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Identification of Gene Candidate of Nucleotide Binding Site (NBS) from Banana *Musa acuminata* Colla var. *Malaccensis* (Riddl.) Nasution and *Musa*, AAA, Cavendish Sub-group

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ABSTRACT
Indonesia possess one of the largest genetic diversities of banana in the world, both cultivated and wild. However, the number of disease especially *Fusarium* wilt has reduce the production of banana. Parthenocarpy, polyploidy and sterility has also become a problem for the development of new varieties that resistant to the diseases. One of the largest plant R-gene families encodes a protein with Nucleotide Binding Site (NBS) domain. The aim of this study was to identify the candidate gene of NBS from *Musa acuminata* var. *malaccensis* and *Musa*, AAA, Cavendish sub-group. In this study, two fragments (P 5-8 and P 5-10) of NBS type gene from Musa *acuminata* var. *malaccensis* and one fragment (P 5-11) from Cavendish were amplified with degenerate primer, cloned and sequenced. Amino acid sequences revealed 97-100% similarity with *RPM1* (P 5-10 and P 5-11) and *RPS2* (P 5-8) from *Musa acuminata* var. *malaccensis*. Contig sequences were deposited in Genbank and assigned number KP 691062 -KP691064. There were four conserved motif sequences i.e., P-loop, kinase-2, kinase-3a and hydrophobic domain. All of the fragments was grouped into non-TIR-NBS-LRR and contained in chromosome number 9. The conserved sequences of NBS gene can be used as a potential genetic marker for disease resistant gene in banana.

Key words: Banana, nucleotide binding site, conserved sequences, *Musa acuminata* var. *malaccensis*, *Musa*, AAA, Cavendish sub-group

INTRODUCTION

Indonesia is one of the centers of banana genetic diversity in the world, both cultivated and wild. However, parthenocarpy, polyploidy and sterility has become a problem for the development of new varieties that resistant to the diseases, especially *Fusarium* wilt. The presence of *Fusarium* wilt caused 1,300 ha of banana in North Sumatra and 300 ha of Cavendish banana plantations in Riau were heavily damaged in 1995 (Nasir and Handayani, 2005). *Fusarium* wilt disease is caused by the fungus *Fusarium oxysporum* f.sp. *cubense*. It is able to live in the soil in the form of chlamydospores and can survive in the soil for long periods, thus it is difficult to control (Widono *et al*., 2003). This fungus will germinate to infect the lateral roots, when it contacts with the roots of banana and forms colonies that clogged the vascular system of plants, causing wilting and death of the banana plant. *F. oxysporum* f.sp. *cubense* race 4 become the most virulent race, therefore, it can infect almost all plants of banana cultivars, including those which are resistant to other races of *Fusarium* wilt (Sun *et al*., 2010).

In order to develop the disease-resistant banana clones, conventionally inhibited by along time generation, parthenocarpy, polyploidy and sterility of the most cultivars. *Musa acuminata* Colla is a wild seeded banana and diploid. In Indonesia, there are 15 varieties of *Musa acuminata* Colla spread from Sumatra to Papua, i.e., *Musa acuminata* var. *acuminata*, var. *alasensis* Nasution, var. *bantamensis*

Nasution, var. *breviformis* Nasution, var. *cerifera* Back. Nasution, var. *flava* (Ridl.) Nasution, var. *halabanensis* (Meijer) Nasution, var. *longipetiolata* Nasution, var. *malaccensis* (Ridl.) Nasution, var. *microcarpa* (Becc.) Nasution, var. *nakaii* nasution, var. *rutilifes* (Back.) Nasution, var. *sumatrana* (Becc. Nasution), var. *tomentosa* (K.Sch.) Nasution and var. *zebrina* (v.Houtte) Nasution (Nasution, 1991). According to Kayat *et al*. (2004), *Musa acuminata* var. *malaccensis* has a high resistance to *Fusarium oxysporum* f.sp. *cubense* race 4. Thus, it can be used as a source of genes for disease resistance.

The largest class of resistance genes (R) is Nucleotide Binding Site-Leusine Rich repeat (NBS-LRR). The NBS regions are important for ATP binding and hydrolysis function. It is involved in the signal transduction triggered by pathogen infection. Conserved amino acid motifs in the NBS region include P-loop (kinase-1), kinase-2, GLPL (kinase-3) and RNBS A, B, C and D motifs (Meyers *et al*., 1999). The LRR region is involved in the interaction of various proteins and recognized the elicitor molecules of pathogens (Fluhr, 2001). In this study, only the NBS region was identified. Sun *et al*. (2010) obtained 20 analog gene fragments in 'Goldfinger' banana with size about 530 bp and included to non-TIR NBS class containing 4 conserved regions of amino acids P-loop, kinase-2, RNBS-B and GLPL hydrophobic amino acids. The research NBS-LRR genes related to resistant has been conducted in tomato and *Arabidopsis* (Pan *et al*., 2000), potatoes (Bendahmaney *et al*., 2002) and sugarcane (Glynn *et al*., 2008). Meanwhile, research on banana consists of wild banana *Musa acuminata* Colla (Miller *et al*., 2008; Peraza-Echeverria *et al*., 2008), Cavendish banana (Li *et al*., 2012), Goldfinger banana (Sun *et al*., 2010) and *Musa* AAB cv *Nendran* (Augustine and Joseph, 2014).

The aim of this study was to identify the gene candidate of Nucleotide Binding Site (NBS) on the accession of *Musa acuminata* colla var. *malaccensis* and *Musa*, AAA, Cavendish sub-group.

MATERIALS AND METHODS

Plant materials: The banana cultivars used in this study consist of a wild banana *Musa acuminata* var. *malaccensis* (LIPI 010) and *Musa*, AAA, Cavendish sub-group (LIPI 090) collected from Banana Germplasm Gardens, Research Center for Biology, Indonesian Institute of Sciences. The DNA material used was, young leaves of the bananas.

DNA extraction: Total DNA was extracted from young leaves by using Cetyltrimethyl Ammonium Bromide (CTAB) method (Dellaporta *et al*., 1983; Syamkumar *et al*., 2003) that has been modified with the addition of Poly Vinyl Pyrrolidone (PVP) at the time of grinding and β-Mercaptoethanol on the extraction buffers.

Amplification of DNA: DNA amplification is conducted using the degenerate primers based on the conserved region of the NBS genes. Three pairs of primers, designed by Sun *et al*. (2010), were used to isolate an analog R gene from the genomic DNA of the *Fusarium* wilt resistant Goldfinger (AAAB) banana as shown in Table 1. The PCR mixture of 15 mL contained 7.5 mL GoTaq® Green Master Mix 2×, 0.75 mL of 20 pmol forward and reverse primers, 1 µL of DNA 50 ng mL^{-1} and Nuclease free water up to 15 mL total volume. The PCR condition included predenaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 94°C for 45 sec, annealing at 44°C (F1+F2); 54°C (F5+F6); 58°C (F9-F10) for 30 seconds respectively and elongation at 72ºC for 60 sec with a post-elongation step at 72°C for 10 min. The PCR process was carried out using Thermal Cycler Takara machine. Amplicons were purified from the gel and ligated with pGEM-T Easy vector (Promega, USA). This mixture consist of 1 µL PCR results (50 ng), 1 µL 25 ng pGEM-T Easy vector, 1 µL of 5 U T4 DNA ligase, 5 µL of 2X rapid ligation buffer and Nuclease Free Water up to 10 mL total volume. The mixture was incubated at 4°C overnight. The ligation result was transformed into *E. coli* JM109 (Promega, USA) by heatshock method according to Sambrook and Russel (2001). Plasmid containing recombinant DNA were extracted using the High Speed Plasmid Mini Kit (GenAid) and sent to the 1st Base sequencing services company for sequencing process.

DNA sequence analysis: The sequence were done by comparing the DNA sequences and a predicted amino acid with the sequence of other plant accessions in the NCBI gene bank database using a BLASTp algorithm. The contig analysis of the DNA sequence was done by Chromas Pro program. The alignment analysis was done with a MUSCLE method and the phylogenetic tree was constructed by the neighbor-joining method from Molecular Evolutionary Genetics Analysis (MEGA5) program (Tamura *et al*., 2011). A thousand of bootstrap replications were used to evaluate the degree of clustering pattern in the phylogenetic tree.

RESULTS

PCR amplification using degenerate primer: The degenerate primers were designed to amplify region between P-loop and the GLPL motifs of NBS class of the resistance gene. Three pairs of degenerate primers were able to produce 11 analogue resistance gene fragments that were isolated from *Musa acuminata* var. *malaccensis* and *Musa*, AAA, Cavendish sub-group. The size of the fragments between ~300-1000 bp

Table 1: Degenerate primer that used to isolate gene of NBS

Degenerate primer	Peptide	Primer sequence	Reference
F1 (F)	P-loop	GGDGTDGGNAARACWAC	Deng *et al*. (2000)
F2 (R)	GLPL	AANGCHAGNGGYAANCC	Deng *et al*. (2000)
F5 (F)	P-loop	GGIGGIGTIGGIAAIACIAC	Peraza-Echeverria *et al*. (2008)
F6 (R)	GLPL	AAGIGCTAAGIGGIAAGICC	Peraza-Echeverria *et al*. (2008)
F9 (F)	P-loop	GGNGGNRTIGGIAARACIAC	Sun *et al*. (2010)
F10 (R)	GLPL	GAGGGCNARNGGNAAICC	Sun *et al*. (2010)

Codes for mix bases: R = A/G, W = A/T, Y = C/T, S = C/G, H = A/T/C, D = A/T/G, N = A/G/T/C, I = Hypoxanthine

as shown in Fig. 1. Primer F5 (F)+F6 (R) produced fragments with the size of ~300-650 bp. Primer F1 (F)+F2 (R) produced fragments with the size of ~500-1000 bp while, F9 (F)+F10 (R) produced ~500-1000 bp fragments. The total of eleven fragments were isolated from the gel and cloned into the *E. coli*.

Sequence analysis of DNA fragments of banana: The PCR product of the 11 fragments have been cloned. All of the fragments contained the inserted fragment between 323-1114 bp in size. Based on the analysis of nucleotide and the deduced amino acid using BLASTp analysis in Genebank, it was found that 3 sequences consisted of P 5-8, P 5-10 and P 5-11 with the size of 770, 1114 and 791 bp, respectively, have similarity of homology more than 80% to the disease resistance protein *RPM1* and *RPS2* on *Musa acuminata* subsp. *malaccensis*. *RPM1* and *RPS2* had been deposited in the Genbank accession that shared 97, 98 and 100% homology with these fragments, respectively as shown in Table 2. Contig sequences were deposited in Genbank and assigned number KP691062-KP691064.

All of three sequences were analyzed on *Musa* Genome BLAST. The results showed that all of three sequences have coverage of 90% and they are located on chromosome number 9 as shown in Table 3. The P 5-8 sequence has similarity to putative disease resistance protein *RPS2*. While, P 5-10 and P 5-11 have similarity to putative disease resistance protein *RPM1* with the percentage of coverage of 92.76, 96.68 and 97.11%, respectively.

Sequence alignment and phylogenetic analysis of the predicted amino acids with protein R-gene and other plants RGA: Sequence alignment between amino acid of three fragments, 16 of NBS-LRR protein from bananas, protein *RPM1* and *RPS2* of *Musa acuminata* var. *malaccensis* and 13 proteins-R from other plants that have been deposited in Genbank showed that there were conservative structure of NB-ARC containing 4 conserved regions of amino acids i.e., P-loop/kinase-1a (GGVGKTT), kinase-2 (LVLDDIW), RNBS-B/kinase-3a (CKVLFTTRS) and hydrophobic amino acids (hydrophobic domain) (GLPL). These motifs are characteristic to NBS regions as shown in Fig. 2. The length

Table 2: Sequence identity between the predicted amino acid sequences of *Musa acuminata* var. *malaccensis*, Cavendish and *Musa* disease resistance protein that has been deposited in Genbank

Fragment	Genebank accession	Query coverage (%)	E-value	Identity (%)	Accession
P 5-8 (KP691062)	Disease resistant protein *RPS2* like (*Musa acuminata* subsp. *malaccensis*)	66	3e-108	97	XM009418398
P 5-10 (KP691063)	Predicted: disease resistance protein *RPM1* like (*Musa acuminata* subsp *malaccensis*)	98	0.0	99	XM009419073
P 5-11 (KP691064)	Predicted: disease resistance protein *RPM1* like (*Musa acuminata* subsp. *malaccensis*)	99	0.0	100	XM009419073

Table 3: Sequence identity between the predicted amino acid sequences of *Musa acuminata* var. *malaccensis* and Cavendish in Musa Genome

Fragment	Coverage (%)	Locus ID	Chromosome	Start	End	Function
P 5-8 (KP691062) (*M. acuminata* var. *malaccensis*)	92.76	GSMUA-Ach r9P02520-001	9	1802617	1803091	Putative disease resistance protein *RPS2* complete
P 5-10 (KP691063) (*M. acuminata* var. *malaccensis*)	96.68	GSMUA-Ach r9P12800-001	9	8304222	8305299	Putative disease resistance protein *RPM1* complete
P 5-11(KP691064) (*Musa*, AAA, Cavendish sub-group)	97.11	GSMUA-Ach r9P12800-001	9	8304527	8305299	Putative disease resistance protein *RPM1* complete

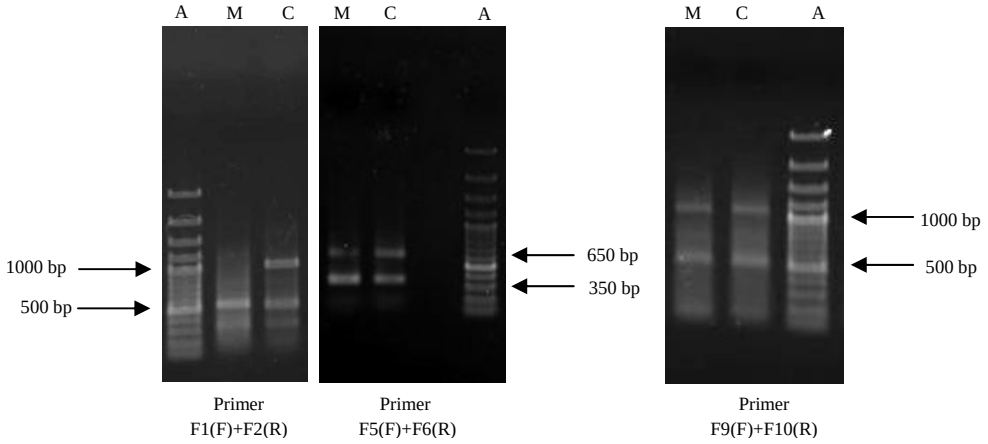

Fig. 1: DNA amplification using the degenerate primer. A: DNA ladder marker 100 bp (Fermentas); M: *Musa acuminata* var. *malaccensis*; C: *Musa*, AAA, Cavendish sub-group

of the amino acids from P-loop region and GLPL is about 200 amino acids. All of RGA bananas and other crop R proteins have four conserved motifs. These results indicated the presence of evolutionary conservation of disease resistance genes. Based on the research by Sutanto *et al*. (2014), there were several differences on the amino acid sequence in the fragments P 5-8, P 5-10 and P 5-11 in the kinase-2 (B) motif, especially at the amino acid Lysine (K), Leucine (L) and Isoleucine (I). On kinase-3a (C) motif, there were differences on the amino acid Cysteine-Lysine-Leucine-Isoleucine-Leucine-Alanine-Serine-Arginine-Serine-Asparagine (CKLILASRSN) and Serine-Arginine-Valine-Valine-Threonine-Arginine-Methionine-Gluthamine (SRVVTTRMQ). Meanwhile, on the hydrophobic region (D) the difference was in the amino acid Serine (S) and Alanine (A) as shown in Fig. 2. The differences of the amino acid sequences in this conservative region may allowed the different responses of different disease resistance.

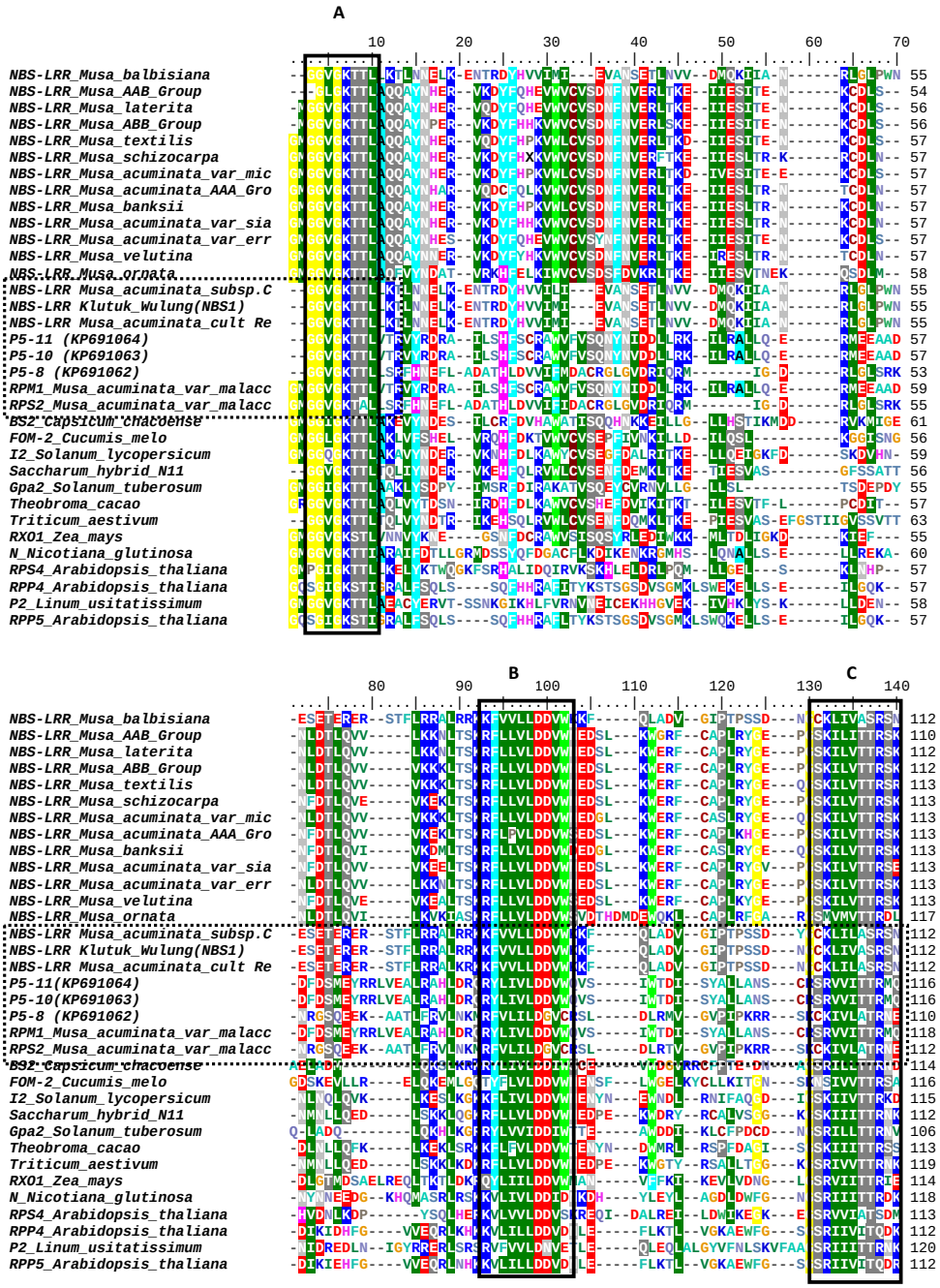

Fig. 2(A-D): Continue

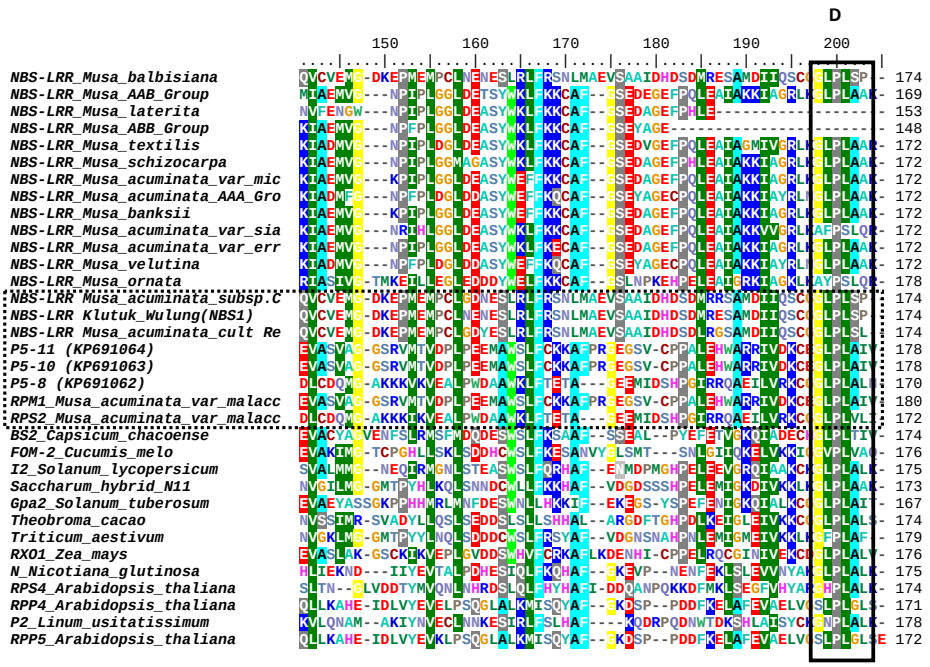

Fig. 2(A-D): Alignment analysis of predicted amino acid sequence with some proteins NBS-LRR of Musa and other R-gene deposited in the NCBI gene bank. Conserved domain area was marked by a bold box in the sequence (A: P-loop/kinase-1a, B: Kinase-2, C: Kinase-3a and D: Hydrophobic domain or GLPL). Comparison of amino acid sequence with *RPM1*, *RPS2* and Sutanto *et al*. (2014) study was marked by dotted box

The results of phylogenetic analysis of the predicted amino acid sequence obtained from this study, the banana protein and protein R from other plants in Genbank as shown in Fig. 3. The phylogenetic analysis showed that R proteins and bananas protein are divided into two groups: TIR-NBS-LRR and non-TIR-NBS-LRR. Among these proteins, protein RPP4, RPP5, RPS4, N and P2 were included to the group of TIR-NBS-LRR while, others were included into the group of non-TIR-NBS-LRR. P5-8 fragment was grouped in *RPS2* protein from *Musa acuminata* var. *malaccensis*. Meanwhile, P 5-10 and P 5-11 fragments were grouped in *RPM1* protein from *Musa acuminata* var. *malaccensis* and *RX01* protein from corn, respectively. These fragments with another banana and protein in monocotyledonous plant, such as; sugarcane, wheat and corn were included in the class of non-TIR-NBS-LRR whereas, the dicotyledonous plant were included in the class of non-TIR-NBS-LRR and class TIR-NBS-LRR.

DISCUSSION

We have isolation and identification members of NBS disease resistance gene family from *Musa acuminata* var. *malaccensis* and *Musa acuminata* cv. Cavendish (AAA). Because the NBS domain is conserved and contain easily identifiable and therefore comparable motifs, The domain is a tractable region for study of R-gene evolution.

According to Sun *et al*. (2010), from 20 RGA that were isolated from Goldfinger banana (AAAB), the predicted amino acid shared 28-54% homology with the known genes *Fom2*, *I2C1*, *I2C2* and *I2*. Agustine and Joseph (2014) isolated 517 bp fragment from banana AAB cv *Nendran* which showed 97-99% homology with NBS-LRR proteins in *Musa* varieties. While, Sutanto *et al*. (2014) mentioned that 17 fragments RGA, which were isolated from three *Fusarium* resistant banana cultivars showed homology from 91.7 up to 98.8% with proteins from NBS-LRR disease resistance in *Musa acuminata* var *malaccensis*, Group AAA, AAB and ABB and of 19.9 up to 35.5% with protein R which was already known.

In this study, three fragment showed 66-99% homology with protein *RPS2* and *RPM1* from *Musa acuminata* var. *malaccensis* (Table 2). In Li *et al*. (2012) study, an increase in the expression of genes in Cavendish cv Nongke no.1 was occurred in complex protein *RPM1/RIN4*. In *A. thaliana*, *RPM1* was associated with resistance to *Pseudomonas syringae* expressing *avrRPM1* or *avrB* (Boyes *et al*., 1998). *AvrB* and *RPM1* caused a hyperphosphorylation on *RPM1* that interact with protein4 (*RIN4*) (Torres *et al*., 2006). Inhibition of expression of *RIN4* by unknown effector of Foc TR4 will activate the *RPS2* pathway. The effector that was secreted by FOC TR4 and its similarity with the *Avr*Rpt2 effector requires further research.

The most important class of R genes is Nucleotide Binding Site Leusine Rich Repeat (NBS-LRR). This R gene contains an N terminal NBS and a C terminal LRR. NBS domain act as intramolecular signal transducer. It is also

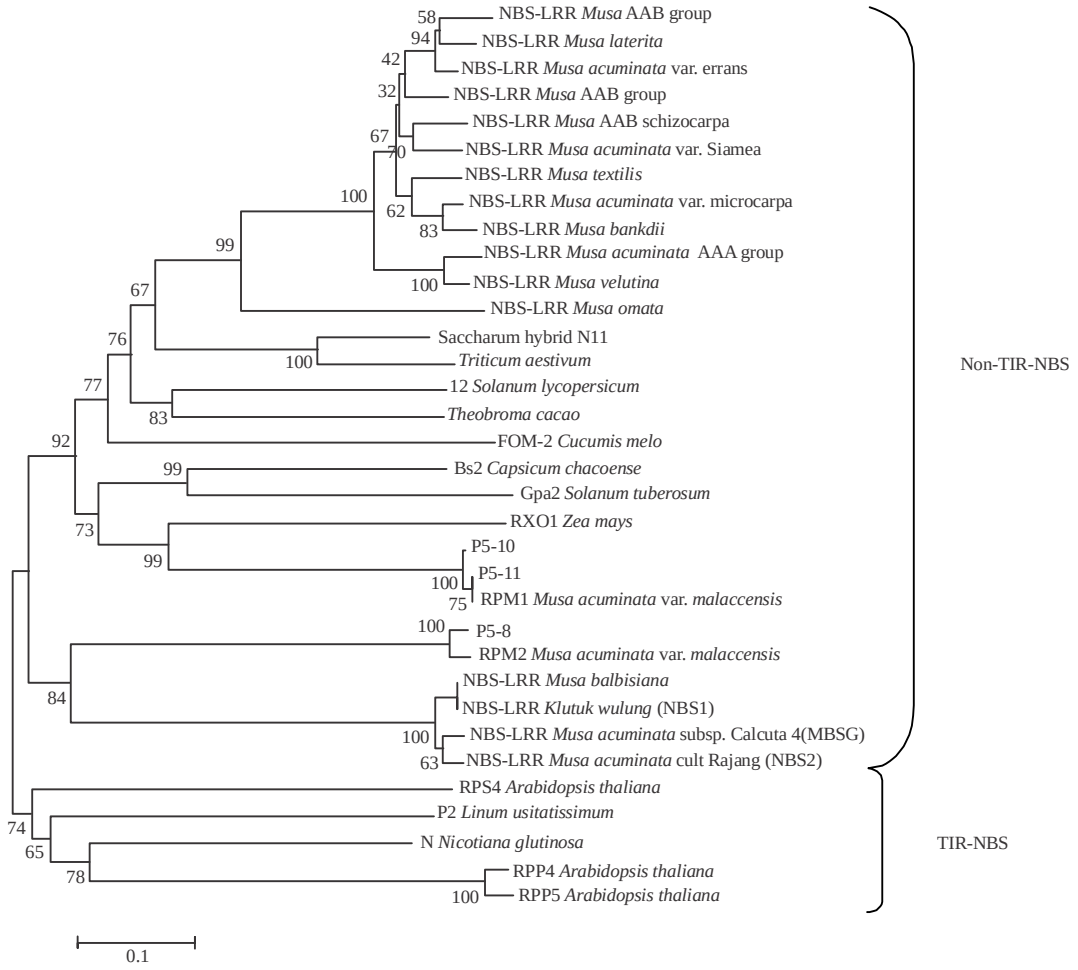

Fig. 3: Phylogenetic tree of the predicted amino acid sequence of a banana and some *Musa* RGA protein and other plant protein based on Neighbour Joining method and alignment based on MUSCLE method. Values on the axis of branching are bootstrap values (1000 replicates). Scale that is under the tree branches represents the length equal to the average amino acid substitutions persitus

involved in pathogen recognition and signal transduction (Ellis and Jones, 1998). The function of the p-loop/kinase-1a and kinase-2 is to bind phosphate in ATP, while, the kinases-3 is to interact with purine (Traut, 1994). The GLPL motif is involved in the binding of ADP. The P-loop motif is also required in nucleotide binding and the mutations in this motif will cause a loss of function of the NBS-LRR proteins. On kinase-2, the last 2 aspartic acid interacts with the third phosphate from ATP and plays a role in the coordination of divalent metal ions that required in the phosphate transfer reaction, for example Mg^{2+} of MgATP (McHale *et al*., 2006).

The absence of the TIR region on the protein-R can be predicted by the presence of RNBS-A motif close to the P-loop and also by the presence of residues of Tryptophan (W) at the end of kinase-2 motif (Meyers *et al*., 1999). In phylogenetic analysis, an evolutionary hypothesis of the loss of TIR areas of NBS-LRR in monocots possibly happened during monocots and dicots divergence (Pan *et al*., 2000). The grouping of non-TIR-NBS-LRR in banana possibly encoded resistance gene with unknown specificity of the product (Peraza-Echeverria *et al*., 2008). Although, other banana protein did not correlate with the disease resistance of banana in general, its potential role in disease resistance process can be tested using the post-genomic era technologies such as, RNA interference (RNAi). This technology can be used to silence the target related to disease resistance (Waterhouse and Helliwell, 2003).

CONCLUSION

We have analyzed three fragments containing four conserved motifs of Nucleotide Binding Site were obtained from *Musa acuminata* var. *malaccensis* (P 5-8, P 5-10) and *Musa*, AAA, Cavendish sub-group (P 5-11). The P 5-8 fragment had similarity with *RPS2* protein of *Musa acuminata* var *malaccensis*. The P 5-10 and P 5-11 had similarity with *RPM1* proteins of *Musa acuminata* var. *malaccensis*. Our

contig sequences were deposited in Genbank and assigned number KP691062-KP691064. Phylogenetically, all fragments belong to the group of non-TIR-NBS-LRR. This result can be used to identify the diversity of NBS-LRR gene from other wild banana. The conserved sequences of NBS-LRR gene can be used as a potential genetic marker for disease resistant gene in banana.

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