21	16	8
NBS- F/NBS-R (IV)	6	0	0	3	0	0
LRR-F/ LRR-R (V)	5	0	0	3	0	0
S2/AS3 (VI)	4	2	1	5	1	0
Total	43	20	8	36	20	10

Table 3. Sequence homology comparisons with highest similarity between consensus sequences, sequences of specific primers and size of the expected products of Musa RGA family.

Musa RGA family	GenBank accession	Identity (%)	E value	Specific primer	Sequences of specific primer		PCR Product sizes (bp)
					Forward primer (5'-3')	Reverse primer (5'-3')	
A (5)	*Vitis riparia* isolate rgVrip126 resistance protein candidate gene AY427192.1	76	1e^{-09}	C1	TGATGTGTGGAATGAGAACGA	CAAGAGCCAGCAATGTTCAA	175
B (3)	*Musa acuminata* AAA NBS-LRR disease resistance protein, cultivar Pisang Bakar, AM931368.1	98	0.00	C2	CGTGGAGAGGCTTACCAAAG	GCCAACCATTTCTGCAATCT	250
C (3)	*Musa ornata* NBS-LRR disease resistance protein AM931420.1	91	0.00	C3	CCTGGAGAGCCTTACGAAAG	GTACTGCGGACCTCAATGGT	194
D (3)	*Musa balbisiana* NBS-LRR disease resistance protein, AM931401.1	98	0.00	C4	CCTGGACAGGCTTACCATAC	AACCATGTCGGCAATCTTTC	247
E (1)	*Oryza sativa* subsp. japonica NBS-LRR-like protein (YR38) pseudogene, AF227002.1	75	3e^{-10}	C5	CAAGAGCCAGCAATGTTCAA	GCAGTGATTTGCAAGCCTTA	248
G (4)	*Musa acuminata* subsp. microcarpa NBS-LRR disease resistance protein, AM931390.1	91	0.00	C6	CGTCGGGAGGCTAACCAAAG	CCTGGTTCTCCGTACCTCAA	198

The number of RGA in RGA family is listed following the family name GenBank accession, identity and E value given in the table is specific to the RGA where primer has been designed.

4

Linear amplification range for each gene was tested on the adjusted cDNA. The number of cycles was optimized for each target so that the PCR product did not reach plateau levels. *Musa* 25S rRNA (AY651067) (5'ACATTGTCAGGTGGGGAGTT-3'; 5'-CCTTTTGTTCCACACGAGATT-3'), was used as an internal control gene since its expression remains relatively constant (Van den Berg et al., 2007). To normalize the transcript levels in each samples, the intensity of the band corresponding to each mNRA was divided by the intensity of the band corresponding to the 25S rRNA.

Statistical analysis

The normalized values were reported as means ± SE and statistical analysis was performed by student's t test. Thresholds for significance were set at $P<0.05$ level.

RESULTS AND DISCUSSION

PCR amplification and cloning of targeted RGA fragment

Seven combinations of 12 degenerate primers which were designed based on conserved motif of resistance genes from other plant species were used to amplify RGAs in the genomic DNA of resistant cultivars namely Karthobiumtham and Rose. Out of the seven combinations, only six combinations namely P6F/P6R, S2/AS3, P12F/P12R, LRR-F/LRR-R, NBS-F/NBS-R and S2/P6R, amplified the expected size in both the cultivars (Table 1). All the amplified products of expected size alone were gel purified and digested with different frequent cutter enzymes (*Rsa*I, *Hae*III and *Hinf*I) which resulted in many fragments, with the sum of molecular weights of all the restriction fragments being much greater than the molecular weight of the original product indicating the presence of a heterogeneous product. This showed that each band consisted of many RGA sequences. The PCR products of all the six primer combinations were cloned separately from each resistant cultivar. Altogether, a total of 79 clones which were showing different restriction patterns of the insert and occasionally, several clones of identical patterns were chosen and further characterized by sequencing and sequence analysis.

Searches of the GenBank database, using the BLASTX algorithm, revealed that out of 79 sequences, only 40 were hit with RGAs or known R-genes from other species. Out of the six primer combinations, I, II, III and VI combinations amplified the RGA sequences. More number of RGA sequences was obtained from the primer combinations I and III in Karthobiumtham (18 RGAs) and Rose (18RGAs) respectively. Hence these two primer combinations may be considered as the most successful in obtaining resistance gene analogues of *Musa* cultivars.

Out of eight sequences obtained from primer combination II, only one clone which was derived from rose was hit with Rosa hybrid cultivar isolate L3P2-8H NBS-LRR

resistance protein gene, partial cds whereas other clone did not match with any of R-genes. Similarly, the sequences obtained from IV and V combinations did not have significant hits with either RGAs or known R-genes from other plant species. All the clones obtained from V combination hit with ubiquitin carboxyl-terminal hydrolase family protein (LOC100259769) of *Vitis vinifera*.

Forty (40) sequences which hit with R genes were further analyzed using ORF finder and it was found that only 18 sequences contained possible frames encoding uninterrupted by stop codons. The remaining clones contained multiple stop codons and are likely to be pseudogenes which might have evolved during the course of evolution (Meyers, 1999). Aarts et al. (1998) also found RGAs with frame shifts and stop codons in *A. thaliana*. Michelmore and Meyers (1998) reported that pseudogenes may be served as reservoirs of potential variation, because they may allow for recombination and gene conversion between alleles or paralogs of functional R genes and therefore represent the possibility of more rapid evolution of new specificities (Ota and Nei, 1994).

Sequence analysis of conserved motifs

Sequences were translated to look for motifs characteristic of plant NBS regions. Multiple alignment of the deduced amino acid sequences of the RGA sequences and the NBS domains of R genes using CLUSTALX revealed the presence of P-loop and GLPL that were used to design primers and four more conserved motifs; RNBS-A (resistance nucleotide binding site) non-TIR (Toll/-Inter-leukin receptor homo-logy), Kinase-2, RNBS-B, and RNBS-C (Figure 1) which are characteristics of the NBS domain encoded by NBS-LRR resistance gene family. Among these conserved motifs, the P-loop, RNBS-B and GLPL motifs are con-served in both TIR and non-TIR NBS-LRR proteins, while RNBS-A non-TIR has been found only in the non-TIR NBS-LRR proteins. The presence of an internal kinase-2 motif and kinase -3a domains, which are independent of the primer sequences, in the PCR clones appears to confirm that these clones correspond to NBS containing genes.

The last residue of the kinase -2 domain can be used to predict whether a RGA would belong to the TIR-NBS or the non-TIR-NBS family. As expected, a tryptophan residue (W) was found in all the sequences carrying the RNBS-A-non-TIR motif. This is in good agreement with the characteristics identified earlier for non-TIR sub class. Cannon et al. (2002) suggest that these sequences correspond to NBS region of non- TIR NBS-LRR sub class of NBS-LRR super-family of genes, as expected for a monocot species. In TIR-NBS-LRR group, a characteristic consensus motif FXXXXF and a highly conserved glycine are present between kinase1a and kinase2 domains, whereas the non-TIR-NBS-LRR group contains the consensus sequence FXXXXW (Pan et al., 2000).
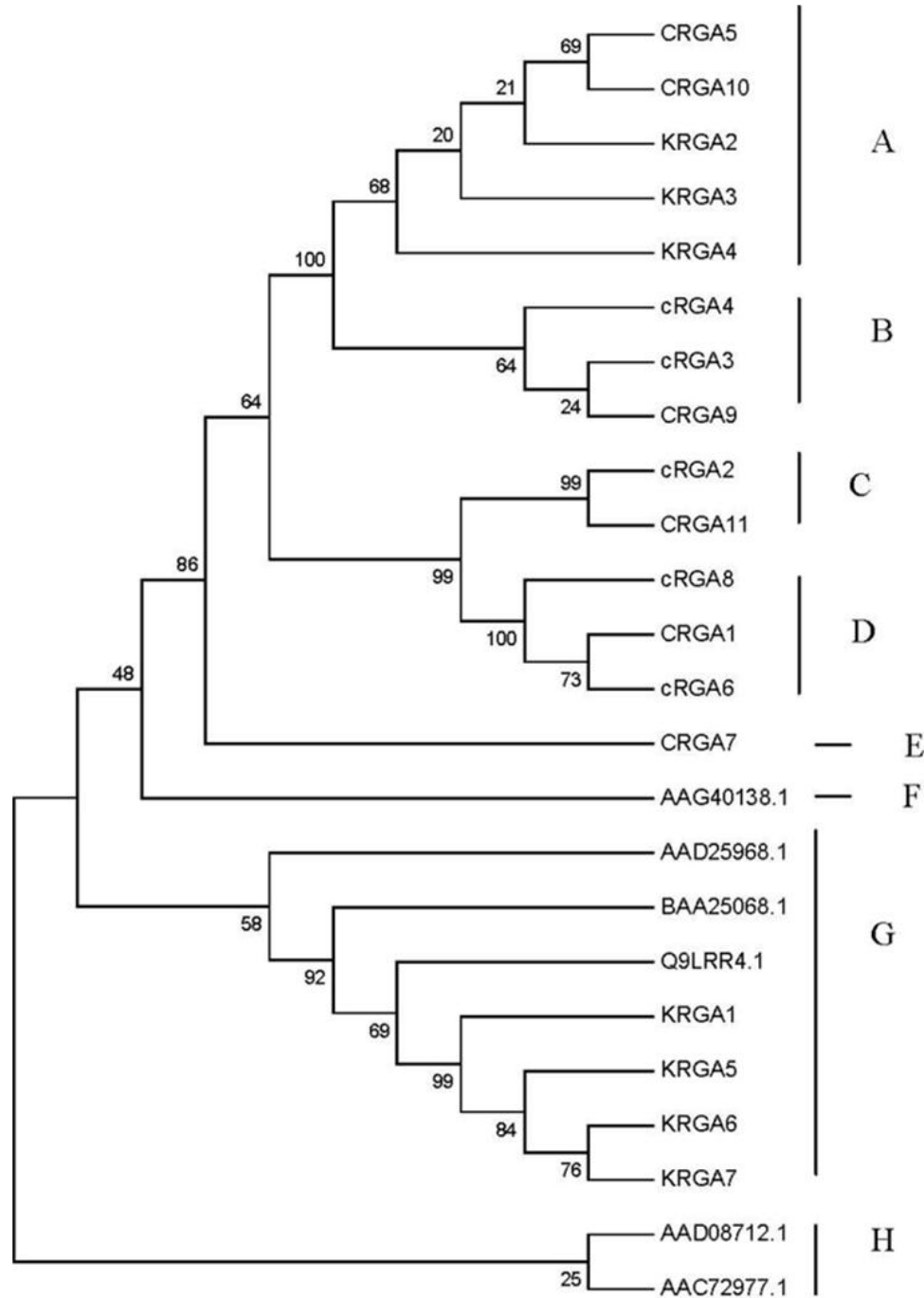
Figure 1. Phylogenetic tree based on the alignment of the consensus aminoacid sequences of *Musa* RGAs and six known resistance genes of other species: *Linum usitatissimum* (AAD25968.1), *Brassica napus* (AAG40138.1), *Oryza sativa* (BAA25068.1), *A.thaliana* (Q9LRR4.1), *Solanum lycopersicum* (AAD8712.1) and *A. thaliana* (AAC72977.1).

The presence of consensus motif FXXXXW in RGAs of the present study further ensures their position in non-TIR-NBS-LRR subfamily. Based on these criteria it was found that all the RGAs obtained in this study are belonged to the non-TIR NBS-LRR class. Similar results

were also obtained by Pei et al. (2007) in *Musa*. It is emphasized that no TIR-type RGA sequences were isolated from any of *Musa* species and this is very well in consistence with earlier reports on the absence of this subclass of R-genes in monocotyledons (Cannon et al.,

2002).

Phylogenetic analysis of *Musa* RGAs

In order to visualize the relative distance of *Musa* sequences to R genes and RGAs from other species, neighbour-joining tree based on the multiple alignment of amino acid was generated. The resulting tree consisted of two major branches, one consisting of TIR NBS-LRR and the other consisting of non-TIR NBS-LRR disease resistance proteins (Figure 2). The phylogenetic tree categorized the *Musa* RGAs into 6 distinct families (A, B, C, D, E, and G) based on the identity of their aminoacid. RGAs obtained from Rose were grouped into 5 families namely (A, B, C, D and E) whereas RGAs of cv. Karthombiutham were grouped into two families (A and G). This showed that RGAs of Rose are having a higher level of divergence than the Karthobiumtham. This revealed that RGA gene family is widely divergent in Rose cultivar. As R-genes are multigene family, their diversity and evolution depends on their genomic organization and selection in response to pathogen pressure (Hammond-Kosack and Jones, 1997).

RGA of *Brassica napus* (Acc.No. AAG40138.1) which is located in between two *Musa* families showed that this RGA might belongs to non-TIR class of NBS family. This was also confirmed based on the multiple alignment tryptophan residues (W) in the RNBS-A motif of *Brassica* RGA. Similarly, RGAs of *A. thaliana* fell under the main branches of non TIR and TIR classes suggesting that dicotyledons are having both non-TIR and TIR classes of NBS-LRR super family of genes as expected RGA of *Oryza sativa* fell in clade F along with the *Musa* cultivar Karthobiumtham RGAs   and also under non-TIR type class.

Expression analysis of *Musa* RGAs

In order to study the expression profile of *Musa* RGAs, specific primers were designed for one representative RGA from each RGA family. These representative RGAs showed high homology to the RGAs isolated from different *Musa* spp and other monocot (*Oryza sativa*) subsp. japonica and dicot (*Vitis riparia)* species (Table 3). Hence, all the specific primers were used for performing the RT PCR in root cDNA of both cultivars. Even though same amount of cDNA template was used for analysis, the expression level of RGAs was varying among the primers as well as cultivars. Out of six specific primers, amplified products were detected only in C1, C2, C3 and C5 while expression of C4 was not observed in uninfected resistant and susceptible cultivars. This suggested that only four RGAs (C1, C2, C3 and C5) are constitutively expressed in both the cultivars. Peraza-Echeverria et al. (2008) revealed that at least three out of

five RGAs from banana were expressed constitutively in root and leaf tissues of resistant and susceptible cultivars. The same kind of trend was also observed in other crops, namely common bean (Rivkin et al.,1999), *B. oleracea* (Vicente and King, 2001), Ginger (Aswati and Thomas, 2007) and *B. napus* (Fourmann et al., 2001). Hammond-Kosack and Jones (1997) suggested that the R-gene products may have a function in plant development and therefore be expressed in healthy, un-inoculated plants, ready to detect any attack.

Moreover, the constitutive expression of C6 was observed only in the resistant not in susceptible. Wang et al. (2006) reported that differences in transcript levels could be correlated with the reaction to susceptibi-lity/resistance of the host plant to pathogen. Gao et al. (2006) found that some RGAs were expressed only after challenge with *Verticillium dahlia* in cotton. Hence all the primers were used for studying the time course expression on nematode inoculated root tissues. The time course expression of these *Musa* RGAs in the *P. coffeae* infested root tissues of resistant and susceptible cultivars was determined by semi-quantitative RT-PCR analysis. The transcript level of C1 and C5 was increased at 4 as well as 6 DAI of nematodes and steadily increased up to 7 DAI in resistant cultivar alone (Figure 3). The peak transcriptional expression was observed on 5 DAI for C1 and 7DAI for C5. While, in susceptible cultivar no significant induction was observed after inoculation for all the RGAs. Similarly, the static level of transcripts was observed in resistant cultivar for C2, C3 and C6 RGAs in resistant culitvar. The expression of C6 in susceptible cultivar was not found even after 7DAI of *P. coffeae* envisaged that this could be considered as pseudogene which may occur due to frame shifts and/or stop codons (Aarts et al., 1998).   These results envisaged that some of the RGAs are constitutively expressed and some of them are over expressed owing to *P. coffeae* infection and some of them may be pseudogenes.

Conclusion

In this study, the level and the time-course accumulation of C1 and C5 RGA transcripts confirmed that these two RGAs had a significant role in resistant cultivar against *P. coffeae.* Currently, we are attempting to clone full-length cDNAs of C1 and C5 to characterize their structure, the mechanism of their response to *P. coffeae*, which will provide further evidence on their role in nematode resistance.

ACKNOWLEDGEMENT

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        P-Loop                  RNBS-non TIR                                    Kinase 2
          10        20        30        40        50        60        70        80        9(
       ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CRGA1   1 --GGVGKTTLAQQAYNHERVKDYFHPKVWLCVSDNFNVERLTKEIIESITENKCD-LSNLDTLQVVVKKKLTSK--RFLLVLDDVWNEDS
cRGA2   1 --GGVGKTTLAQQAYNHERVQDYFQLKVWVCVSRNFNVERLTKDIIESLKQEKCD-LNNLDTLQQVVKEGLTSK--RFLLVLDDVWNDDS
cRGA3   1 --GGVGKTTLAQLVYNDERVMAQFDGRFWVYVSENFSIIEIIRSIVRSPRN-----CSDLQALRLQLQALLSWK--RYLLVLDDVWNENE
cRGA4   1 --GGVGKTTLAQLVYNDERVMAQFDGRFWVYVSENFSIIEIIRSIVRSPRN-----CSDLQALRLQLQALLSWK--RYLLVLDDVWNENE
cRGA6   1 --GGVGKTTLAQQAYNHERVKDYFHPKVWLCVSDNFNVERLTKEIIESITENKCD-LSNLDTLQVVVKKKLTSK--RFLLVLDDVWNEDS
CRGA5   1 --GGVGKTTLGQLVYNDERVMAQFDGRFWVYVSENFSIIEIIRSIVRSPRN-----YSDLQALRLQLQALLSWK--RYLLVLDDVWNENE
CRGA7   1 --GGVGKTTLAQ-LYNHERVKDYFHPKVWLCVSDNFNVERLTKEIIESITENKCD-LSNLDTLQVVVKKKLTSK--RFLLVLDDVWNEDS
cRGA8   1 --GGVGKTTLAQQAYNHERVKDYFHPKVWLCVSDNFNVERLTKEIIESITENKCD-LSNLDTLQVVVKKKLTSK--RFLLVLDDVWNEMS
CRGA9   1 --GGVGKTTLAQLVYNDERVMAQFDGRFWVYVSENFSIIEIIRSIVRSPRN-----CSDLQALRLQLQALLSWK--RYLLVLDDVWNENE
CRGA10  1 --GGVGKTTLGQLVYNDERVMAQFDGRFWVYVSENFSIIEIIRSIVRSPRN-----YSDLQALRLQLQALLSWK--RYLLVLDDVWNENE
CRGA11  1 --GGVGKTTLAQQAYNHERVQDYFQLKVWVCVSRNFNVERLTKDIIESLKQEKCD-LNNLDTLQQVVKEELTSK--RFLLVLDDVWNDDS
KRGA1   1 GPGGVGKTTLAQQAYIHERVQDYFQLKVWLCVSDNFNVERLTKAIIENLNQKNCD-LNNLDTLQEVVKEKLTSK--RFLLVLDDVWNEDS
KRGA2   1 GPGGVGKTTLAQQAYIHERVQDYFQLKVWLCVSDNFNVERLTKAIIENLNQKNCD-LNNLDTLQEVVKEKLTSK--RFLLVLDDVWNEDS
KRGA3   1 --GGVGKTTLAQLVYNDERVMAQFDGRFWVYVSENFSIIEIIRSIVRSPRN-----YSDLQALRLQLQALLSWK--RYLLVLDDVWNENE
KRGA4   1 --GGVGKTTLAQLVYNDERVMAQFDGRFWVYVSENFSIIEIIRSIVRSPRN-----YSDLQALRLQLQALLSWK--RYLLVLDDVWNENE
KRGA5   1 --GGVGKTTLAQLVYNDERVMAQFDGRFWVYVSENFSIIEIIRSIVRSPRN-----YSDLQALRLQLQALLSWK--RYLLVLDDVWNENE
KRGA6   1 GYGGVGKTTLAQQAYNHERVKDYFHPKVWLCVSDNFNVERLTKEIIESITENKCD-LSNLDTLQVVVKKKLTSK--RFLLVLDDVWNEDS
KRGA7   1 GPGGVGKTTLAQQAYNHERVQDYFQPKVWLCVSDNFNVERLTKDIIEFITEEKCD-LSNLDTLQVVVKKKLNSK--RFLLVLDDVWNEDS
Q9LRR4.1 1 GIGGVGKTTLSQLLYNDQHVRSYFGTKVWAHVSEEFDVFKITKKVYESVTSRPCE-FTDLDVLQVKLKERLTGTGLPFLLVLDDLWNENF
BAA25068.1 1 GNGGIGKTTLAQLVCKDLVIKSQFNVKIWVYVSDKFDVVKITRQILDHVSNQSHEGISNLDTLQQDLEEQMKSK--KFLIVLDDVWEIRT
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             Kinase3a- RNBS-                        RNBS-C
         110       120       130       140       150       160       170       180
       ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CRGA1   96 RYGE---------QGSKILVTTRSRKIADMVG--NPIPLDGLDEASYWKLFKKCAFGSEDVGEFPQLEAIAGMIVSRLKGLPLAL
cRGA2   96 RSAV---------PGSKILVTTRSKKIAEMVG--NPIPLYGLDNASYWEFFKTCAFGSEDAGEFPQQEAIARKIASRLKGLPLAL
cRGA3   92 RCGK---------QGSKIITTTRSETVARIMGTVTLHKMPILSFEHCWLLFEQRAFRLVREEEKPRFVEIGRQIVERCGGLPLAL
cRGA4   92 RCGK---------QGSKIITTTRSETVA-----------------------------------------------------------
cRGA6   96 RYGE---------QGSKILVTTRSRKIADMVG--NPIPLDGLDEASYWKLFKKCAFGSEDVGEFPQLEAIAGMIVSRLKGLPLAL
CRGA5   92 RCGK---------QGSKIITTTRSETVARIMGTVTLHKMPMLSFEHCWLLFPQRAFRLVREEGKLDSWQIG------TSGLPLAL
CRGA7   95 RYGE---------QGSKILVTTRSRKIADMVG--NPIPLDGLDEASYWKLFKKCAFGSEDVGEFPQLEAIAGMIVSRLKGLPLAL
cRGA8   96 RYGE---------QGSKILVTTRSRKIADMVG--NPIPLDGLDEASYWKLFKKCAFGSEDVGEFPQLEAIAGMIVSRLKGLPLAL
CRGA9   92 RCGK---------QGSKIITTTRSETVARIMGTVTLHKMPILSFEHCWLLFEQRAFRLVREEGKPRFVEIGRQIVERCGGLPLAL
CRGA10  92 RCGK---------QGSKIITTTRSETVARIMGTVTLHKMPMLSFEHCWLLFEQRAFRLVREEGKLDSWQIG------TS------
CRGA11  96 RSAV---------PGSKILVTTRSKKIAEMVG--NPIPLYGLDNASYWEFFKTCAFGSEDAGEFPQQEAIARKIASRLKGLPLAL
KRGA1   98 RYGE---------PGSKILVTTRSKKIAEIVG--NPIPLGGLDEVSYWKLFKQCAFGSEDAGEFPQLEDIARQIAYRFNGLPLAL
KRGA2   98 RYGE---------PGSKILVTTRSKKIAEIVG--NPIPLGGLDEVSYWKLFKQCAFGSEDAGEFPQLEDIARQIAYRFNGLPLAL
KRGA3   92 RCGK---------QGSKIITTTRSETVARIMGTVTLHKMPMLSFEHCWLLFEQRAFRLVREEEKPRFVEIGRQIVERCGGLPLAL
KRGA4   92 RCGK---------QGSKIITTTRSETVARIMGTVTLHKMPMLSFEHCWLLFEQRAFRLVREEEKPRFVEIGRQIVERCGGLPLAL
KRGA5   92 RCGK---------QGSKIITTTRSETVARIMGTVTLHKMPMLSFEHCWLLFEQRAFRLVREEEKPRFVEIGRQIVERCGGLPLA-
KRGA6   98 RYGE---------QGSKILVTTRSRKIADMVG--NPIPLDGLDEASYWKLFKKCAFGSEDVGEFPQLEAIAGMIVSRLKGLPLAL
KRGA7   98 RYGE---------PGSKILVTTRSKKIAEMVG--NPIPLGGLDEASYWKLFKKCAF-------------------------------
Q9LRR4.1 100 IHAA---------QGSQILVTTRSQRVASIMCAVHVEHNLQPLSDGDCWSLFMKTVFGNQEPCLNREIGDLAERIVHKCRGLPL--
BAA25068.1 99 RPNDQVNSSQEEATGNMIIILTTRIQSIAKSLGTVQSIRKLEALRDDDIWSLFKVHAFGNDKHDSSPGLQVLGKQIASELRGNPL--
```

Figure 2. Multiple alignments of the consensus aminoacid sequences of the 20 RGAs and NBS domain of R-genes constructed with Clustal X. These R-gene including *A.thaliana* RPP13 (Q9LRR4.1) and *Oryza sativa* Xa1(BAA25068.1). The locations of conserved motifs were included at the top of the results.
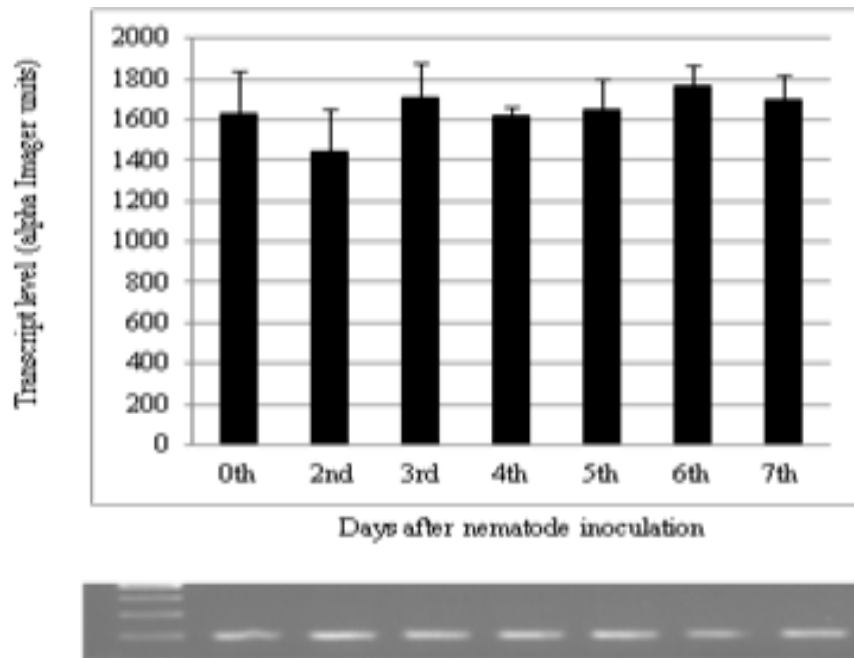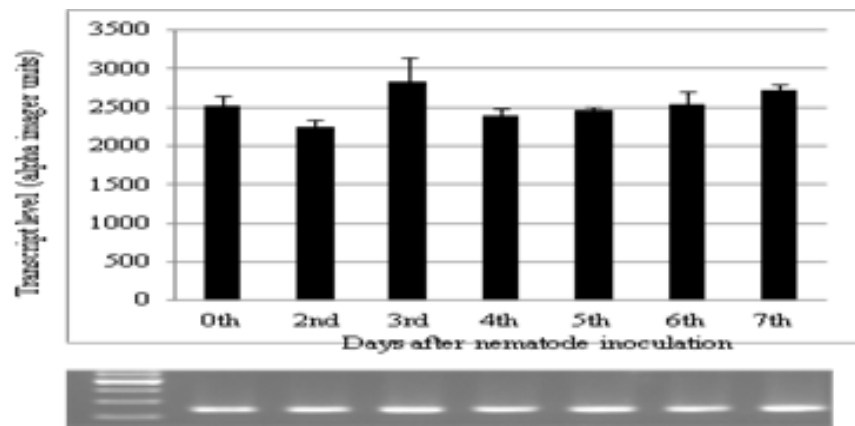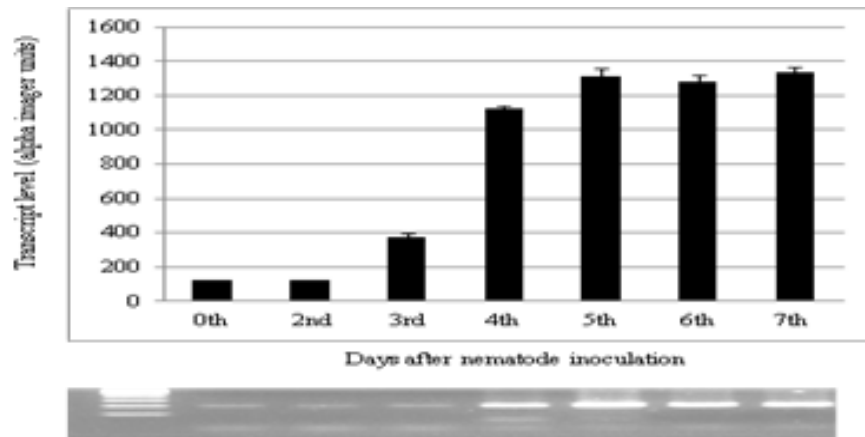
A

C1
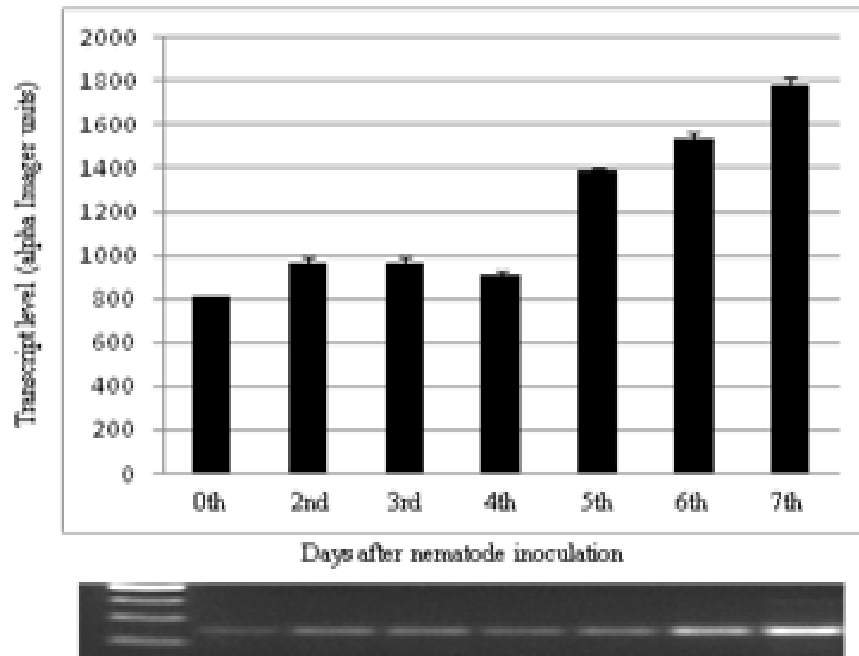
C2

Figure 3. Expression profiles of *Musa* RGAs C1, C2, C3, C5 and C6 at 0, 2, 3, 4, 5, 6, 7 days after *P. coffeae infection* in A) resistant cultivar (Karthobiumtham) B) susceptible cultivar (Nendran).
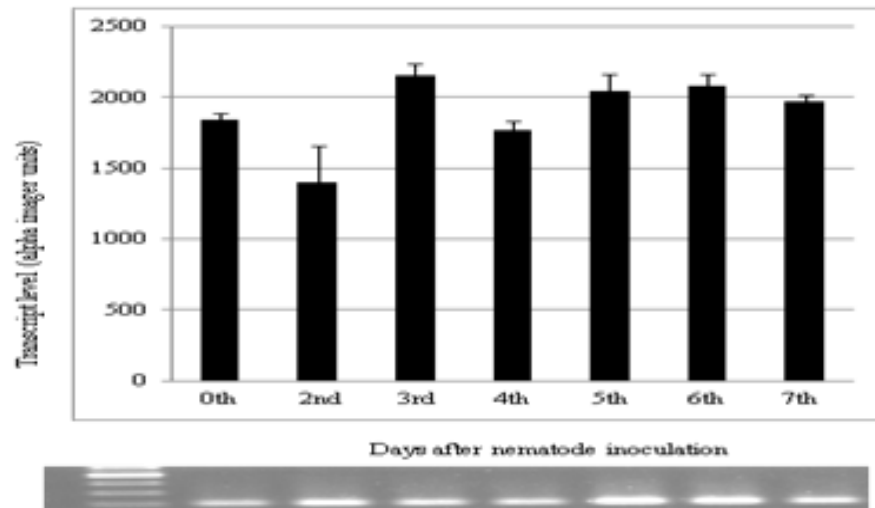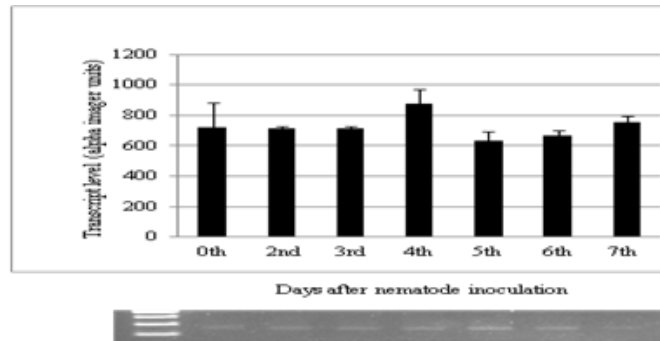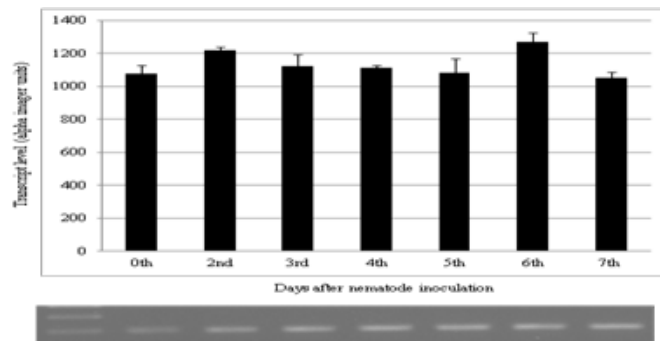
9

C3
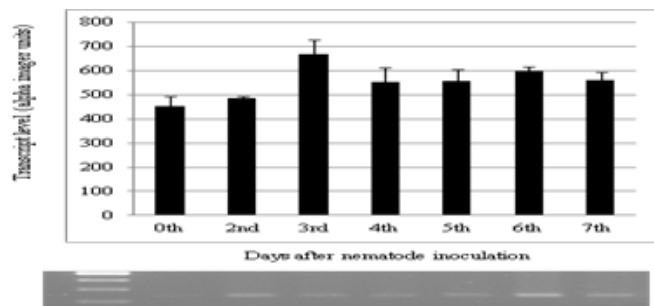

C4

C5

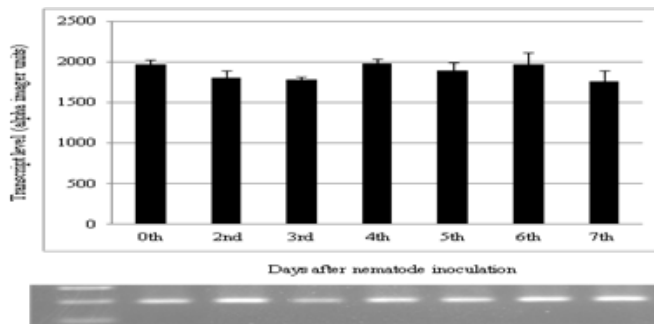
Figure 3. Contd.

10

B

C1

C2

C3

C4

Figure 3. Contd.

11

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