Emir. J. Food Agric. 2014. 26 (6): 508-518
doi: 10.9755/ejfa.v26i6.17219
http://www.ejfa.info/

Regular Article

PLANT MOLECULAR BIOLOGY

Isolation and characterization of Resistance Gene Analogue (RGA) from Fusarium resistant banana cultivars

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Abstract

Isolation and characterization of resistance genes from local banana cultivars was important in order to support the development of *FOC* resistant banana cultivars. Resistance gene analogues (RGAs) were isolated and characterized form three fusarium resistant banana cultivars using degenerate primers based on NBS domains. From 91 fragments sequenced, 17 fragments were positively NBS-type sequences and encoded as *MNBS1-MNBS17*. Phylogenetic analysis of *MNBS* deduced amino classified into three groups; the first group consisted of 14 members (*MNBS1-MNBS14*) with 97.4% identity, and the other three groups consisted of one member (*MNBS15*, *MNBS16* and *MNBS17*, respectively) with 28.5% identity. All *MNBS* sequences were categorized as non-TIR-NBS-LRR. Comparison and phylogenetic analysis of *MNBS* with other known *RGA* and *R* genes showed that deduced amino acid *MNBSs* shared 91.7-98.8% identity with *Musa NBS-LRR* and 19.9-35.5% identity with known *R* genes. Among them, *MNBS17* shared 50.5% identity with RGC2 (ABY75802) that assosiated to FOC race 4 resistant *Musa* species.

Key words: Disease resistance, Banana, Nucleotide binding site, Resistance Gene Analogue

Introduction

Banana and plantain are important fruit crops in the world, but they face the complexity of pests and diseases. One of the major fungal disease is Panama disease caused by *Fusarium oxysporum* f.sp. *cubense* (*FOC*). This disease is one of the devastating fungal diseases in banana. It is difficult to control because the innoculum is easily spreads in the field and persists in the soil for over 20 years without losing the virulence (Agrios, 2005). More importantly, the majority of commercial banana cultivars cultivated in the world such as Cavendish and plantain subgroups are reported suceptible to this pathogen (Ploetz, 2006).

Fusarium wilt control in banana plantation by using cultivation practices such as chemical control, soil treatments, crop rotation and organic amendments could reduce the severity of the disease. However, its commercial application is limited (Pegg et al., 1993). The use of resistant cultivars is the best alternative for controlling FOC and conventional banana breeding for FOC resistance has been actively reported (Rowe and Rosales, 1993; Tomekpe et al., 2004; Sebuliba et al., 2006). Technically, it faces the problems of flower sterility and ploidy of commercial cultivars (Roux et al., 2004). Breeding program through genetic engineering is a promising technique, but little information is available on the genetic basis of R gene on banana for resistance to *FOC* TR4 (Li et al., 2012). However, transgenic banana cultivars have been reported by number of researchers around the world. Paul et al. (2011) reported transformed apoptosis-inhibition-related genes in 'Lady Finger' banana. Based on the evaluation under glasshouse conditions, transformed banana plants showed resistant to *FOC* race 1. Mohandas et al. (2013) used antimicrobial peptide (*Ace-AMP1*) gene to confer resistance to *FOC* race 1 on

Received 09 June 2013; Revised 14 December 2013; Accepted 22 January 2014; Published Online 25 March 2014

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'Rasthali'. The evaluation was carried out in the screenhouse.

Functional disease resistance (*R*) genes isolated so far encode resistance to bacterial, viral, fungal, oomycete, nematode and insect pathogens (Dangl and Jones, 2001). The majority plant *R* genes encode nucleotide binding site-leucine rich repeat (NBS-LRR) proteins (McHale et al., 2006). The NBS-LRR immune receptors recognize specific pathogen-effector proteins and produce defence-related signaling to induce defence-related expression (Caplan et al., 2008).

The conserved NBS-LRR contains at least three domains, such as the variable N-terminal domain, the nucleotide binding site (NBS), and the C-terminal LRR motif. The NBS domain consists of p-loop/kinase-1, kinase-2, kinase-3a and hydrophobic (GLPL) and highly conserved in diverse organisms (Traut, 1994). Based on the N-terminal motifs, the NBS-LRR class of *R* genes is grouped into two distinct subclasses. The subclass I TIR-NBS-LRR, showed homology to the Drosophila Toll and mammalian Interleukin-1 receptor (TIR) proteins at their N-terminal region (Meyers et al., 1999). The subclass I is restricted to dicot and gymnosperm species (Tarr and Alexander, 2009). The subclass II non-TIR-NBS-LRR, exhibited a coiled-coil (CC) motif at the N-terminal region, instead of the corresponding TIR. This type R gene is widely distributed in both monocot and dicot species (McHale et al., 2006).

Based on NBS-LRR domain from known R genes, degenerate primers have been designed and used to isolate resistance gene analoque (RGA) from various plants such as soybean (Kanazin et.al., 1996), lettuce (Shen et al.,1998), lens (Yaish et al., 2004), wild apple (Baek and Choi, 2013) using PCR approach. The same approach was used to isolate RGAs from banana cultivars and wild species by Pei et al. (2007), Peraza-Echeverria et al. (2008), Azhar and Heslop-Harrison (2008), Sun et al. (2009), and they have isolated and characterized 12 RGAs from China's banana, 5 RGAs from segregated line of *M. acuminata* ssp. *malaccensis*, 135 RGA from cultivars and wild species, 5 RGAs from 'Gold Finger', respectively.

This present study was based on the PCR amplification and characterization of NBS-LRR class of RGA from three banana cultivars. Evaluation of their nucleotide diversity and phylogenetic relationship with known *R* genes from other plants were also carried out.

Materials and Methods

Plant materials

Three banana cultivars were used as sources of genomic DNA. They were Indonesian banana cultivars (Klutuk Wulung [BB] and Rejang [AA]) and introduced from ITC collection (Calcuta-4 [AA]). All selected cultivars were shown to be resistant to *FOC* tropical race-4 at the previous studies (Molina et al., 2009; Sutanto et al., 2012). The leaves were collected from one-month-old acclimatized planlets obtained from *in vitro* micropropagation culture.

DNA extraction

Genomic DNA were extracted from young fresh leaves and purified using modified CTAB method (Das et al., 2009) based on Doyle and Doyle (1987). Fresh leaf tissues (~0.1 g) were ground to powder and transferred to the pre-warmed extraction buffer [2% (w/v) CTAB, 1.4 mol/L NaCL, 20mmol/L EDTA, 100 mmol/L Tris-HCL (pH 8.0) and 2% (v/v) β-mercaptoethanol]. The suspension was extracted twice with equal volume of chloroform:isoamyl alcohol (24:1). The supernatant was precipitated with two volume of pre chilled (-20ºC) 95% ethanol and sodium acetate (0.3 M). The pellet was washed twice with 70 % ethanol. Dried pellet was dissolved in an appropriate volume of double distilled sterile water.

Degenerate primers and PCR conditions

Four primer combinations of degererate primers designed based on NBS-LRR containing P-loop/kinase-1, kinase-2, kinase-3a and hydrophobic domains were used to amplify genomic fragments of NBS-LRR from banana. The primer combinations tested were: F5(F)+F6(R), F9(F)+F6(R), F5(F)+F10(R), and F9(F)+F10(R). Information on the denerate primers used were presented in Table 1.

Table 1. Degenerate primers used for PCR amplification of NBS-LRR fragments from banana genomic DNA.

Primer	Primer sequence	Conserveddomain	Reference
F5(F)	GGIGGIGTIGGIAAIACIAC	P-loop	Peraza-Echeverria *et al.* 2008
F6(R)	AAGIGCTAAGIGGIAAGICC	Hydrophobic domain/GLPL	Peraza-Echeverria *et al.* 2008
F9(F)	GGNGGNRTIGGIAARACIAC	P-loop	Sun et al. 2009
F10(R)	GAGGGCNARNGGNAAICC	Hydrophobic domain/GLPL	Sun et al. 2009

Degenerate code: I= inosine; R= A/G; N=A/C/G/T

PCR amplification was carried out in a 25 µL total volume containing 1 µL of genomic DNA, 5.0 µL of 5X KAPA2G buffer, 0.5 µL of 25 mM $MgCl_2$, 0.5 µL of 10 mM dNTP mix, 1.25 µL of each forward and reverse primers, 0.5 unit of KAPA2G fast Taq polymerase, and double distilled water. PCR amplification was performed in a thermal cycler (Perkin Elmer) with an initial denaturing step of 95°C for 3 min, followed by 35 cycles of 95°C for 10 sec, 55°C for 10 sec and 72°C for 3 sec, and a final extension at 72°C for 10 min. PCR products were electrophoresed on a 1.5% TBE agarose gel at 80 V for 25 min, stained with ethidium bromide and visualized on a UV transilluminator.

Cloning PCR product and sequencing

Amplification products with clear and distinct band were purified using Gel/PCR DNA Fragments Extraction Kit (Geneaid, Taiwan). Purified products were ligated into the pGEM-T Easy Vector Systems (Promega) overnight and then transformed into DH-5α competent cells by the 42°C heat shock method. Potential transformants were selected on an LB plate containing ampicillin (50 µg/ml), X-gal and IPTG. The cultures were incubated at 37°C overnight. Single white colony was picked and inoculated into liquid LB medium containing ampicillin and grown overnight cultivation with vigorous shaking. Recombinant plasmids were extracted and purified using High-Speed Plasmid Mini Kit (Geneaid, Taiwan) and sent to 1st BASE for sequencing.

Sequence BLAST, alignment and phylogenetic analysis

Vector sequences were removed manually from the raw sequence data of RGA clones. The clone sequences were compared with the sequences in the non-redundant database at NCBI GenBank using the BLAST. ClustalW (Larkin et al., 2007) was used to align the deduced amino acid sequences of the PCR-derived genomic fragments of banana with the known *R* genes from *Arabidopsis thaliana* (*RPP1* and *RPP5*), tomato (*Prf* and *Mi-1,2*), peper (*Bs2*), potato (*Gpa2*), wheat (*Yr10*), flax (*L6* and *M*), and *Nicotiana tabbacum* (*N*). The alignments were graphically displayed using GeneDoc (Nicholas et al., 1997). A neighbour-joining phylogenetic tree, drawn with MEGA 5.0 (Tamura et al., 2011) was subsequently generated to reveal relationship between the compared sequences. Bootstrap analysis was employed with 1000 replications.

Result

PCR amplification of targeted RGA

Primer combinations F9(F)+F6(R) produced one distinct clear band with amplification product size of ~550 bp in all three cultivars (Rejang, Calcuta-4 and Klutuk Wulung), while the combination of F9(F)+F10(R) produced two bands, with the product size ranged from ~550 bp to ~1100 bp (Figure 1). Product with the size of ~550 bp was the expected DNA fragment size of the P-loop until GLPL motifs of NBS domain. However, two primer combinations F5(F)+F6(R) and F5(F)+F10(R) produced no amplification product. PCR product obtained from the combination of F9(F)+F6(R) was used for further analysis.

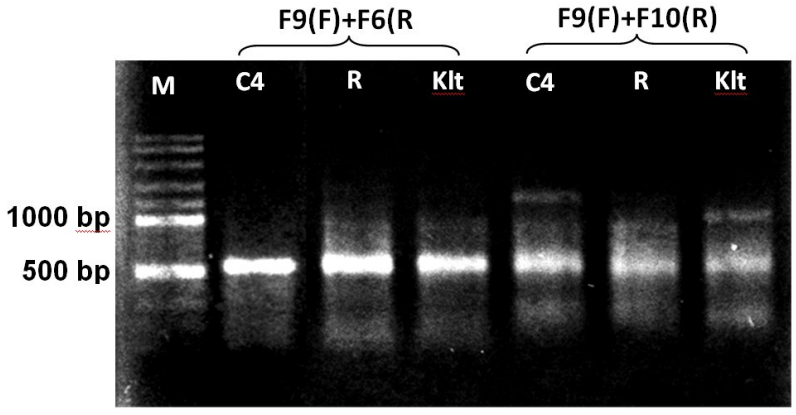

Figure 1. Amplification products obtained by PCR using two sets of primer combinations and tempelate of genomic DNA of three banana cultivars. Lane M: 100bp DNA ladder, Lane 2-7: banana cultivars (C4=Calcuta-4, R= Rejang and Klt=Klutuk Wulung).

Sequence analysis of banana RGAs

Out of 95 clones sequenced, 91 clones produced readable sequencing results at 68-959 bp in length. However, only 16 sequences with uninterupted open reading frame (ORF) typical of NBS were further analyzed. Those fragments, *MNBS1-MNBS16* with the ranged size of 523-628 bp, contained P-loop, kinase-2, kinase-3a and *hydrophobic domain* (GLPL). The remaining 75 sequences were BLAST analyzed to the *Musa* genome (http://banana-genome.cirad.fr/) and revealed three remaining sequences with coverage above 60% and sequence identity above 95% were showed in Table 2. One clone D6_K14 with 98.44% homolgy to *putative disease resistance protein RGA3*. This sequence was truncated NBS with only has P-loop and kinase 2 motifs and encoded by *MNBS17* for further analysis. Nucleotide sequence identity among 17 banana RGA range from 52.2% to 98.5%, while identity of their deduced amino acid range from 18.3% to 100%.

BLASTP analysis of deduced amino acid of RGAs revealed that *MNBS1-MNBS14* showed a high amino acid sequence identity (96%) to *Musa NBS-LRR* (ABY75803), while *MNBS 15* shared a 95% sequence identity with CAP66325, *MNBS16* shared 92% sequence identity with ACK44408, and *MNBS17* shared 97% sequence identity with AFR46148 (Table 3).

Diversity analysis of Musa RGAs.

Phylogenetic analysis of deduced amino acid showed that 17 RGAs resulted from this study were devided into four groups. Fragments with >97% identity of deduced amino acid were consider to be identical. Fourteen *RGA*s (*MNBS1-MNBS14*) were in the same group with 97.4% sequence identity and considered as group I. The *MNBS15*, *MNBS16* and *MNBS17* showed only 28.5% sequence identity and considered as separate groups (group II, III and IV, respectively) (Figure 2).

Table 2. BLAST result of three remaining clones to the *Musa* genome (http://banana-genome.cirad.fr/) with above 60% of querry coverage and above 95% of sequence identity.

No	Clone	E value	% Coverage	% Identity	Locus ID	Chr.	Start	End	Function
1	G3_R1+	1.00E-125	60.50	99.14	GSMUA_Achr6P24960_001	chr6	25839390	25851104	GSMUA_Achr6T24960_001~ exostosin family protein, putative, expressed
2	**D6_K14**	**1.00E-134**	**90.78**	**98.44**	**GSMUA_Achr4P26160_001**	**chr4**	**25543881**	**25547093**	**GSMUA_Achr4P26160_001~ Putative disease resistance protein RGA3**
3	E3_R21	6.00E-35	78.64	97.53	GSMUA_Achr3P03680_001	chr3	2390433	2395935	GSMUA_Achr3T03680_001~ Serine/threonine-protein phosphatase

Table 3. Comparison of deduced amino acid sequences among *MNBSs* with *Musa NBS-LRR* deposited in the NCBI GenBank database.

MNBS	GenBank accessions	*Query coverage* (%)	*E value*	Identity (%)
MBS1-MNB14	Resistance gene candidate NBS-type protein, partial [*Musa acuminata* subsp. *malaccensis*], RGC3 (ABY75803)	99	3e-115	96
MNBS15	NBS-LRR disease resistance protein, [*Musa* AAB Group] (CAP66325)	99	6e-109	95
MNBS16	NBS resistance protein, RGA-J [*Musa* ABB Group] (ACK44408)	80	9e-107	92
MNBS17	NBS-LRR protein, partial cds [*Musa* AAB Group] (AFR46148)	93	4e-53	97

Number in brakets indicated accession numbers in GenBank

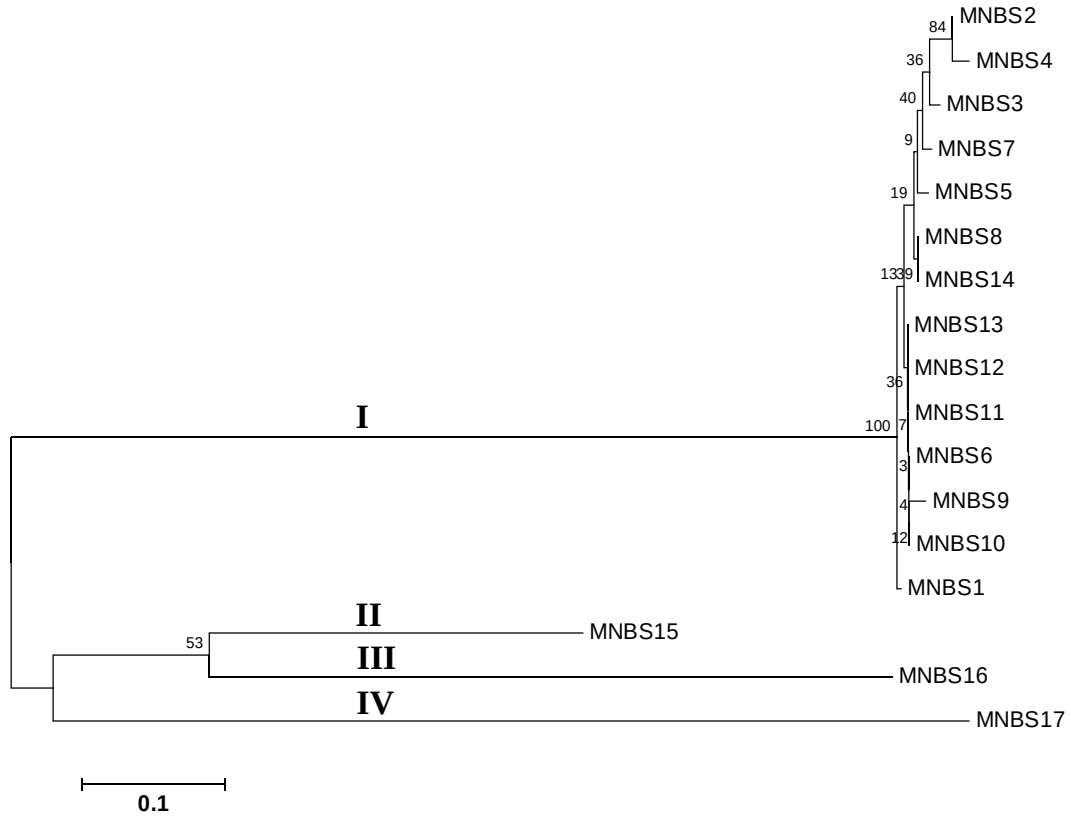

Figure 2. Phylogenetic tree of deduced amino acid sequences of *Musa* RGAs based on the neighbor-joining method. The numbers on the branches indicate bootstrap values (1000 replications). Bootstrap (%) refers to the percentage of trees in which the members form a clade. Branches corresponding to *Musa* RGA families are labeled as I, II, III and IV.

Multiple sequence alignment of Musa RGAs and known R genes

Based on multiple alignment of the deduced amino acid of *Musa MNBS* and other R genes revealed that the P-loop/kinase-1a (G**[V/M/I]** GKTT), kinase-2 (**[L/V][L/I]**DDV**[W/D]**), kinase-3a and hydrophobic domain (GLPL) motifs of the NBS domain were highly conserved among *Musa MNBS* sequences and the other known *Musa* NBS-LRR protein such as: *Prf* , *Mi-1*,*2*, *Bs2*, *Gpa2*, *Yr10*, *RPP1*, *RPP5*, *L6, M*, and *N* proteins (Figure 3). Among them *RPP1*, *RPP5*, *L6, M*, and *N* belong to TIR-NBS-LRR, while the others belong to non-TIR-NBS-LRR. The TIR-type and non-TIR-type of NBS-LRR proteins were distinguished by specific motif at the N-terminal region and amino acid residue at the end of kinase-2. For non-TIR-type the residue at the end of kinase-2 is tryptophan (W) and for TIR-type is aspartate (D) (Tarr and Alexander, 2009). Multiple alignment analysis of deduced amino acid sequences also generated genetic identity matrix as shown in Table 4. This matrix showed the identity of each accession to others.

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doi: 10.9755/ejfa.v26i6.17219
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Table 4. Genetic identity matrix (%) derived from multiple alignment of the deduced amino acid sequences of *Musa* RGAs and other NBS domain of R genes using ClustalW2.

Accessions	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
MNBS1		95.4	96.0	96.0	96.0	96.6	94.8	96.0	96.0	96.6	96.6	96.6	96.6	96.0	24.7	19.5	20.4	25.3	20.2	**23.1**	96.0	19.7	22.5	19.9	25.2	22.5	22.2	17.2	19.2	23.0	19.0	25.3
MNBS2	.....		97.1	97.7	95.4	96.0	95.4	95.4	95.4	96.0	96.0	96.0	96.0	95.4	24.1	18.9	19.4	24.7	19.7	**20.2**	96.0	19.7	23.7	20.5	26.9	22.0	23.4	17.2	18.6	23.6	19.5	25.3
MNBS3	.....	.....		97.1	97.7	96.6	97.1	97.1	96.0	96.6	96.6	96.6	96.6	97.1	23.6	18.9	19.4	23.6	20.2	**20.8**	97.1	18.5	23.7	20.5	26.3	21.4	22.8	16.7	18.0	23.0	18.4	24.7
MNBS4	.....	.....	.....		96.6	97.1	96.6	96.6	96.6	97.1	97.1	97.1	97.1	96.6	23.6	18.9	18.3	25.3	20.2	**20.8**	98.3	19.7	23.7	21.1	25.7	22.0	22.8	17.2	18.6	23.6	20.1	25.3
MNBS5	.....	.....	.....	.....		97.1	96.6	97.7	96.6	97.1	97.1	97.1	97.1	97.7	23.6	18.9	19.4	23.6	20.2	**21.4**	98.8	18.5	23.1	19.9	26.3	22.0	22.8	17.2	18.0	23.0	19.0	24.7
MNBS6	.....	.....	.....	.....	.....		98.3	99.4	99.4	100.0	100.0	100.0	100.0	99.4	24.7	18.3	20.4	24.7	20.2	**21.4**	97.1	18.5	23.1	19.9	26.3	22.0	22.8	17.2	18.0	23.0	19.0	24.7
MNBS7	.....	.....	.....	.....	.....	.....		98.9	97.7	98.3	98.3	98.3	98.3	98.9	23.6	18.3	19.4	23.6	19.7	**20.8**	96.5	17.9	23.1	19.9	25.7	20.8	22.2	16.7	18.0	23.0	17.8	24.7
MNBS8	.....	.....	.....	.....	.....	.....	.....		98.9	99.4	99.4	99.4	99.4	100.0	24.1	18.9	19.4	24.1	20.2	**21.4**	97.7	18.5	23.1	19.9	26.3	22.0	22.8	17.2	18.0	23.0	18.4	24.7
MNBS9	.....	.....	.....	.....	.....	.....	.....	.....		99.4	99.4	99.4	99.4	98.9	24.1	17.8	19.4	24.1	19.7	**20.8**	96.5	17.9	22.5	20.5	25.7	22.0	22.2	16.7	18.6	22.4	19.0	24.1
MNBS10	.....	.....	.....	.....	.....	.....	.....	.....	.....		100.0	100.0	100.0	99.4	24.7	18.3	20.4	24.7	20.2	**21.4**	97.1	18.5	23.1	19.9	26.3	22.0	22.8	17.2	18.0	23.0	19.0	24.7
MNBS11	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....		100.0	100.0	99.4	24.7	18.3	20.4	24.7	20.2	**21.4**	97.1	18.5	23.1	19.9	26.3	22.0	22.8	17.2	18.0	23.0	19.0	24.7
MNBS12	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....		100.0	99.4	24.7	18.3	20.4	24.7	20.2	**21.4**	97.1	18.5	23.1	19.9	26.3	22.0	22.8	17.2	18.0	23.0	19.0	24.7
MNBS13	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....		99.4	24.7	18.3	20.4	24.7	20.2	**21.4**	97.1	18.5	23.1	19.9	26.3	22.0	22.8	17.2	18.0	23.0	19.0	24.7
MNBS14	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....		24.1	18.9	19.4	24.1	20.2	**21.4**	97.7	18.5	23.1	19.9	26.3	22.0	22.8	17.2	18.0	23.0	18.4	24.7
MNBS15	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....		49.1	35.5	94.4	30.6	**25.4**	23.1	47.4	31.2	31.3	33.3	28.9	29.9	21.1	18.6	22.6	26.3	23.4
MNBS16	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....		35.5	51.5	32.0	**32.0**	19.5	91.7	30.8	33.7	31.4	28.4	31.7	18.3	20.7	24.3	24.9	21.9
MNBS17	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....		32.3	94.6	**50.5**	17.2	33.3	32.3	34.4	29.0	25.8	35.5	17.2	16.1	26.9	21.5	29.0
CAP66325	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....		30.1	**26.6**	24.9	51.5	31.8	32.5	32.8	29.5	29.3	21.7	19.2	23.7	28.0	24.0
AFR46148	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....		**43.4**	20.2	31.8	30.1	35.5	28.1	28.9	34.7	20.2	18.0	23.7	22.0	24.9
ABY78502	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....		21.4	31.8	25.4	27.7	27.5	23.7	32.9	18.5	17.4	20.2	21.4	22.0
ABY75803	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....		19.1	23.1	20.5	25.7	22.5	22.8	17.3	18.0	23.1	19.7	24.9
ACK44408	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....		30.1	33.7	31.0	28.9	29.9	20.8	19.8	25.4	24.9	23.7
AF202179	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....		54.8	41.5	33.0	42.5	20.2	18.6	25.4	24.3	25.4
AF195939	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....		47.0	31.3	42.8	19.3	19.9	23.5	24.1	25.3
LEU65391	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....		31.6	49.7	19.3	18.7	21.1	22.2	21.1
AF149114	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....		32.3	21.4	19.2	19.7	25.4	21.4
AAC67238	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....		22.2	22.2	22.2	26.4	22.8
AAC72977	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....		44.2	29.1	38.3	28.6
AAF08790	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....		30.8	40.7	30.8
Q40253	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....		40.0	81.7
Q40392	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....		37.7
AAB47618	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	

Remark: Numbers in the heading row indicated accessions of *Musa* RGAs and R genes from GenBank. 1: *MNBS1*, 2: *MNBS2*, 3: *MNBS3*, 4: *MNBS4*, 5: *MNBS5*, 6: *MNBS6*, 7: *MNBS7*, 8: *MNBS8*, 9: *MNBS9*, 10: *MNBS10*, 11: *MNBS11*, 12: *MNBS12*, 13: *MNBS13*, 14: *MNBS14*, 15: *MNBS15*, 16: *MNBS16*, 17: *MNBS17*, 18: NBS-LRR *Musa* (CAP66325), 19: NBS-LRR *Musa* AAB (AFR46148), 20: RGC2 *Musa* (ABY78502), 21: RGC3 *Musa* (ABY75803), 22: RGA-J *Musa* ABB (ACK44408), 23: *Bs2* Pepper (AF202179), 24: *Gpa2* Potato (AF195939), 25: *Prf* Tomato (LEU65391), 26: *Yr10* Wheat (AF149114), 27: *Mi-1.2 Solanum* (AAC67238), 28: *RPP1 Arabidopsis* (AAC72977), 29: *RPP5 Arabidopsis* (AAF08790), 30: *L6 Linum* (Q40253), 31: *N Nicotiana* (Q40392), 32: *M Linum* (AAB47618).

Emir. J. Food Agric. 2014. 26 (6): 508-518
doi: 10.9755/ejfa.v26i6.17219
http://www.ejfa.info/

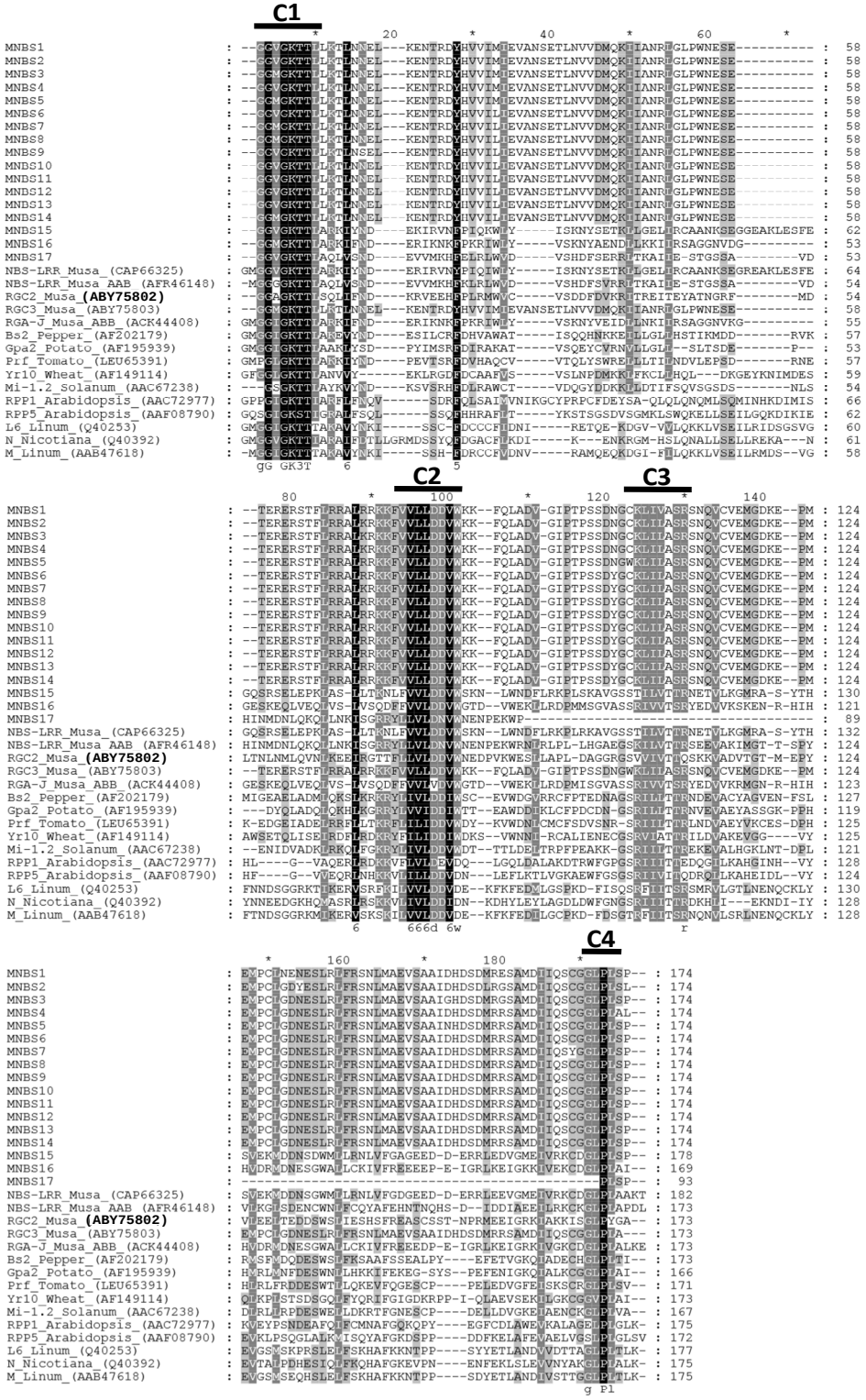

Figure 3. Multiple alignments of deduced amino acid sequences of *MNBSs* with other proteins of different plant R genes and *Musa NBS-LRR* from GenBank DNA database. The location of conserved domain are shown above the sequence (C1: kinase 1a or P-loop, C2: kinase 2, C3: kinase 3a, and C4: hydropobic domain or GLPL).

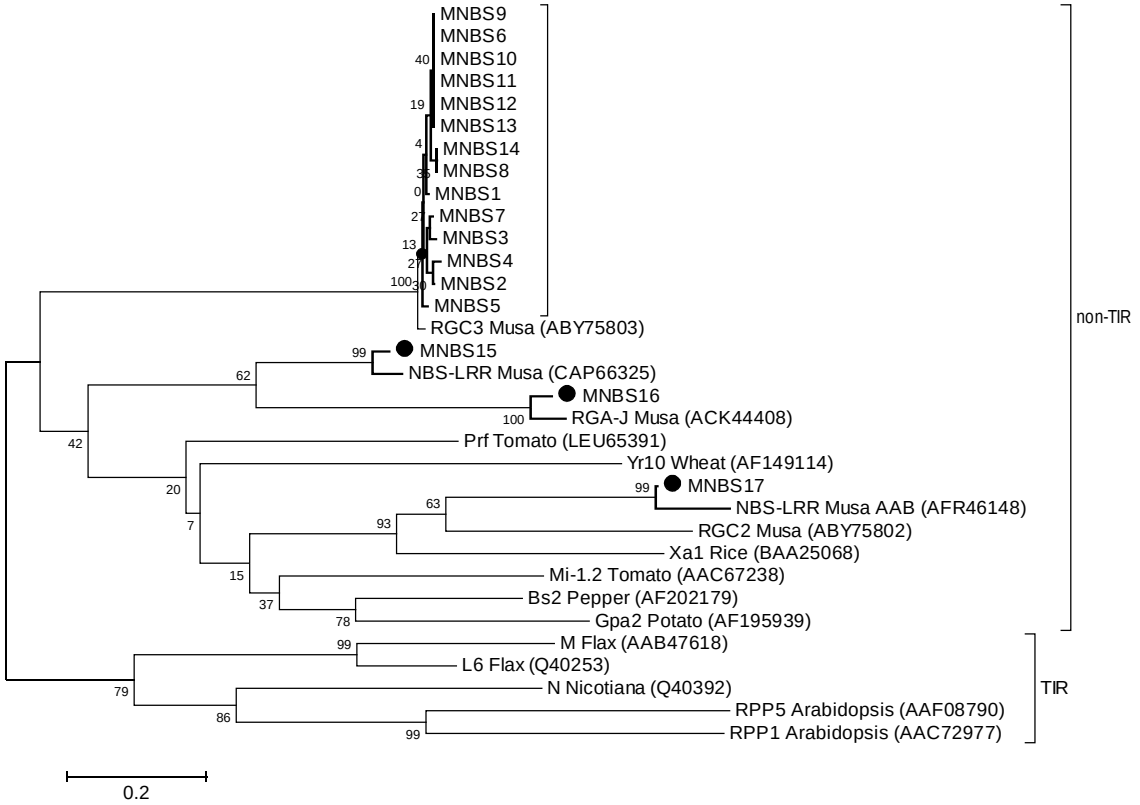

Figure 4. Phylogenetic tree of the deduced amino acid sequences of *Musa* RGAs and other R genes based on multiple alignment using Clustal W and the tree was generated using neighbor-joining method. The numbers on the branches indicate bootstrap values (1000 replications). Bootstrap % refers to the percentage of trees in which the members form a clade.

Phylogenetic analysis of *Musa* RGAs and other R genes.

Phylogenetic tree of deduced amino acid sequence of *Musa* RGA (*MNBSs*) characterized in this study, with five *Musa NBS-LRRs* and 10 known R proteins was shown in Figure 4. Two major branches designated as TIR-NBS-LRR and non-TIR-NBS-LRR were revealed. The first class, non-TIR-NBS-LRR, was divided into two groups. *MNBS1-MNBS14 were* in the same group with *Musa RGC3* (ABY75803) with 96-98% identity, while *MNBS15*, *MNBS16* and *MNBS17* were in the same group with *Musa NBS-LRR* (CAP66325), *Musa RGA-J* (ACK44408), *Musa NBS-LRR* (AFR46148), *Musa RGC2* (ABY75802), *BS2*, *GPA2*, *Mi-1,2*, *Prf* and *Yr10* with 18-95% identity. The second class, TIR-NBS-LRR, consisted of *RPP1*, *RPP5*, *M*, *L6* and *N* shared 16-29% sequence identity with *MNBSs*.

Discussion

Plants have developed sophisticated mechanisms to recognize and protect againts pathogens. The interaction between hosts and pathogens elicits both localized and systemic responses (Meyers et al., 2003). Plant disease resistance genes encode R proteins that represent the first plant defense against infection of many pathogens. The pathogens recognized by the R protein are usually highly specialized for host plant specific (Friedman and Baker, 2007).

Disease resistance trait is frequently controlled by a specific recognition between plant disease resistance (*R*) and pathogen avirulen (*Avr*) genes (Jones, 2001). Successful recognition of *Avr* product of pathogen by *R* poduct of host plant triggers variour defense mechanisms which include the hypersensitif response (HR), and results in a compatible interaction and leading to resistance of the plant (Baker et al., 1993).

In this study, we have sequenced 94 clones derived from amplified PCR products. Sixteen sequences encoded by *MNBS1-MNBS16* were typically RGA with uninterrupted ORFs and one sequence, *MNBS17*, was truncated RGA with only P-loop and kinase-2 motifs. The characteristic of motifs in the known NBS (Meyers et al., 2003) were also found in *MNBS* proteins.

Two highly conserved motifs were found in *MNBS* deduced amino acid sequence: kinase-1a and

kinase-2. The kinase-1a (GG[**V/M**]GKTT) was also called P-loop or Walker A motif, formed a glycine (G)-rich flexible loop containing an invariant lysine (K) residue involved in binding the phosphates of the nucleotide (Walker et al., 1982). This motif were shown to bind ATP in NBS-LRR resistace protein *I-2* and *M1* from potato (Tameling et al., 2002), suggesting that the *Musa MNBS* protein may bind ATP. The kinase-2 or Walker B motif (**[L/V][L/I]**DDV**[W/D]**) has an invariant aspartate (D) that coordinate divalent metal ion (Mg^{2+}) required for phospho-transfer reactions (Traut, 1994).

The absence of TIR-NBS-LRR was not only occured in *Musa*, but also reported in other monocots such as: rice (Zhou et al., 2004), wheat (Zhang et al., 2011), maize (Xiao et al., 2006), barley (Madsen et al., 2003), sorghum (Cheng et al., 2010), and sugarcane (Que et al., 2009). The TIR-NBS-LRR RGAs have been eliminated from majority monocots over time during evolution, although no mechanism has been described how to explain the elimination process. Pan et al. (2000) proposed a model suggesting that NBS-LRR evolution had involved two stages. During stage 1, plant genom had a few NBS-LRR genes, while during stage 2, both the TIR and the non-TIR groups underwent amplification and diversification in the dicot species. According to sequence analysis done by Cannon et al. (2002), non-TIR-type NBS genes appeared to be much more diverse than TIR-type NBS, and it was therefore tempting to speculate that non-TIR-type NBS genes were more ancient than the TIR-type. However, recent study on NBS evolution done by Yue et al. (2012) showed that the NBS domains found in early land plants (Coleochaetales, liverworts, and bryophytes) were closer to the TIR-type NBS domain at sequence level and they concluded that TIR-type NBS genes had developed earlier than non-TIR-type.

Refer to genetic identity matrix shown in Tabel 3, all deduced amino acid of *Musa MNBSs* shared 91.7-98.8% identity with *Musa NBS-LRR* proteins deposited in GenBank database, and 19.9-35.5% identity with known *R* genes such as *Bs2*, *Gpa2*, *Prf*, *Yr10* dan *Mi-1.2*, suggesting that *Musa MNBSs* might have the function of resistance gene. Among them, the identity bentween *MNBS17* and RGC2(ABY75802) were relatively high (50.5%). RGC2 expression was found to be associated only to *FOC* race 4 resistance line of *M. acuminata* spp. *malaccensis* (Peraza-Echeverria et al., 2008). This indicated that it might be a possible function of resistance to fusarium wilt of banana.

Conclusion

We have isolated and characterized 17 RGAs from *FOC* resistance banana cultivars, which will add to the existing RGA information in the GenBank, and it would be potentially used for molecular markers for screening resistance genes from banana. Further studies on the expression of RGAs are required to find out if the RGAs play a role in *FOC* resistance or in other diseases.

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