Cloning and Characterization of Resistance Gene Analogues (RGAs) from Piper Nigrum L. cv. Semongok Aman and Piper Colubrinum Link

Lau Ee Tiing, Hwang Siaw San, Lily Eng, and Paulus Amin Det

***Abstract*—Resistance gene analogues (RGAs) have been isolated from many crops such as banana, cereals, sugarcane, wheat and tomato. Cloning and characterization of RGAs offer potential in breeding for disease resistant crops through marker-assisted selection. Resistance genes from a broad range of plant species are known to share similarities in amino acid structural motifs. A pair of degenerate oligonucleotide primers designed from conserved motifs of P-loop and GLPL regions, common to many resistance genes, were used to amplify the Nucleotide-Triphosphate Binding Site (NBS) regions of RGAs from two *Piper* species, *Piper nigrum* L. cv. Semongok Aman and *Piper colubrinum* Link. A total of ten (10) partial RGAs fragments designated as *PcRGAt5*, *PcRGAt6*, *PcRGAt7*, *PcRGAt8* and *PcRGAt9* amplified from *Piper colubrinum* Link.; *PnRGAt10*, *PnRGAt11*, *PnRGAt12*, *PnRGAt13* and *PnRGAt14* amplified from *Piper nigrum* cv. Semongok Aman have been sequenced and characterized. The cloned partial RGAs with sequence length ranged from 498bp to 540bp encoding proteins with the size ranging from 165 amino acids to 179 amino acids. The predicted *Piper* RGA peptides contained the conserved motifs of Kinase-1 or the P-loop (GMGGVGKT), Kinase-2 (VLDDVW) and the hydrophobic Kinase-3 domain or GLPL. The obtained peptide sequences showed 42% to 47% similarity with published disease resistance proteins from various plant species such as *Prunus persica* (peach species) NBS resistance proteins, *Malus baccata* (apple species) and *Prunus kansuensis* (wild peach species) NBS-LRR-like proteins, *Manihot esculenta* (cassava) RCa9, RCa10.2 and RCa10.6 NBS type resistance proteins. An Un-weighted Pair Group Method with Arithmetical Averages (UPGMA) phylogenetic tree has been generated from the alignment of predicted *Piper* RGA peptides with the 6 published disease resistance proteins. The clustering analysis revealed that NBS-containing resistance genes comprise a large gene family in *Piper* species.**

***Index Terms*—Clustering analysis, conserved motifs, disease resistant, *piper nigrum* L. cv. semongok aman, *Piper colubrinum* link., resistance gene analogues (RGAs).**

I. Introduction

Black pepper is the world's most widely used spice for food flavoring. It is the fruit of the tropical climbing woody vine, *Piper nigrum* L., belonging to the family Piperaceae and native to South-western India. Vietnam, India, Brazil, Indonesia and Malaysia are the five main producers of black pepper in the world. In Malaysia, black pepper is mainly cultivated in the State of Sarawak and it contributes significantly to the rural population as a source of income.

Black pepper is susceptible to various pathogens which cause considerable yield loss every year. The major diseases found in pepper are foot rot caused by *Phytophthora capsici*, black berry caused by *Colletotrichum capsici* and *Colletotrichum gloeosporioides* and slow decline due to a disease complex caused by root-knot nematodes (*Meloidogyne* spp.) and the fungi, *Fusarium* spp.

Piper colubrinum Link is a woody shrub and is a wild relative of *Piper nigrum* L. It is native to the northern part of South America. This species is important because of its resistance to *Phytophthora capsici* and nematodes [1], [2], [3].

Grafting *Piper nigrum* L. shoots onto *Piper colubrinum* Link. Rootstocks had been carried out as a way of trying to solve the problems of soil-borne pathogens in pepper cultivation in Brazil, Malaysia and India [2], [4]. The grafted plants indicated high initial success but poor survival later due to the problem of delayed graft incompatibility [5], [6].

Wide hybridization between *Piper colubrinum* Link and *Piper nigrum* L. had been attempted in Malaysia, but there had been no success due mainly to the different ploidy levels and that the two species are cross incompatible [6]. With the development of biotechnological approaches, there is a lot of potential in transferring desirable resistance genes from *Piper colubrinum* Link. onto *Piper nigrum* L.

Genes conferring resistance to various pathogenic bacteria, fungi, nematode or viruses have been isolated from a variety of plant species [7], [8], [9]. Majority of the plant resistance genes encode cytoplasmic receptor-like proteins that contain Nucleotide-Triphosphate Binding Site (NBS) and Leucine-Rich Repeat (LRR) domains [10].

Motifs of these domains are well conserved in plant resistance genes and have been named as Resistance Gene Analogues (RGAs) [11] or Resistance Gene Candidates (RGCs) sequences [12]. Genetic analysis has associated the sequences of this group of genes to known gene loci that confer resistance to bacteria, fungi, nematode or viruses [11] - [17].

Examples of members of plant resistance genes from NBS-LRR gene family are *Prf*, *I2CI* and *Mi* from tomato [18], [19], [20], *N* from tobacco [21], *RPM1* from *Arabidopsis* [22], *M* and *L6* from flax [23], [24], *RGC2* from lettuce [25], *Xa1* from rice [26] and *Cre3* from wheat [27].

Plant resistance genes appear to encode components of

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signal transduction pathways and have been found to share significant homologies in DNA, amino acid and structural domains [7], [9]. The high degree of sequence conservation among the NBS-LRR domains of plant resistance genes has permitted the design of degenerate oligonucleotide primers for use in Polymerase Chain Reaction (PCR) amplification and cloning of RGAs from plant species.

In this study, we aimed to clone and characterize the RGAs in *Piper* species using degenerate oligonucleotide primers designed for recognition of conserved NBS domains.

II. Materials and Method

A. Sampling

Young leaves of *Piper nigrum* L. cv. Semongok Aman and Piper *colubrinum* Link. were collected from the Pepper Breeding Section of Agriculture Research Centre, Department of Agriculture Sarawak at Semongok, Kuching, Sarawak.

The leaf samples were wrapped with aluminum foil, kept in separate plastic bags, labeled and preserved in liquid nitrogen prior to storage at -80 ℃.

B. Chemical Reagents and Solutions

The RNA isolation buffer contained 2% (w/v) cetyl trimethylammonium bromide (CTAB), 1% (w/v) polyvinyl-pyrrolidone (PVP), 0.1M Tris-hydrochloric acid (HCl) with pH 8.0, 25mM ethylenediaminetetraacetic acid (EDTA) with pH 8.0, 1.4M sodium chloride (NaCl) and 1% (v/v) beta-mercaptoethanol (added prior to use).

Chemical solutions prepared for RNA isolation comprised 0.1% (v/v) diethyl- pyrocarbonate (DEPC) treated water, 3M sodium acetate (NaOAc) with pH 5.2, phenol:chloroform: isoamylalcohol (25:24:1 v/v), chloroform, cold isopropanol (-20 ℃), cold absolute ethanol (-20 ℃) and 75% (v/v) ethanol.

C. Total RNA Extraction and Purification

Total RNAs were isolated from the young leaf tissues of *Piper nigrum* L. cv. Semongok Aman and *Piper colubrinum* Link. using modified ion detergent CTAB-based extraction method [28].

DNA contamination was eliminated using RQ1 RNase-free DNase (Promega, USA). The total 10µl DNase reaction volume contained 1X RQ1 RNase-free DNase reaction buffer [40mM Tris-HCl with pH 8.0, 10mM magnesium sulfate ($MgSO_4$), 1mM calcium chloride ($CaCl_2$)] and 1 unit of RQ1 RNase-free DNase per 1µg of total RNA.

The reactions were carried out at 37 ℃ for 30 minutes, followed by heat inactivating the DNase at 65 ℃ for 10 minutes with 0.1 volume of RQ1 DNase stop solution [20mM ethylene glycol tetraacetic acid (EGTA) with pH 8.0].

The purified RNA was treated with equal volume of phenol:chloroform:isoamylalcohol (25:24:1, v/v), followed by equal volume of chloroform and recovered through 1 volume of cold isopropanol (-20 ℃) precipitation prior stored at -80 ℃.

The qualities of the isolated total RNAs were analyzed through gel electrophoresis. The electrophoresis apparatus were pre-treated with 0.1% (v/v) DEPC treated water. The RNA samples were electrophoresed on 1.2% (w/v) agarose gels for an hour at 100V in 0.1% (v/v) DEPC treated 1X TBE (90mM Tris-borate, 2mM EDTA with pH 8.0, 1X SYBR® Safe DNA gel stain) buffer.

The stained gels were visualized using Safe Imager™ 2.0 Blue-Light Trans- illuminator and documented using the AlphaImager® HP Gel Documentation System (Alpha Innotech Corporation, South Africa).

The isolated total RNAs were quantified with the Gene-Quant 1300 UV Spectrophotometer (GE Healthcare Bio Science, UK) at wavelengths of 230nm, 260nm and 280nm with a dilution factor equal of 100.

D. Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis

First-strand cDNA templates were prepared from the isolated total RNAs using ImProm-II™ Reverse Transcription System (Promega, USA).

The total 20µl reverse transcription volume contained 1X ImProm-II™ reaction buffer, 3mM magnesium chloride ($MgCl_2$), 0.5mM each dNTP mix, 1U/µl recombinant RNasin® ribonuclease inhibitor, 1µl ImProm-II™ reverse transcriptase (Promega, USA) and 0.5µg of oligo$(dT)_{15}$ primer per 1µg of total RNA.

The reverse transcriptions were carried out with 5 minutes of initial RNA incubation at 70 ℃ to remove RNA secondary structures, followed by 5 minutes of annealing at 25 ℃ and 60 minutes of first-strand extension at 42 ℃. The reactions were terminated by heat inactivating the ImProm-II™ reverse transcriptase at 70 ℃ for 15 minutes to prevent the reverse transcriptase from binding to newly synthesized first-strand cDNA.

A pair of degenerate oligonucleotide primers designed to amplify from the P-loop to the GLPL domain was chosen for Polymerase Chain Reaction (PCR). The forward primer from the P-loop was CNL298F with the nucleotide sequence 5'-GGN ATG GGN GGN GTN GGN AAR AC-3' encoding the amino acid sequence GMGGVGKT and the reverse primer was NBSIR, 5'-CGT CTT TGC MGC NAR NGG NAA NCC-3' [29].

PCR amplifications were carried out using the Applied Biosystem Thermal Cycler with Gradient Features. The total 25µl of PCR reaction mixture contained 1X PCR buffer [20mM Tris-HCl with pH 8.4, 50mM potassium chloride (KCl)], 1.5mM $MgCl_2$, 0.2mM each dNTP mix, 0.4µM forward and reverse oligonucleotide primers, 1 unit *Taq* DNA polymerase (Invitrogen, USA) and 1.0µl of first-strand cDNA templates.

PCR cycles were performed with 5 minutes of initial denaturation at 94 ℃, followed by 35 cycles of 1 minute of denaturation at 94 ℃, 1 minute of annealing at 55 ℃ and 2 minutes of extension at 72 ℃, and ending with 5 minutes of final extension at 72 ℃.

E. Cloning of Partial RGA cDNAs

The RT-PCR amplified partial *RGA* fragments were analyzed using 1X SYBR® Safe DNA gel stained 1.5% (w/v) agarose gels and purified using PureLink™ Quick Gel Extraction Kit (Invitrogen, USA).

Gel slices containing the PCR amplicons were dissolved with 3 gel volumes of kit supplied Solubilization Buffer L3 at 50 ℃ for 15 minutes. To bind cDNAs, the reactions were applied to Quick Gel Extraction Columns. Supernatants were discarded through centrifugation at 16,627*g* for 1 minute at room temperature. The cDNAs bound on the membrane of the columns were washed with kit supplied Wash Buffer W1 and eluted into the recovery tubes by Elution Buffer E5.

The purified PCR amplicons were cloned using pGEM®-T Easy Vector System I (Promega, USA). The total 10µl ligation volume contained 1X rapid ligation buffer, 50ng pGEM®-T Easy vectors, 3 Weiss units T4 DNA ligase and 3µl of PCR amplicons. Ligation was carried out overnight at 4 ℃ prior transformation of recombinant plasmid DNAs into high efficiency *Escherichia coli* JM109 competent cells.

Transformed plasmid DNAs were purified using PureLink™ Quick Plasmid Miniprep Kit (Promega, USA) through centrifugation. Bacterial cultures were lysated with kit supplied Cell Lysis Buffer CLC and neutralized using Neutralization Solution NSC.

To bind plasmid DNAs, the neutralized lysate were pipetted into PureYield™ Minicolumn and centrifuged at 16,627*g* for 15 seconds at room temperature. The flow-through was discarded. The plasmid DNAs bound on the silica-membranes of the columns were initially washed with kit supplied Endotoxin Removal Wash (ERB) buffer followed by Column Wash Solution CWC and finally eluted using Elution Buffer EBB. The purified recombinant plasmids DNAs were sequenced.

F. Sequence Analysis of Predicted Partial RGA Peptides

The sequenced partial RGA fragments were edited using CHROMAS 2.30 to remove cloning vector sequences. Individual RGA sequences were translated and searched against GenBank non-redundant protein sequences using Basic Local Alignment Search Tool (BLASTX) for sequence homology searches.

Multiple alignments of *Piper* RGA peptides with published NBS type disease resistance proteins from various plants were performed using ClustalW algorithm. The output of alignment analysis was displayed via BoxShade server of the European Molecular Biology Network (EMBnet) retrieved from http://www.ch.embnet.org/software/BOX_form.html.

The conserved motifs of Kinase-1 or the P-loop (GMGGVGKT), Kinase-2 (VLDDVW) and the hydrophobic Kinase-3 domain or GLPL were predicted from the alignments. Cluster analyses of *Piper* RGA peptides with published NBS type disease resistance proteins were carried out using Molecular Evolutionary Genetics Analysis (MEGA) software [30].

A phylogenetic tree was constructed through Un-weighted Pair Group Method with Arithmetical Averages (UPGMA) method with bootstrap test (1000) for evaluating the reliability of the inferred UPGMA trees.

III. Results and Discussions

High quality total RNAs were successfully isolated from the young leaves of *Piper nigrum* L. cv. Semongok Aman and *Piper colubrinum* Link. Fig. 1 shows the 1.2% (w/v) gel electrophoresis of isolated RNAs. The distinct 25S and 18S ribosomal RNA bands were viewed on 1X SYBR® Safe DNA gel stained agarose gel.

Ribosomal RNA represents more than 90% of the total RNAs [31]. Any degradation occurring during the RNA preparations can be easily viewed as smearing or indistinct bands. Therefore, the presence of distinct ribosomal RNA bands in Fig. 1 indicated little or no RNA degradation occurred during isolation process.

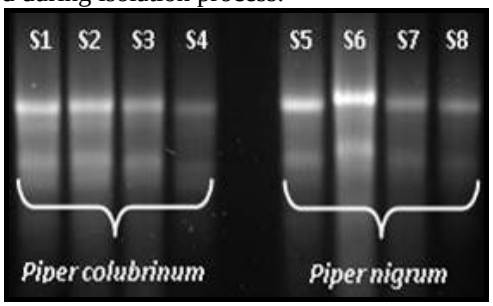

Fig. 1. 1.2% (w/v) Agarose gel electrophoresis of total RNAs isolated from the young leaves of *piper nigrum* L. Cv. Semongok Aman and *piper colubrinum* link. using modified ion detergent ctab-based extraction method [28]; Lanes S1 to S4, isolated total rnas of *piper colubrinum* link.; lanes S5 to S8, isolated total rnas of *piper nigrum* L. Cv. semongok aman.

Table I shows the spectrophotometric evaluation of RNA samples. The RNA yields ranged from 24.2 µg to 61.0 µg of total RNAs per gram of fresh leaf tissues. The high purity of isolated RNAs was determined by the absorbance ratios of A_{260}/A_{230} and A_{260}/A_{280}.

The A_{260}/A_{230} absorbance ratio indicates polysaccharide or polyphenolic contamination; and the A_{260}/A_{280} absorbance ratio indicates protein contamination [32], [33].

The A_{260}/A_{230} absorbance ratios of isolated RNAs were within 1.7 to 2.0; indicating weak contamination of polysaccharides and polyphenolics [34], [35], [36]. Meanwhile, the A_{260}/A_{280} absorbance ratios of the isolated RNAs ranging from 1.6 to 2.0, suggested less contamination of protein substances during RNA preparations [34], [35], [37].

TABLE I: Spectrophotometric Evaluations of Total RNAs Isolated From the Young Leaves of Piper Nigrum L. cv. Semongok Aman and Piper Colubrinum Link. Using Modified ION Detergent CTAB-Based Extraction Method

RNA Samples	[1]Absorbance ratios		[2]Yield (µg/g)
	$A_{260/230}$	$A_{260/280}$	
S1	1.790	1.801	56.2
S2	2.000	1.859	63.2
S3	1.837	1.824	49.6
S4	2.608	1.583	26.6
S5	1.784	1.930	61.0
S6	1.350	1.929	43.2
S7	1.043	2.017	24.2
S8	1.593	1.957	27.4

[1]Results are presented as the mean of 3 absorbance readings.
[2]Yields of isolated total RNAs per gram of fresh young leaf tissues used.

The intactness of isolated RNAs was confirmed through reverse transcriptase-PCR (RT-PCR). Fig. 2 shows the 1.5% (w/v) gel electrophoresis of amplified partial *Piper* RGA fragments. RT-PCR amplicons with approximate size of 500bp were viewed as distinct fragments on 1X SYBR® Safe DNA gel stained agarose gel.

A total of ten positive clones were sequenced and

designated as *PcRGAt5*, *PcRGAt6*, *PcRGAt7*, *PcRGAt8*, *PcRGAt9* (amplified from *Piper colubrinum* Link.); and *PnRGAt10*, *PnRGAt11*, *PnRGAt12*, *PnRGAt13*, *PnRGAt14* (amplified from *Piper nigrum* L. cv. Semongok Aman).

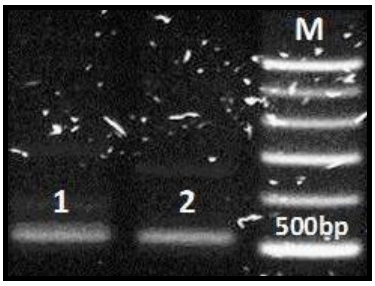

Fig. 2. 1.5% (w/v) Agarose gel electrophoresis of RT-PCR amplifications of *Piper* RGA fragments using degenerate oligonucleotide primer pair CNL298F/NBSIR; lane1, partial rga fragment of *piper nigrum* L. Cv. semongok aman; lane 2, partial rga fragment of *piper colubrinum* link.; lane m, 100bp dna ladder (promega, usa).

The cloned partial *Piper* RGA sequence lengths ranged from 498bp to 540bp encoding proteins with size ranging from 165 amino acids to 179 amino acids.

BLASTX database search programme indicated partial peptide sequences of *Piper* RGAs were 42% to 47% similar to the published disease resistance proteins of various plants, i.e. *Prunus persica* (peach species) NBS resistance proteins, *Malus baccata* (apple species) and *Prunus kansuensis* (wild peach species) NBS-LRR-like proteins, *Manihot esculenta* (cassava) RCa9, RCa10.2 and RCa10.6 NBS type resistance proteins.

Table II shows the sequence homology tests of partial *Piper* RGA peptides against the GenBank non-redundant protein sequences.

TABLE II: BLASTX SEQUENCE HOMOLOGY SEARCHES OF PARTIAL PIPER RGA PEPTIDES AGAINST THE GENBANK NON- REDUNDANT PROTEIN SEQUENCES

Plant species	Protein types	Query Coverage	Identity (%)
Prunus persica	NBS resistance (AEB61537.1)	99%	42
Malus baccata	NBS-LRR-like (AED99146.1)	99%	47
Prunus kansuensis	NBS-LRR-like (ADO66738.1)	99%	43
Manihot esculenta RCa9	NBS resistance (AAO38220.1)	98%	42
Manihot esculenta RCa10.2	NBS resistance (AAP30045.1)	97%	41
Manihot esculenta RCa10.6	NBS resistance (AAP30049.1)	97%	45

The presence of conserved motifs, i.e. Kinase-1 or the P-loop (GMGGVGKT), Kinase-2 (VLDDVW) and the hydrophobic Kinase-3 domain or GLPL, as shown in Fig. 3, proves that it is effective to isolate and clone RGAs from *Piper* species based on the domains conserved among the known plant resistance genes.

The P-loop in NBS domain of plant RGAs has been reported to interact directly with the phosphate of the bound nucleotides and has been well characterized for adenosine triphosphate (ATP) and guanosine triphosphate (GTP) binding proteins [38], [39], [40], [41], [42]. Mutations of key residues in the P-loop can led to partial loss of the resistance gene (*N* gene) function in tobacco [7].

The Kinase-2 motif of RGAs was reported to be involved in coordinating the ion magnesium (Mg^{2+}) required for phosphor-transfer reactions [41]. Protein arrangements occurring in the Kinase-2 to GLPL motifs are corresponding to the specificity of the NBS downstream signaling activation of coil-coiled domains [29].

Thus, the P-loop, Kinase-2 and the GLPL motifs of *Piper* RGAs detected in the present study are believed to play an important role for ATP binding and hydrolysis; and in signal transduction, triggered by the presence of the pathogen [43].

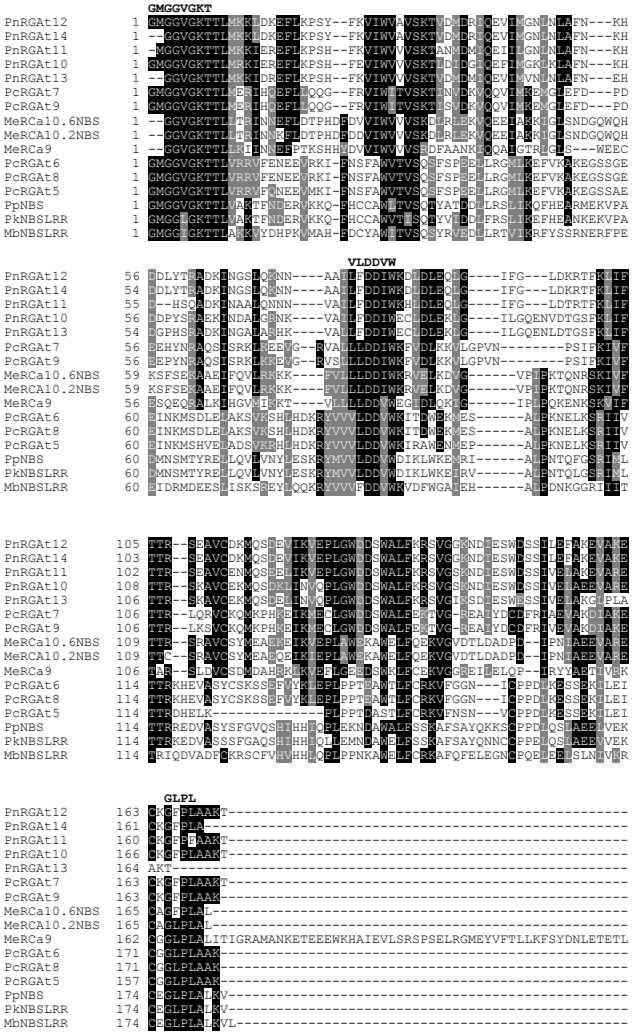

Fig. 3. Multiple alignments of predicted partial *piper* rga peptides with 6 published disease resistance proteins from genbank, i.e. *prunus persica* (peach species) nbs resistance proteins (accession no: aeb61537.1); *malus baccata* (apple species) and *prunus kansuensis* (wild peach species) nbs-lrr-like proteins (accession no: aed99146.1 and ado66738.1); *manihot esculenta* (cassava) rca9, rca10.2 and rca10.6 nbs type resistance proteins (accession no: aao38220.1, aap30045.1 and aap30049.1) using clustalw algorithm. identical amino acids are in reverse color. conservative substitutions are shaded using online boxshade programme. sequence comparison shows the presence of conserved motifs of kinase-1 or the p-loop (gmggvgk), kinase-2 (vlddvw) and the hydrophobic kinase-3 domain or glpl, indicated by bolded letters on top of the alignments.

Fig. 4 shows the Un-weighted Pair Group Method with Arithmetical Averages (UPGMA) phylogenetic tree constructed from multiple alignments of the cloned partial

Piper peptide sequences with 6 published NBS type disease resistance proteins carried out via Molecular Evolutionary Genetics Analysis (MEGA) software [30].

The PcRGAt7 and PcRGAt9 isolated from the *Piper colubrinum* Link. were found clustered closely with the five partial RGA peptides, i.e. PnRGA10, PnRGA11, PnRGA12, PnRGA13 and PnRGA15 amplified from the *Piper nigrum* L. cv. Semongok Aman.

The other three members of *Piper colubrinum* Link. RGA proteins, i.e. PcRGAt5, PcRGAt6 and PcRGAt8 were clustered close to the *Prunus persica* (peach species) NBS resistance proteins, *Malus baccata* (apple species) and *Prunus kansuensis* (wild peach species) NBS-LRR-like proteins.

These results indicate that NBS-containing resistance genes comprise a large gene family in *Piper* species. Thus, further studies are needed to isolate more novel *Piper* RGAs, study the full-length gene sequences and evaluate the role of these genes in disease resistance.

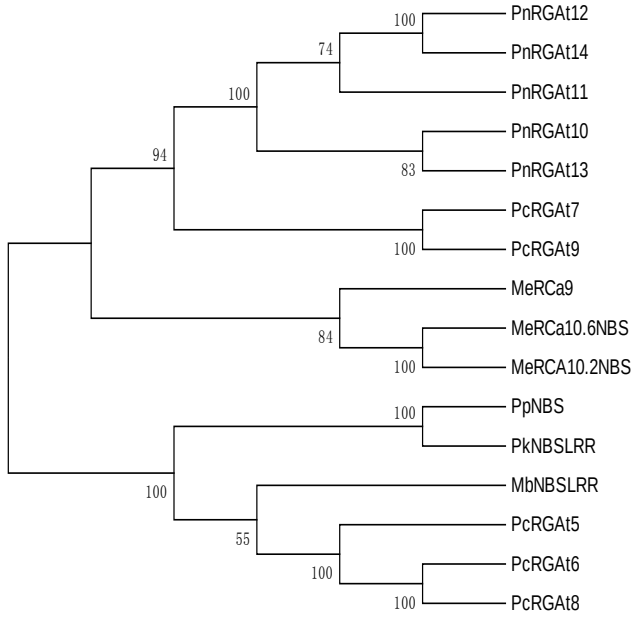

Fig. 4. UPGMA phylogenetic tree generated from the alignment of predicted partial *piper* rga peptides with 6 published disease resistance proteins from genbank, i.e. *prunus persica* (peach species) nbs resistance proteins (accession no: aeb61537.1); *malus baccata* (apple species) and *prunus kansuensis* (wild peach species) nbs-lrr-like proteins (accession no: aed99146.1 and ado66738.1); *manihot esculenta* (cassava) rca9, rca10.2 and rca10.6 nbs type resistance proteins (accession no: aao38220.1, aap30045.1 and aap30049.1) using mega software. alignments were made using the default parameters of the clustalw algorithm. bootstrap values are indicated next to the relevant node.

IV. Conclusion

In conclusion, RGAs in *Piper* were successfully cloned using degenerate primer pair CNL298F/NBSIR designed for identifying the nature and diversity of NBS domains in *Musa* spp. (banana) resistance genes [29].

The UPGMA dendrogram (Fig. 4) shows that PcRGAt7 and PcRGAt9 of Piper *colubrinum* Link. were clustered closely to the RGA members of *Piper nigrum* L.

The other three members of *Piper colubrinum* Link., PcRGAt5, PcRGAt6 and PcRGAt8 were found to be more related to the *Prunus* species and *Malus baccata* NBS-type proteins rather than PcRGAt7 and PcRGAt9. These results reveal that NBS-type resistance genes comprise a large gene family in *Piper* species.

A few pairs of degenerate primers designed for recognition of NBS domains in other plant resistance genes, *viz*. LM638/REV7 [44], S2/A2 [45], CNL298F/M1445R [29], 3F2/I3RI [43] and RGA51F/RGA51R [46] have been used to clone more novel RGAs in *Piper* species.

Besides that, gene specific primers used in conjunction with GeneRacer^{TM} PCR primers were designed based on the partial *RGAs* genes obtained in the present study to discover the full-length sequences and evaluate the role of these *Piper* resistance genes in disease resistance.

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