LCMS-BASED PROTEOMIC ANALYSIS OF SUSCEPTIBLE *Musa-Meloidogyne incognita* INTERACTION AND CHARACTERISATION OF PARTIAL *NBS-LRR RESISTANCE* GENE IN *Musa acuminata* spp.

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LCMS-BASED PROTEOMIC ANALYSIS OF SUSCEPTIBLE *Musa-Meloidogyne incognita* INTERACTION AND CHARACTERISATION OF PARTIAL *NBS-LRR RESISTANCE* GENE IN *Musa acuminata* spp.

ABSTRACT

Plant-parasitic nematode (PPN) infestation is burdening banana market and economy worldwide. To overcome this problem, an efficient nematode management approach needs to be developed. Since most cultivated bananas are sterile, the chances of obtaining improved traits using conventional breeding programmes are close to none. To complement this limitation, producing transgenic banana lines with an improved trait is seen as an ideal alternative to manage nematode infestation in bananas. Therefore, an overall aim of the studies conducted in this thesis was to obtain an in-depth understanding of the molecular basis of a compatible banana-nematode interaction. Therefore, a proteomics experiment was designed to profile a compatible interaction between Grand naine cultivar (ITC 1256) and *Meloidogyne incognita*. Two time points were chosen for this experiment namely 30 and 60 days-after-inoculation (dai). A high-throughput Liquid Chromatography Mass Spectrometry (LCMS) Proteomics platform was used to profile banana root proteins involved during the interaction at both time points. A total of 2065 proteins were recovered from this experiment. Statistical analysis carried out on the peptide feature data obtained revealed that 112 proteins recovered at 60-dai time point showed significant abundance changes (ANOVA, $p \leq 0.05$) between *M. incognita*- inoculated and control root tissues. These proteins were divided into ten predicted function groups namely DNA replication, defence, energy-related, catalytic, structural component, carrier, stress response, metabolism, oxidation-reduction and biosynthesis. The proteomics data also revealed that a protein involved in plant defence namely pathogenesis-related protein 1 (ITC1587_Bchr9_P26466) was present at a

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significantly lower abundance level in *M. incognita*-inoculated root tissues when compared to control root tissues. However, the involvement of *NBS-LRR Resistance* (*R*) gene was not captured by LCMS proteomics platform. Therefore, a molecular-based strategy was adopted to isolate and characterise the gene at both genomic and transcript levels. A pair of degenerate primers was used to target the conserved regions of the *R* gene. This study had successfully isolated 73 clone sequences. These isolated clones were found to show high sequence similarities with *Resistance* gene in other *Musa* spp. with E-values ranging from 0.00 to 2e^{-14}. A phylogenetic Neighbour-Joining tree constructed based on the aligned deduced NBS-LRR Resistance amino acid sequences of eight *Musa* species together with the isolated cloned sequences revealed that there were six types of *NBS-LRR Resistance* gene in *Musa* spp. This study also found that 30% of the isolated cloned sequences to have their open reading frame (ORF) encoding the Resistance protein interrupted. This phenomenon may lead to the occurrence of premature stop codon that led to the formation of pseudogenes.

Keywords: *Meloidogyne incognita*, *Musa acuminata*, proteomics, LCMS, *NBS-LRR Resistance* gene

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ANALISIS PROTEOMIK 'LCMS' BAGI INTERAKSI RENTAN *Musa-Meloidogyne incognita* DAN PENCIRIAN GEN *RINTANGAN NBS-LRR* SEPARA DALAM SPESIS-SPESIS *Musa acuminata*

ABSTRAK

Jangkitan nematod parasit tumbuhan membebani pasaran dan ekonomi pisang seluruh dunia. Untuk mengatasi masalah ini, pendekatan pengurusan nematod yang cekap perlu dibangunkan. Disebabkan majoriti pisang yang ditanam adalah mandul, kemungkinan untuk mendapatkan pisang yang mempunyai trait-trait yang lebih baik melalui teknik pembiakan pisang yang lazim adalah hampir mustahil. Untuk melengkapi kekangan ini, penghasilan pisang transgenik dengan trait yang ditambah-baik dilihat sebagai alternatif ideal untuk mengatasi jangkitan nematod terhadap pokok pisang. Oleh itu, sasaran keseluruhan penyelidikan yang dijalankan dalam tesis ini adalah untuk memperolehi satu pemahaman mendalam tentang asas molekular suatu interaksi serasi antara pisang – nematod. Oleh itu, satu eksperimen proteomik telah direkabentuk untuk memprofil suatu interaksi serasi antara pisang Grand naine (ITC 1256) dengan *Meloidogyne incognita*. Dua titik masa iaitu 30 dan 60 hari selepas jangkitan (hsj) telah dipilih. Sebuah pelantar 'Liquid Chromatography Mass Spectrometry' (LCMS) berkapasiti tinggi telah digunakan untuk memprofil protein-protein akar pisang yang terlibat semasa interaksi ini pada dua titik masa tersebut. Sejumlah 2065 protein telah diperolehi dalam kajian ini. Analisis statistik yang dijalankan terhadap ciri peptida yang diperolehi mendedahkan bahawa 112 protein menunjukkan perubahan kelimpahan yang signifikan (ANOVA, $p \leq 0.05$) antara tisu terkawal dan tisu terjangkit *Meloidogyne incognita* pada titik masa 60- hsj. Protein-protein ini dibahagikan kepada sepuluh kelas ramalan fungsi biologi iaitu replikasi DNA, pertahanan, kaitan tenaga, pemangkin, komponen struktur, pembawa, tindak balas stres, metabolisma, pengoksidaan-penurunan dan biosintesis.

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Data proteomik juga mendedahkan bahawa tahap kelimpahan satu protein yang terlibat dalam pertahanan tumbuhan iaitu protein berkaitan patogen 1 (ITC1587_BChr9_P26466) telah berkurang secara signifikan dalam tisu akar terjangkit *M. Incognita* apabila dibandingkan dengan tisu akar terkawal. Walaubagaimanapun, penglibatan gen *Rintang* '*NBS-LRR'* tidak dapat dicerap dengan menggunakan teknologi proteomik LCMS ini. Oleh itu, strategi berasaskan molekul telah digunakan untuk mengasing dan mencirikan gen pada tahap transkrip dan genomik. Sepasang primer tidak khusus digunakan untuk menyasar jujukan-jujukan terabadi gen *R* ini. Kajian ini berjaya mengasingkan 73 klon jujukan. Klon-klon yang diasingkan ini menuunjukkan persamaan jujukan yang tinggi dengan gen *Rintangan* dalam spesis *Musa* lain dengan nilai-E dalam lingkungan julat antara 0.00 ke $2e^{-14}$. Satu pepohon filogenetik "Neighbour-Joining" telah dibina berasaskan jujukan asid amino gen *Rintang NBS-LRR* dalam lapan spesis *Musa* berserta klon-klon jujukan yang telah diasingkan mendedahkan bahawa gen *Rintangan NBS-LRR Musa* boleh dibahagikan kepada enam kumpulan. Kajian ini juga mendapati 30% daripada klon yang diasingkan mempunyai kerangka bacaan terbuka ('ORF') protein Rintang yang terganggu. Fenomena ini mungkin membawa kepada kewujudan kodon penamat tidak matang yang menjurus kepada pembentukan gen pseudo.

Kata kunci: *Meloidogyne incognita, Musa acuminata*, proteomik, LCMS, Gen *Rintang NBS-LRR*

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LIST OF SYMBOLS AND ABBREVIATIONS

&	And
$	Dollar
=	Equals to
<	Less Than
≤	Less Than or equal to
-ve	Negative
%	Percentage
µA	Microampere
µg	Microgram
µL	Microlitre
µM	Micromolar
1st-D	First Dimension
2-DE	Two-Dimensional gel electrophoresis
2nd-D	Second Dimension
A	Adenine
A260/A230	Absorbance at 260nm over absorbance at 230nm
A260/A280	Absorbance at 260nm over absorbance at 280nm
AGE	Agarose gel electrophoresis
ANOVA	Analysis of Variance
Avr	Avirulence
B	Beta
BLAST	Basic local alignment search tool
bp	Base Pair
BSA	Bovine serum albumin

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C	Cytosine
cDNA	Complementary deoxyribonucleic acid
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CTAB	cetyl trimethylammonium bromide
cv.	Cultivar
dai	Days After inoculation
dH2O	Distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ELF	egg-laying female
et al.	at alii
EtBr	ethidium bromide
E-value	expectation value
g	earth's gravitational acceleration
g	gram
G	Guanine
GN	Grand naine
h	hour
H_3BO_3	boric acid
HCl	hydrochloric acid
i.e	id est
IAA	iodoacetic acid
IEF	isoelectric focusing

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INIBAP	international network for the improvement of banana and plantain
IPG	immobilised pH gradient
IPTG	isopropyl β-D-1-thiogalactopyranoside
J2	juvenile stage two
Kb	kilobase pair
KCl	potassium chloride
kg	kilogram
L	litre
LB	lysogeny broth (Luria-Bertani)
LC-MS	Liquid Chromatography-mass spectrometry
LRR	leucine-rich repeat
m/z	mass-to-charge ratio
MALDI	Matrix-Assisted Laser Desorption/Ionisation
Max	maximum
MEGA	Molecular Evolutionary Genetics Analysis
mg	milligram
$MgCl_2$	magnesium chloride
Mi	*Meloidogyne incognita*
Min	minute
mL	millilitre
mM	milimolar
mM	milimolar
MQH_2O	Mili-Q water
MS	mass Spectrometry
MS	Murashige and Skoog
MW	molecular weight

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N	nitrogen
$Na_2S_2CO_3$	sodium carbonate
$Na_2S_2O_3$	sodium thiosulfate
NaCl	sodium chloride
Na-EDTA	sodium ethylenediaminetetraacetic acid
NaOCl	sodium hypochlorite
NaOH	sodium chloride
NBS	nucleotide binding site
NCBI	National Centre for Biotechnology Information
NEB	New England Biolabs
ng	nanogram
No.	number
ºC	degree celsius
PCA	principal component analysis
PCR	polymerase chain reaction
pH	power hydrogen
pI	isoelectric point
PPN	plant parasitic nematode
R	resistance
R^2	Squared of correlation coefficient
RE	restriction enzyme
RKN	root-knot nematode
RNA	ribonucleic acid
Rpm	rotary per minute
sdH_2O	sterile distilled water
SDS	sodium dodecyl sulphate

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SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
sec	second
sp.	species (singular)
spp.	species (plural)
T	Thymine
TBE	Tris Borate EDTA
TIR	Toll/Interleukin-1-receptor
TOF	time-of-flight
Tris-HCl	hydrochloric tris
U	unit
U	Uracil
UV	ultraviolet
V	volt
v/v	volume per volume
Vh	volthour
W	watt
w/v	weight per volume
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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CHAPTER 1: INTRODUCTION

1.1 Background

Banana (*Musa* spp.) was recorded as the world's most produced crop in 2013 (FAOstat, 2014) with a total volume of global gross export reaching 16.5 million tonnes in 2012 (FAOstat, 2014). However, pest and disease pressures constraint its production worldwide. Plant-parasitic nematodes (PPNs) are amongst the many pathogens infesting this giant herb, incurring approximately USD100 billion worth annual crop losses (Ibrahim *et al.*, 2011). Besides *Radopholus similis* and *Helicotylenchus* spp., banana productions were significantly affected by *Meloidogyn*e spp. infestation particularly in the absence of *R. similis* (De Waele & Davide, 1998; Quénéhervé *et al.*, 2009). *Meloidogyne* spp. (root-knot nematodes) were found abundant on bananas planted in the tropics especially in Asian countries (Olubunmi & Rajani, 2004; Quénéhervé *et al.*, 2009; Sayed Abdul Rahman *et al.*, 2014) and in dry sub-tropical countries (Jaizme-Vega *et al.*, 1997). At least five root-knot nematode (RKN) species have been reported on *Musa* in the warm and tropical areas with *M. incognita* and *M. javanica* being most commonly found (Gowen *et al.*, 2005; Wang & Hooks, 2009). PPN-infected plants will result in decreased bunch weight and eventually fruit production, hence, an effective control measure to manage its infestation is needed.

At present, control measures at macro level adopted to combat their infestations are ineffective while the only effective solution is the non-executable usage of toxic nematicides (Gowen & Quénéhervé, 1990; Speijer & De Waele, 1997; De Waele & Elsen, 2007). These setbacks instigated agricultural scientists worldwide to focus on improving the crop by manipulating the naturally-developed nematode resistance/tolerance mechanisms established in plants. This is possible as nematode

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resistance and tolerance sources were found present in *Musa* gene pool (Pinochet, 1996; Speijer & De Waele, 1997). Since conventional crossbreeding programmes using elite cultivars are not practical due to the sterility of cultivated bananas (Heslop-Harrison & Schwarzacher, 2007), development of transgenic plant lines is seen as a practical approach (Bird & Bird, 2001). In this light, the isolation of *resistance* (*R*) gene from *Musa* spp. is seen as an effort in paving the road towards the development of such material.

Plants employ a network of complex mechanisms to defend themselves from pathogen infections. One such line of defence is based on a dominant disease *resistance* (*R*) gene that mediates defence mechanisms against pathogens possessing the corresponding *Avirulence* (*Avr*) genes. Such genetic interaction will trigger a chain of signal-transduction events that activates the defence mechanisms to arrest pathogen growth. For the past 20 years, the knowledge of the central role of *R* genes in mediating pathogen resistance (Pedley & Martin, 2003) has triggered a worldwide screening initiative that lead to the cloning of these genes from numerous plant species (Dangl & Jones, 2001). Several nematode resistance (*Nem-R*) genes expressing nucleotide-binding site leucince-rich repeats (NBS-LRR) proteins (van der Biezen & Jones, 1998) have been isolated in various plants (Williamson & Kumar, 2006; Mehta *et al.*, 2008), all conferring resistance against sedentary endoparasites (Williamson & Kumar, 2006).

In Malaysia, banana is the second most widely cultivated fruit (Lim, 2015). This cash-crop however, is exposed to various types of pathogens such as nematodes. On a survey conducted in banana plantations in Peninsular Malaysia during June 2004 to January 2006, *M. incognita* was found to be predominantly infesting the *Cavendish* banana (Sayed Abdul Rahman, 2014), contrasting the widely reported prevalence of

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Radopholus similis on banana plants worldwide (Thorne, 1961; Gowen *et al*., 2005). Despite the economic importance of banana and the subsequent harmful effects of *M. incognita,* knowledge and information in the interaction between these two organisms are still scarce. This reflects the need of investigation and researches to be conducted in this area for an in-depth understanding in plant-nematode interaction.

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CHAPTER 2: LITERATURE REVIEW

2.1 Banana and plantains (*Musa* spp.)

Bananas and plantains are perennial monocotyledonous herbs that grow in humid tropical and subtropical regions (Pillay & Tripathi, 2007). Both the words 'banana' and the genus name '*Musa'* were originated from Arabic words 'banan' meaning finger and 'mouz' meaning banana, respectively (Boning, 2006). The centre of origin and domestication of banana was reported to be in South-East Asia, specifically from India to Polynesia (Simmonds, 1962) including the Malay Peninsula, Indonesia, the Phillipines and New Guinea (Simmonds & Shepherd, 1955). It was also reported that banana dispersal out of Asia was due to human movement (Daniells *et al*., 2001). *Musa acuminata* was reported to originate from Malaysia while *Musa balbisiana* originated from Indochina (Ortiz & Vuylsteke, 1994). This perennial crop (FAO, 2003) made of false stem (pseudostem) consisting of leaf sheaths and a true stem called corm with roots (Pillay & Tripathi, 2007). The corm produces suckers (Department of Health and Ageing Office of the Gene Regulator, 2008) that emerge from a single mat.

2.1.1 Banana structure

Banana plant consists of two main parts (aerial and the underground root system) that were generated from meristematic tissues (Figure 2.1) (Summerville, 1939). These meristematic tissues will develop into a stem and a root system which can be termed as a corm (Simmonds, 1959). The root system started with a root axis that develops from a rhizome. This root axis will produce primary lateral root from which a secondary lateral root grows. An axis with its primary and secondary lateral roots is considered as a root system (Gowen, 1995).

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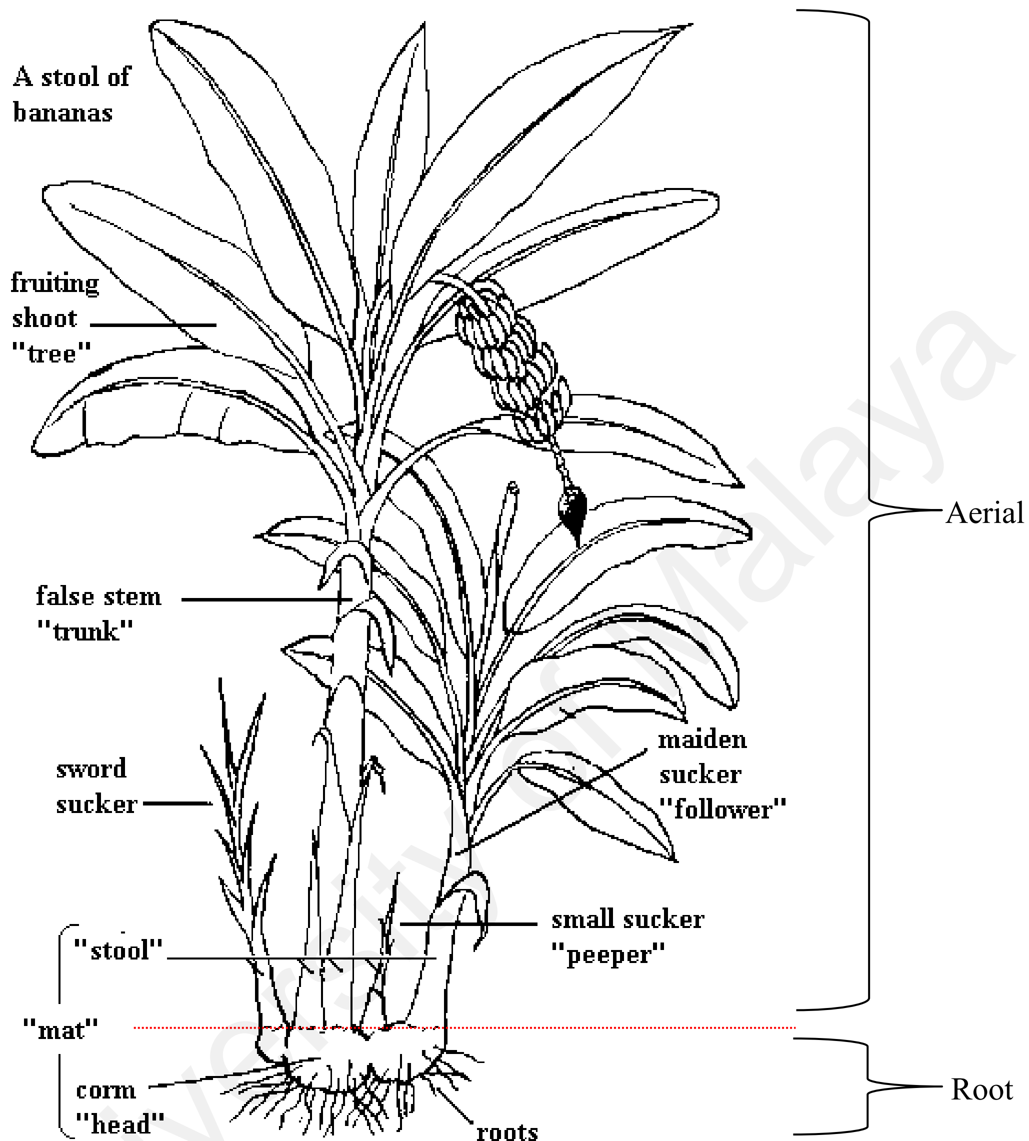

Figure 2.1: A banana mat with aerial & underground structures (divided by the dotted red line) adapted from http://www.uq.edu.au/_School_Science_Lessons/BaProj.html

2.2 Taxonomy

The taxonomy of approximately 50 banana species within the genus *Musa* is yet to be resolved because of the widespread of vegetative reproduction and natural occurrence of many hybrid plants (Department of Health and Ageing Office of the Gene

Technology Regulator 2008). Generally, bananas are categorised into two groups namely the cooking bananas and dessert bananas. The former group requires cooking prior to eating while the latter can be directly consumed.

Majority of the cultivated bananas existing today were the results of interspecific (AB, AAB, etc.) and intraspecific (AA, BB) crosses between two wild diploid (2n=2x=22) species namely *Musa acuminata* (AA) and *Musa balbisiana* (BB) (Simmonds & Shepherd, 1955). The overview of *Musa*ceae family classification is shown in Figure 2.2.

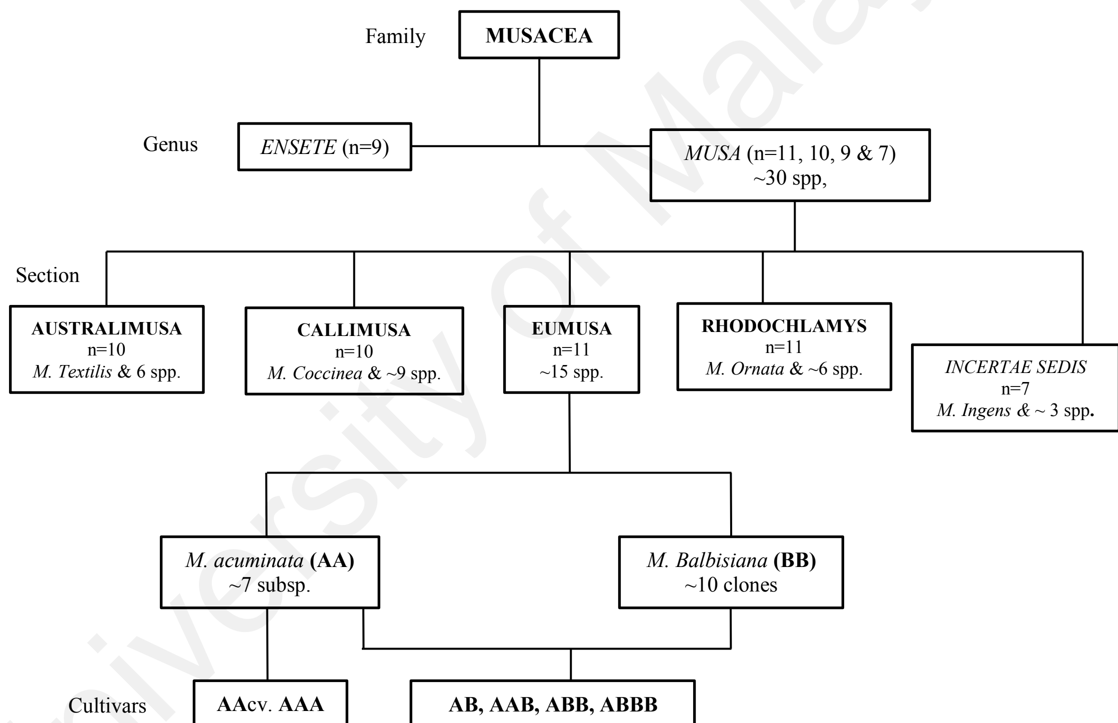

Figure 2.2: Classification of Family *Musa*ceae based on Pillay and Tripathi (2007). Current *M. acuminata* and *M. balbisiana* could be traced back to Section: Eumusa and Genus: *Musa*

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2.3 Economic Value

Bananas and plantains are among the most important crops in developing countries due to their fast growing nature as perennial crops that enable harvesting at any time of the year (FAO, 2003). World's banana production was estimated at over 85 million metric tonnes annually with Ecuador being the largest banana exporter in the world. In 2011, the global banana exports reached 15.4 million tonnes and increased to 16.5 million tonnes (7.3%) a year later (FAOSTAT, 2014). Plantains were mainly produced in Africa and Latin America, while the other types of cooking bananas were grown in Africa and Asia. Latin America was reported as the leading region for Cavendish production, followed by Asia (FAOSTAT, 2014). The world's leading producer of Cavendish bananas is India, followed by Ecuador, China, Colombia and Costa Rica. These five countries contributed for over half of global Cavendish production (FAO, 2003; Heslop-Harrison & Schawarzacher, 2007).

In Malaysia, banana is one of the popular fruits and covers more than 11% of the total fruit area (Hassan, 2004). In 1997, Malaysia's banana trade revenue was reaching USD 5.5 million (Jamaluddin, 1999) and seven years later, this amount was increased to USD 8 million (Hassan, 2004; Hassan *et al*., 2008). However, pest and diseases had been a constant threat to Malaysian banana production. Extensive researches were conducted to manage infections by pathogens such as fungi, viruses and plant-parasitic nematodes (PPN).

2.4 Plant-parasitic nematode (PPN) in bananas and its economic importance

Plant-parasitic nematodes (PPN) are multicellular microscopic animals that are spindle-shaped and tapered at both ends (Pearse *et al*., 1987). This translucent organism withdraws nutrients from plant cells using a dagger-like organ called stylet (Pearse *et*

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al., 1987). According to Thorne (1961), PPNs are grouped in the order Tylenchida from the class Secernentea. This order includes majority of the known ectoparasitic and endoparasitic nematodes (Stover, 1972). Ectoparasitic nematodes usually remain in the soil and use their stylet to pierce the outermost plant cell layers to obtain food. On the other hand, endoparasitic nematodes reside in plant tissues and can be divided into two groups based on their mobility in plants. They could either be migratory or sedentary.

Migratory endoparasitic nematodes move intracellularly in root tissues and destructively causing lesions as they feed on plant cells. On the contrary, sedentary endoparasitic nematodes migrate intercellularly in root tissues towards the tip of the vascular bundle and enter the vascular bundle from there. The nematodes would then move towards the differentiation zone of the bundle to establish their feeding sites. Both the movement of migratory nematodes and feeding site establishment of sedentary nematodes cause major destruction to banana plantations worldwide, resulting in varying degrees of losses in fruit yields. The amount of loss depends on the nematode species involved and their association to other soil pathogens as well as the susceptibility level of a given banana cultivar (Davide, 1996). Banana nematodes usually attack the root and corm tissues affecting the plant growth and yield by disrupting the anchorage and nutrient uptake of the plants (Speijer & De Waele, 1997). It was reported that in 2000, global crop production loss due to nematode infestation reached up to USD 121 billion. For bananas, nematode infestation had incurred 20% yield loss annually especially in countries such as Cuba, South Africa, Philippine and India where bananas were grown for commercial purposes (De Waele & Elsen, 2007).

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2.4.1 Management strategies of nematode infestation

Although the use of nematicides would result in yield increase, nematicides are expensive and would cost more than US$1 billion per year as seen in country like USA in the year 1982. PPNs would normally travel up to few feet each year as they move from current host to the roots of the nearest host (Turner & Rosales, 2003). However, nematode distribution over fields or from one field to another is frequently aided by machinery, water, feet of animals, seedling plants and nursery stock. On the other hand, dispersal over long distances occurred through the movement of nursery stock, seedling plants, tubers, rhizomes, and other plant material especially when the soil is transplanted (Thorne, 1961). Many farmers have established agricultural practices including crop rotation. This is because planting the same crop for a few years on the same soil will result in high nematode populations and crop rotation is only effective if the alternate crops are not susceptible to nematodes infestation. However, crop rotation with proper investigation is necessary to avoid plants that are susceptible towards nematodes (Thorne, 1961).

In a crop management system, a prompt and accurate diagnosis of plant diseases is important so that the most effective control measures were introduced at an early stage of disease development (Miller & Martin, 1988). Normally, most plant roots including banana show no symptoms such as galling during the earlier stage of juvenile stage 2 (J2) infection (Sayed Abdul Rahman *et al*., 2010). Reliance of symptoms is often inadequate as the disease may already be well ongoing when the first symptom appeared. *In planta* disease diagnosis and pathogen detection are usually accurate but are slow and laborious, not relevant to a large-scale application. For plant-parasitic nematode, typical steps involved staining of the selected root tissues, nematodes isolation, slide preparation and microscopy observation (Sayed Abdul Rahman *et al*.,

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2010; McCartney *et al*., 2003; Qiu *et al*., 2006; Sundelin *et al*., 2009). The advancement in the molecular biology techniques such as Polymerase Chain Reaction (PCR) was proven to distinguish a single nematode at the species level (Qiu *et al*., 2006). Besides that, pathogens also produced proteins and toxins to assist their infection, long before the symptoms appear. These molecules may also be detected using this molecular biology and proteomics approaches (Padliya & Cooper, 2006; Padliya *et al*., 2007).

2.5 Plant-nematode interaction

Sedentary endoparasitic nematodes are among the most economically important nematodes together with the genera *Heterodera* and *Globodera* (cysts nematodes) and *Meloidogyne* (root-knot nematode) (Williamson & Gleason, 2003). Generally, the interaction between the nematode and the host plant starts when they use their hollow, protrusible stylet to penetrate plant cell wall. They will then inject the gland secretion containing parasitism gene into the cells and also withdraw nutrients from the cytoplasm (Davis *et al.*, 2000). These stylet secretions have a direct role in infection and parasitism of plants, and developmental changes in the secreted proteins occur during the parasitic cycle (Hussey *et al*., 1990; Davis *et al*., 2000)

According to Williamson and Gleason (2003), genes involved in plants defence against pathogens were expressed after a compatible plant-nematode interaction. This mechanism however was first described by Flor (1947) in his work with rust (*Melamspora lini*) in flax (*Linum usitatissimum*). He hypothesised a Gene-for-Gene Theory of Complete Resistance proposing that for resistance (incompatibility) to occur complementary pairs of dominant genes must be present in the host and pathogen. These genes are termed as *Resistance* (*R*) gene for the host and Avirulence (*Avr*) gene for the pathogen. Altering either of these genes leads to compatibility (disease). The

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mechanism of resistance is most likely to involve interaction between the Avr protein (an elicitor) and the *R*-gene product (the receptor). Chan *et al*. (2007) claimed that more than 60 types of *R* genes have been isolated from various plants against various pathogens. At present, five classes of *R* genes have been identified which are the nucleotide binding site-leucine-rich repeat (NBS-LRR), protein kinase, extracellular leucine-rich repeat transmembrane (eLRR-TM), LRR, and toxin reductase (Hammond-Koasack & Jones 1997; Luo *et al*., 2000; Dong *et al*., 2001; Miller *et al*., 2008). The motifs of the N- terminal of NBS-LRR Resistance protein can be divided into two subfamilies which are Toll-like Interleukin-1 Receptor (TIR) proteins showing homology with *Drosophilla Toll* and mammalian Interleukin-1 Receptor, whereas another one is non-TIR-NBS-LRR, that contains a coiled-coil (CC) motif at the N-terminal region (Pei *et al*., 2007). Extensive studies have been conducted on the NBS-LRR-type *R* genes because its only known function is in disease resistance (Dangl & Jones, 2001; Meyers *et al*., 2005).

Several *NBS-LRR Resistance* genes from *Musa* spp. have been isolated. The first report was made in the year 2007 by Pei *et al*. (2007) followed by Mohamed and Heslop-Harrison (2008) in the subsequent year. Both studies used degenerate primers to amplify conserved regions of *NBS-LRR Resistance* gene in wild and cultivated *Musa* species. Both studies found that all the isolated *Resistance* gene sequences belong to the non-TIR-NBS type and no representative of the TIR-type *Resistance* gene was isolated. In order to find banana *Resistance* gene functioning against its pathogen of economic importance, transcriptomic analysis were carried out on banana plants against fungal invasion. Currently, transcriptomic analysis on banana-pathogen relationship were conducted only on *Fusarium oxysporum* f. sp. cubense tropical race 4 (Li e*t al*., 2012) and *Mycosphaerella musicola* (Passos *et al*., 2013). However, at present, no known

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literature describing transcriptomic analysis was available for banana-nematode interaction. In terms of plant-nematode interaction, a few numbers of nematode resistance (*Nem-R*) genes have been mapped and cloned. These included the *Hs1pro-1,* the first (*Nem-R*) gene to be isolated conferring resistance to the beet cyst nematode (*Heterodera schachtii Schmidt*) as well as the *Mi* gene from tomato. Both *Resistance* genes conferred effective resistance against several plant-parasitic nematode species (Williamson, 1998). However, an experiment conducted to transfer the *Mi*-mediated resistance from tomato into tobacco had been unsuccessful due to the inability of the gene to confer resistance at high temperature environment (Williamson, 1998). Kersten *et al.* (2002) deduced that although the *R*-gene sequences are similar in both tomato and tobacco, the resulting Resistance protein might undergo modification in tobacco hence its ineffectiveness. Although a large amount of data had been generated utilizing the transcriptomic technology, full representation of this complex biological network is yet unanswered due to limitations of these approaches. Therefore, proteome profiling is seen as a complement technology to fill the gaps in both genomic and transcriptomic approaches (Mehta, 2008).

2.6 Proteomics

The word proteome is derived from "the PROTEins expressed by the genOME or tissue" coined by Marc Wilkins while doing his PhD. research at Australia Macquarie University (Wilkins *et al.,* 1997). Therefore, proteomics is defined by a large-scale study of proteins (Pandey & Mann, 2000) or the systematic analysis of all protein population in a tissue, cell or subcellular compartment (Chen & Harmon, 2006). Before the rise of proteomics, microarray studies of mRNA expression were given much attention. However, it was then known that gene expression was regulated at different levels and a number of informations were not encoded by DNA (Quirino *et al.,*

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2010). According to Pandey and Mann (2000), proteomics is the complementary of genomics because the gene product, protein, is the active agents in cells whereas the gene expression does not reflect the consequential protein abundance. Kersten *et al.* (2002) supported the notion and further added that sequence information is insufficient to provide significant information of the biology of organisms. These drawbacks were also expected to be seen in the transcriptomics analysis (Carpentier *et al.,* 2007). The RNA molecules are converted into mRNA and later could undergo various processes of post-transcriptional protein modifications such as phosphorylation, glycosylation, protein cleavage and multi-protein complex. These processes will later influence the biological function of proteins that once not coded by the genome (Kersten *et al.,* 2002). It is noteworthy that proteomics can be divided into two sub-groups namely gel-based procedures and non-gel based procedures. The former include conventional two dimensional electrophoresis and DIGE, the latter include LC-MS, metabolic isotopic labelling (SILAC) and chemical labelling (ICAT) (Monteoliva & Albar, 2004).

In 2001, plant proteomics was still at its infancy (van Wijk, 2001) compared to other model organism such as human, *Eschericia coli* amd yeast (Chen & Harmon, 2006). However, after 22 years from the 'birth' of proteomics field, an increasing amount of plant proteomics works were recorded. As reported in ISI Web of Knowledge up to May 2014, 365 out of 5179 reports in the *Proteomics* journal corresponded to plant, representing 7% of the reports (Jorrín-Novo *et al*., 2015). In plant proteomics, most researches conducted revolved around three major areas namely plant species, plant organs and tissues; as well as biological processes occurring in plants (Jorrín-Novo *et al*., 2015). Hu *et al*. (2015) had thoroughly reviewed the impact of proteomic approaches to understand the mechanism of stress response in plant organs and tissue. By taking the benefit of the advancement in quantitative proteomics, they

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summarised that the approach had improved the recovery of total proteomes and sub-proteomes from lesser amounts of starting material, protein-protein interactions at the cellular level as well as post-translational modifications (PTMs). Wu *et al*. had emphasised the important of PTMs to regulate protein function, subcellular localisation and protein activity and stability. They reviewed various studies verifying plant responses to abiotic stress at the PTMs level and the necessity to identify and quantify the modifications to a detailed protein functional characterisation to improve the current knowledge. D'Ambrosio *et al*. (2013) had conducted proteomic analysis on apricot fruit flesh during ripening and Wu *et al*. (2014) integrated proteomics and transcriptomics platforms in order to analyse fruit ripening stages and development in mangoes. Besides that, researches in plant proteomics also involved plant-pathogen interactions. Li *et al*. (2013) reported differential protein abundances in banana root cells inoculated with *Fusarium oxysporum* f. sp. *cubense* tropical race 4 while Palomares-Rius *et al*. (2011) had reported a proteomics study of in-root interactions between soil-borne fungus *Fusarium oxysporum* f. sp. *ciceris* race 5 with root-knot nematode *Meloidogyne artiellia* in chickpeas.

Proteomics analysis involves two-dimensional electrophoresis as the separation techniques followed by Mass Spectrometry. The first separation technique is based on isoelectric point (1st-D) while the second separation technique is based on protein sizes (2nd-D). These techniques separate proteins based on two different parameters which are isoelectric point and size. These techniques were able to separate up to several thousands of protein spots (Westermeier & Naven, 2002). Since the immobilised pH gradient strips (IPG) were developed, variability in experimental conditions had decreased because of the availability of the IPG strips in various lengths and pH ranges enable specific protein targeted according to their characteristics (Gorg *et al*., 2000).

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Various protein visualisation methods are available. The most common techniques are the Colloidal Coomassie blue (CCB) and silver staining. However, although CCB is inexpensive and technically applicable, it is less sensitive compared to silver staining method. However, the only disadvantage of using silver staining is that spectrometry analysis will be interrupted by silver ions. Therefore, an alternative to that was fluorescent-detection-based proteomics such as Difference Gel Electrophoresis (DIGE). However, despite sensitive and reliable, this method is expensive (Monteoliva and Pablo Albar, 2004). Therefore, non-gel based proteomics such as Liquid Chromatography- Mass Spectrometry (LC-MS) proteomics is seen as an alternative to circumvent problems faced in gel-based proteomics.

2.7 Liquid Chromatography-Mass Spectrometry (LC-MS) proteomics

The 21st century witnesses the blooming of gel/label free proteomics technology with the aid of mass spectrometry and their ability to couple with genomics information (Quirino *et al*., 2010). A single separated protein in a solution or thousands of proteins existing in a complex mixture can be easily identified using a mass spectrometer (Sparkman, 2000). This technique works by chemically fragmentised the protein/peptide into charged particle (ion) by gaining or losing their charge by protonation, deprotonation or electron injection. Subsequently, the ionised molecules are electrostatically propelled inside the instrument and detected according to their charge to mass ratio. Among the most popular ionisation methods are matrix-assisted laser desorption/ionization (MALDI) and electron-spray (ESI) (Siuzdak, 1996). The latter is being employed in this very study.

The emergence of protein sequence databases such as Uniprot and NCBI Protein Database had shaped the world of proteomics with their information about protein

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sequences obtained by the translation of open reading frames. This bioinformatics tool allows the comparison of data obtained by mass spectrometry to these protein databases to identify the proteins or peptides present in a sample (Quirino *et al*., 2010). The contribution of this tool has become more prominent with the popularity of traditional method such as Sanger sequencing (Shendure & Ji, 2008) as well as the development of new high throughput DNA sequencing technologies like the next-generation sequencing (NGS) (Metzker, 2010) enables complete genome sequencing to become possible to many more species.

2.8 Objective of study

The overall objective of this study was to obtain an in-depth understanding on a compatible banana-nematode interaction at the molecular level. The specific objectives were:

i. to obtain host's protein abundance profiles (via proteomics) that reflect plant's response against nematode infestation;
ii. to identify host's proteins involved during plant-nematode interaction at 30- and 60- day post inoculation (dpi);
iii. to isolate and characterise partial *NBS-LRR Resistance* (*R)* transcripts from Grand naine plantlets infested with *Meloidogyne incognita*;
iv. to isolate and characterise partial *NBS-LRR R* gene from genomic DNA of Grand naine, Berangan and Malaccensis plantlets;
v. to assess the diversity of NBS-LRR family by carrying out a phylogenetic analysis of the isolated *NBS-LRR Resistance* gene sequences and compare them with *NBS-LRR Resistance* gene sequences available in the GenBank.

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CHAPTER 3: MATERIALS AND METHODS

3.1 Gel-based proteomics

This experiment was carried out on eight Grand Naine plantlets using four plantlets inoculated with 1000 *M. incognita* juvenile type 2 (J2) and the remainder plantlets served as control samples. All plantlets were harvested 30 days after inoculation (dai) and subjected to conventional proteomics platform which included 1st - and 2nd – dimensional electrophoresis

3.1.1 Plant tissue culture

Banana cv. Grand naine plantlets used in this study were maintained and propagated according to Jalil *et al*. (2003) by subculturing the plantlets in a standard Murashige and Skoog (MS) (1962) basal medium supplemented with 4.1 µM biotin, 5.7 µM indoleacetic acid (IAA), 5.4 µM naphtaleneacetic acid (NAA), 87 µM sucrose, 2 g/L gelrite with 5 mg/L 6-Bensylamunopurine (BAP). Ten millilitres of the MS mixture (pH 6.12-6.15) were aliquoted into a 15cm x 2.5cm culture tubes and autoclaved at 121°C for 15 min. The cultures were allowed to propagate on the MS media for approximately two months and then transferred into rooting media (4.59 g/L normal MS including vitamins, 1 mL/L ascorbic acid, 30 g/L sucrose, 2 g/L gelrite and 0.5 g/L active charcoal) with pH adjusted to 6.15 at 60°C. The plantlets were grown in the rooting media until reaching a four-leaf stage prior to transplantation with photoperiod of 12 hour light and 12 hour dark.

3.1.2 Transplantation

The root system of tissue cultured plantlets was thoroughly washed under running tap water to remove traces of rooting media prior to transplantation into soil to

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prevent fungal infection. The plantlet was transplanted into a 10 cm-diameter –wide poly-bag containing autoclaved soil (2 sand: 1 peat) (All Purpose Mix BABA, Malaysia) and left to acclimatise eight weeks in a growth room (27°C) with 12-hour light and 12-hour dark photoperiod. Fertilizer (Hypotonic Vitalizer,eco$^{+ ™}$) was applied to the plantlet once a week starting from the second week after transplantation. Soil used in all experiments was first autoclaved at 121°C for 20 min.

3.1.3 Inoculum preparation

Meloidogyne incognita (Malaysian population) culture was obtained from Malaysian Agricultural Research and Development Institute (MARDI) courtesy of Mr. Mohd Nazarudin Anuar, and maintained in tomato plants. Nematode egg masses were hand-picked from dissected root fragments under a stereo microscope (10 x 100 magnification level) placed in a 50mL beaker containing dH_2O. Nematode juveniles were hatched as described in Speijer and De Waele (1997) by adding a few drops of 1% sodium hypochlorite (w/v) in a period of one to two weeks with proper aeration. The number of J2 used as inoculants was estimated by averaging the triplicates of the number of J2 individuals obtained per mL and dH_2O volume was adjusted to the inoculation level used.

3.1.4 Single-Inoculation Experiment

Single inoculation experiment was carried out according to Sayed Abdul Rahman *et al*. (2010). Briefly, the acclimatised plant was transferred into a 1.5 L pot filled with autoclaved soil. One of the primary roots was selected for inoculation and was placed across a 5 cm diameter Petri dish. Note that two slits were made at the sides of Petri dish prior to placing the selected plant root. J2 nematodes were inoculated at single inoculation target site as depicted in Figure 3.1 using a 1000 µL pipette. This

target site was then covered with the soil and the root fragment was left to acclimatise for 3 days prior to inoculation.

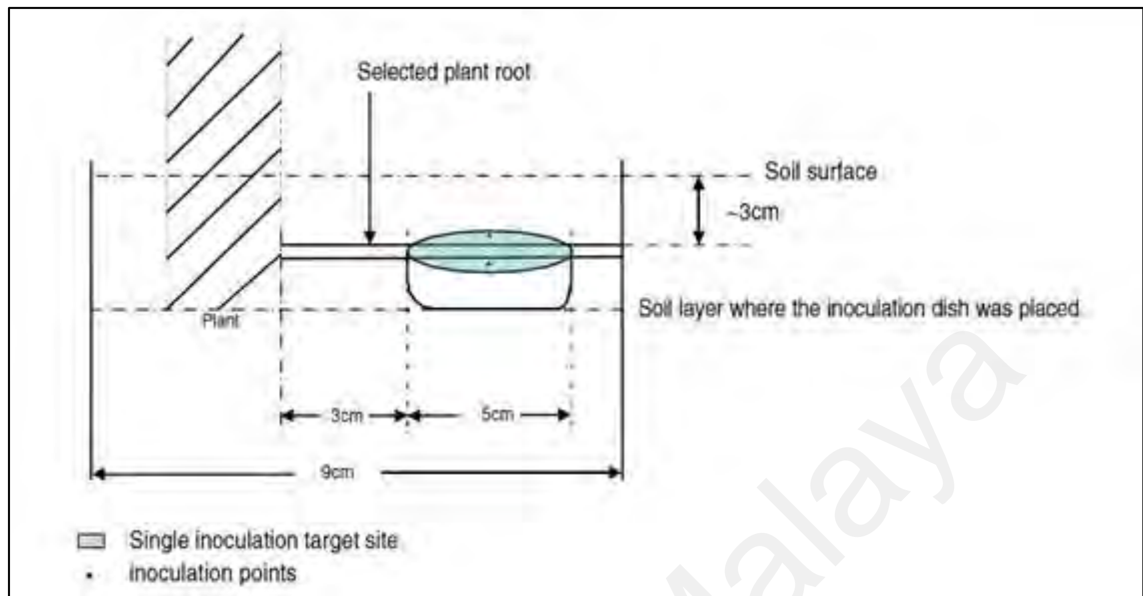

Figure 3.1: Schematic diagram of the single inoculation method. Adopted from Sayed Abdul Rahman *et al.* (2010).

3.1.5 Harvesting the inoculated and control root fragments

The targeted root fragment was excised at 30 dai and thoroughly washed under the running tap water and left air-dried. Subsequently, the root fragment was placed in a fresh 1.5 mL microcentrifuge tube and snapped-frozen in liquid nitrogen (N_2) prior to storage at -80°C until future use.

3.1.6 Protein Isolation

Protein isolation was carried out following phenol extraction methanol/ammonium acetate precipitation method as described in Carpentier *et al.* (2005). To avoid cross-contamination, a set of mortar and pestle was dedicated for each root sample and the non-inoculated root sample was first subjected to grinding followed by the inoculated sample. The mortar and pestle was baked at 250°C for 12 hours and let to cool prior to protein extraction. A ten millilitres of Extraction Buffer containing 100 mM Tris-HCl (pH 8.3), 5 mM EDTA, 100 mM KCl, 1% DTT, 30% Sucrose, 1 tab

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Complete Mini Protease Inhibitor (Roche Applied Science, Germany) and MQH_2O was prepared. Later, 500 µL of the Extraction Buffer were aliquoted into a fresh 2 mL microcentrifuge tube and placed in ice to prevent protease activation that was released by root tissues during grinding. Subsequently, approximately 50 mg of root fragment were ground into a fine powder in the presence of liquid nitrogen in a pre-cooled mortar and pestle. Later, 50 to 150 mg of fine root powder were transferred into the 2 mL microcentrifuge tube containing 500 µL Extraction Buffer and later vortexed for 30 seconds. Five hundred microlitres of buffered phenol (Sigma, USA) were added into the mixture and vortexed for 10 min at 4°C. Subsequently, the sample was centrifuged for 3 min at 5900 x *g* at 4°C and the resulting phenolic phase was pipetted into a fresh 2mL microcentrifuge tube. This phenolic phase was then re-extracted with 500 µL Extraction Buffer and centrifuged again for 3 min, 5900 x *g* at 4°C. Subsequently, the phenolic phase was transferred into a fresh 2 mL microcentrifuge tube and the proteins were left to precipitate in methanol containing 100 mM ammonium acetate overnight at -20°C. After precipitation, the sample was centrifuged for 60 min at 15 600 x *g* at 4°C to precipitate the proteins. The resulting supernatant was removed and the pellet was rinsed with 2 mL Rinsing Solution (0.2% DTT in cold acetone) and left in -20°C for one hour followed by a centrifugation step for 30 min at 15 600 x *g* at 4°C. The rinsing step was repeated once again and the sample was centrifuged using the same parameters. Subsequently, the rinsing solution was discarded and the pellet was air-dried. Finally, the pellet was dissolved in 100 µL Lysis Buffer (7 M urea, 2 M thiourea, MQ H_2O, 4% CHAPS, 1% IPG-buffer pH 4-7 and 1% DTT). For a cleaner protein sample, the sample was centrifuged for 30 min, 15 600 x *g* at 18°C to sediment the impurities. Finally, the resulting supernatant was transferred into a fresh 1.5 mL microcentrifuge tube and stored in -80°C as aliquots.

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3.1.7 Protein Quantification

Protein quantification was carried out using 2D Quant Kit (GE Healthcare, USA) according to the manufacturer's protocol. This assay is based on the specific binding of copper ions to protein. Dissolved protein sample was resuspended in a copper-containing solution and unbound copper was measured with a colorimetric agent. The colour density was inversely related to the protein concentration. The assay has a linear response to protein in the range of 0-50 µg (2D Quant Kit manual). Briefly, a standard curve was generated using the provided 2 mg/mL Bovine Serum Albumin (BSA) standard solution in order to quantify 7 µL of isolated protein sample. Six fresh 1.5 mL microcentrifuge tubes were prepared to contain BSA concentrations of 0 µg, 10 µg. 20 µg, 30 µg, 40 µg and 50 µg.

Briefly, protein sample together with BSA standard solution were precipitated with 500µL precipitant solution and subjected to brief vortexing prior to 3 min incubation at room temperature. Subsequently, 500 µL of co-precipitant solution were added into each tube and the mixture was briefly mixed by vortexing. The tubes were then centrifuged for 5 min at 15 600 x *g* at room temperature to pellet the protein and the resulting supernatant was discarded. The tubes were briefly centrifuged to collect excess liquid. Subsequently, 100 µL of copper solution and 400 µL of dH_2O were added to each tube. The tubes were briefly vortexed to dissolve the precipitated protein and thoroughly mix with the copper ion. The protein sample was instantaneously mixed with 1mL of working colour solution (1 part colour reagent B: 100 parts colour reagent A) followed by rapid inversion. The mixture was then incubated for 15-20 min at room temperature prior to quantification at 480 nm wavelength. A linear correlation graph was generated based on the obtained data using the XY scatter function available in

Microsoft Excel (Microsoft Office®2010). The standard curve was then used to quantify the concentration of the isolated protein.

3.1.8 First Dimension protein separation (1st-D)

Fifty-microgram proteins were electrophoresed and focused on an Ettan IPGphor III IEF System using 3 mm wide, 24 cm long Immobilised pH Gradients (IPG) strips with a pH range of 4-7. The strip was first rehydrated overnight in Rehydration Buffer [6 M Urea, 2M Thiourea, 0.5% CHAPS, 10% glycerol, 0.002% bromophenol blue, MQ H_2O, 0.5% IPG-buffer (pH 4-7) and 0.28% DTT] according to Westermeier & Naven (2002). The placement of the strips on the manifold was carried out according to manufacturer's protocol. Briefly, the rehydrated strips were placed into the manifold channels with the gel side facing upwards. Approximately 108 mL (Immobiline DryStrip Cover Fluid, Sweden) were poured to evenly cover the manifold tray. Two pre-cut paper wicks were assigned for each strip each of which was first moistened with 150 µL of MQH_2O. Each paper wick was placed overlapping both ends of the strip. The movable electrode was then positioned at the middle of each paper wick and the cams were locked. The sample loading cup was then placed approximately 1 cm below the electrode without interfering the protrusion structures present on the manifold channels.

Protein sample used in this experiment was first centrifuged for 30 min at 15 600 x *g* at 18°C prior to loading to remove insoluble materials that could interfere with the sample entry. IEF was carried out at 20°C with current limit of 50 µA /strip (Westermeier & Naven, 2002). The IEF started with a step- and-hold phase at 300 V for 3 h followed by a gradient step at 1000 V for 6 h, another gradient step at 8000 V for 3 h and finally a step-and-hold phase at 8000 V (24000 Vh). Upon completion of IEF procedure, the strips were either stored in -80°C or directly subjected to the 2nd-

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Dimension separation. Note that in this procedure, the samples were labelled according to the number on the IPG strips that served as identification (ID) numbers. The ID number for each samples and their replicates were summarised in Table 3.1

Table 3.1: Sample replicates with the corresponding sample ID.

Inoculation level	Replicates	Sample ID
0 J2	30 dai 0 J2a	35
	30 dai 0 J2b	36
	30 dai 0 J2c	34
1000 J2	30 dai 1000 J2a	37
	30 dai 1000 J2b	38
	30 dai 1000 J2c	39

3.1.9 Second Dimension protein separation (2nd-D)

3.1.9.1 Preparation of Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Lab cast SDS-PAGE gels (12.5%, 1.5 mm) were prepared using Ettan^{TM} DALTsix Gel Caster according to the manufacturer's protocol. The monomer solution of 12.5% acrylamide gels [30% acryl/bisacryl (Bio-Rad, USA), 1.5 M Tris buffer (pH 8.8), 10% v/v SDS, 10% v/v APS and 100% v/v TEMED] was poured through the filling channel and each gel was covered with 50% of water- saturated isobutanol. The gels were then left to polymerise overnight.

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3.1.9.2 Strips Equilibration and Gel Electrophoresis

The gel strips that had been subjected to IEF were first equilibrated in 200 mL Equilibration Buffer (EB) [6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue and 50 mM Tris pH 8.8] prior to 2nd-D SDS-PAGE. The strip was first equilibrated in an EB containing 1% DTT for 15 min to ensure that the disulphide bonds between amino acids were broken. Subsequently, the strip was equilibrated in the 2nd EB containing 4.5% Iodoacetamide (IAA) for the same duration. IAA alkylates thiol groups on the proteins, preventing their re-oxidation during electrophoresis. Besides, IAA would also alkylates residual DTTs hence preventing point streaking and other silver staining artefacts (Stochai *et al*., 2006). Finally, the strips were placed in the gel cassettes and sealed with agarose sealing solution (0.5% agarose, 0.002% bromophenol blue, 1 X Running Buffer).

The proteins were electrophoresed in the presence of an anodal buffer and a cathodal buffer diluted from 10X Running Buffer (250 mM Tris base and 1.92 M Glycine) for 45 min at 12 W (2 W per gel) and 5 h later at 100 W using an Ettan™ DALTSix Electrophoresis System. Finally, the acrylamide gels were fixed overnight in a fixing solution (40% ethanol, 10% acetic acid, MQH_2O)

3.1.10 Silver staining and gel scanning

The fixed SDS-PAGE gel was stained using a silver staining method described by Switzer *et al*. (1979). The fixed gel was first washed with Washing Solution I (30% ethanol) for 20 min followed by another 20 min washing step with Washing Solution II (20% ethanol). The gel was then subjected to 20 min washing step with MQ H_2O and sensitized with 0.02% $Na_2S_2O_3$ for 1 min. Subsequently, the gel was rinsed three times with MQH_2O for 20 seconds and later were stained with silver solution (0.2% $AgNO_3$

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and 0.02% formaldehyde) for 25 min. The gel was then rinsed three times with MQH_2O for 20 seconds and developed in a developing solution (3% Na_2CO_3, 0.0005% Na_2S2O_3 and 0.05% formaldehyde) for 4 min and rinsed with MQH_2O for 20 seconds before the Blocking Solution (0.5% glycine) was applied. Finally, the gel was washed with MQH_2O for 10 min before being stored in 1% acetic acid solution prior to gel scanning. The stained gel was removed from the glass cassettes and scanned using LabScan 5.0 software (GE Healthcare, UK) and saved in Melanie format for analysis with resolution value between 150-200 dots per inch (dpi). Precautious steps were taken to ensure no air bubbles formed underneath the gel.

3.1.11 Gel statistical analysis

Analyses of the stained gels were conducted using ImageMaster 2D Platinum Volume 7.0. The spots were first detected based on parameters that include Smooth value of 3, Saliency value of 10 and Min Area value of 31.

The experimental variations across gels were determined by Spot Correlation Analysis using Scatter Plot function available in the software. This analysis evaluate the relationship between the spot values from two gels by searching for the linear dependence between the spot values of one gel (variable X) and the corresponding spot values in the reference gel (variable Y). Gel pair with correlation value of 0.8 to 1.0 signified low replicate variations and correlation between gels increases as the correlation value approaches 1.0.

Next, the gels were subjected to Factor Projection Plot to analyse the clustering behaviour of the protein spots. Subsequently, the protein spots were subjected to Kolmogorov-Smirnov analysis to verify if the two samples belong to the same

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population. Protein spots with the value of 1 and showing significant abundance different at $p \leq 0.05$ for Analysis of Variance (ANOVA) were manually analysed using Spot Histogram Analysis.

3.2 LC-MS proteomics

This experiment was carried out on 16 Grand Naine plantlets with four plantlets, each inoculated with 1000 *M. incognita* J2 and the remaining four served as control plantlets. Two time points were used in this experiment namely 30- and 60- dai and similar experimental set-up was used for each time point.

3.2.1 Plant tissue culture material

Grand Naine plantlets used in this experiment were prepared following the procedures described in Sections 3.1.1 and 3.1.2.

3.2.2 Nematode inoculation on Grand Naine root fragments

Nematode samples were first prepared as described in Section 3.1.3. Single Inoculation Experiment was conducted following Sayed Abdul Rahman *et al*. (2010) as described in Section 3.1.4.

3.2.3 Harvesting of the control and inoculated root fragments

Inoculated root fragments and control samples were harvested as described in Section 3.15 and lyophilised at -100ºC for 40 h according to Carpentier *et al*. (2007). Lyophilised root samples were then stored at room temperature until future use.

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3.2.4 Protein isolation

Banana root proteins were isolated as described in Carpentier *et al.* (2005) with slight modifications. The lyophilised banana root fragment weighing approximately 12 to 20 mg was ground in liquid nitrogen. The resulting fine powder was suspended in 850 µL ice-cold Extraction Buffer [EB; 1 M Tris–HCl (pH 8.5), 0.5 M EDTA, 0.1 M KCl, 6.5 mM DDT, 1 mM PMSF and 0.7 M sucrose] and briefly vortexed. Following this, 850 µL ice-cold Tris-buffered phenol (pH 8.0) were added to the sample and vortexed for 10 min at 4 °C. Later, the sample was centrifuged for 3 min at 8000 x *g* at 4 °C and the resulting phenolic phase was collected. The collected sample was re-extracted with 850 µL of EB and further vortexed for 30 sec. The sample was centrifuged again as previously described as above and the resulting phenolic phase was collected into a fresh 2mL microcentrifuge tube. Proteins were precipitated by adding 5 volumes methanol containing 0.1 M ammonium acetate and the sample was then left overnight at −20°C. Subsequently, the sample was centrifuged for 60 min at 4°C with 13000 X g to pellet the protein. The supernatant was then discarded and rinsed with cold acetone containing 0.2% DDT and incubated for 1 hour at −20°C. Samples were later rinsed again with cold acetone containing 0.2% DDT and centrifuged for 30 min at 13,000 x *g* at 4 °C. Finally, the pellet was air-dried and resuspended in 100 µL Lysis Buffer (8 M urea, 5 mM DTT). To sediment the impurities, the sample was centrifuged for 60 min at 13000 x *g* at 18 °C. The sample was either stored in -80°C freezer or subjected to quantification as described in Section 3.1.7.

3.2.5 Protein quantification

Protein quantification was carried out as described in Section 3.1.7.

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3.2.6 Peptide digestion

Twenty micrograms of isolated proteins were incubated with 0.02 M DTT for 15 min and later with 0.05 M Iodoacetamide (IAA) to prevent reformation of disulphide linkage between the peptides for 30 min in the dark. Next, the sample was diluted 4 times with 100 mM ammonium bicarbonate (ABC) and later digested with 0.2 µg/µL of Trypsin at 37 °C overnight. The sample was then acidified with trifluoroacetic acid (TFA) to the final concentration of 0.1% and de-salted using Pierce C18 solid phase extraction column according to the manufacturer's protocol (Thermo Fisher Scientific, Belgium). Peptides were eluted with 40 µL of 70% ACN and the solvents were subsequently dried in a speedvac. The digested peptide was then dissolved in 5% ACN and 0.1% formic acid and separated via Liquid chromatography- mass spectrometry (LC-MS) in KUL facility for SYstems BIOlogy based Mass (SYMBIOMA) spectrometry, K.U. Leuven, Belgium.

3.2.7 Peptide separation and MS analysis

The UPLC-MS/MS analysis was performed on a Q Exactive Orbitrap mass spectrometer (Thermo Scientific, USA) following Vanhove *et al*. (2015). Briefly, five microliter of sample containing 1 µg of peptides were injected and separated on an Ultimate 3000 UOLC system (Dionex; Thermo Scientific, USA) equipped with a C18 PepMap100 pre-column (5 µm, 300 µm x 5 mm; Thermo Scientific, USA) and an EasySpray C18 column (3 µm, 75 µm x 15 cm; Thermo Scientific, USA) using a gradient of 5% to 20% ACN in 0.1% formic acid (FA) for 10 min followed by a gradient of 10% to 35% ACN in 0.1% FA in 4 min and then a final gradient from 35% to 95% ACN in 0.1% FA for 2.5 min. Positive ion mode with a nanospray voltage 0f 1.5kV and source temperature of 250°C was set to operate the mass spectrometer. External calibrant used was ProteoMAss LTQ/FT-Hybrid ESI Pos. Mode Cal Mix

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(MSCAL5-1EA SUPELCO, Sigma-Aldrich, USA) and the lock mass 445.12003 as an external calibrant. The instrument was operated in a data-dependent acquisition (DDA) mode with a survey MS scan at a resolution of 70000 (FWHM at m/z 200) for the mass range of m/z 350-1800 for precursor ions. This was followed by MS/MS scans of the top 10 most intense peaks with +2, +3, and +4 charged ions above a threshold ion count of 16000 at a 35000 resolution using a normalised collision energy (NCE) of 29 eV with an isolation window of 3.0 m/z and dynamic exclusion of 10 s. All data were acquired with Xcalibur 2.2 software (Thermo Scientific, USA).

3.2.8 Statistical analyses

Quantitative analysis of the peptides was performed using Progenesis LC–MS version 4.0 (Nonlinear Dynamics). The reference run sample was automatically selected by the software and the total ion chromatogram files were automatically aligned. The sensitivity of the peak picking limits was put to default (Sensitivity: Automatic; Minimum retention time window: 0 min; Maximum allowable charge: 20). With these settings, the software generated an aggregate run that contained all ions from the analysed runs. Peptides with charges from two to five were retained in the filter step, and the data was then normalised by calculating abundance ratios to a reference run. Feature tables of the different fractions were combined to give a complete overview of peptide quantification of all samples. Data were subjected to principal component analysis (PCA) to analyse the clustering behaviour of the samples. Only proteins with ANOVA p-value ≤ 0.05 and abundance change of more than 1.5-fold were selected for further analyses.

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3.2.9 Peptide homology search

Peptide homology search was performed using Mascot platform (Matrix Science, country) against an in-house (SYIMBIOMA) banana database containing *Musa* A and *Musa* B genomes. The search parameters allowed one peptide miss cleavage and mass tolerance of 0.8 Da on MS/MS fragments. Carbamidomethylation on cysteine was assigned as fixed modification and oxidation of methionine was regarded as a variable modification.

3.2.10 Protein function retrieval

Protein names and its biological functions were determined based on their accession numbers obtained from *Musa* A and B databases in GreenPhyl v4 (http://www.greenphyl.org/cgi-bin/index.cgi) and Uniprot (http://www.uniprot.org/) databases.

3.3 Isolation of partial NBS-LRR *Resistance* gene from *Musa* spp.

3.3.1 Nematode treatment

Approximately 5000 *M. incognita* stage two juvenile (J2) were inoculated onto the roots (De Waele and Speijer, 1997) of three 2-month-old Grand naine plants. The plants served as the hosts for these nematodes for five months in a growth room with 16 h light/8 h dark cycle. These hosts were watered and fertiliser was applied accordingly during this period.

3.3.2 Nucleic acid isolation

3.3.2.1 Genomic DNA isolation from banana leaf tissues

Genomic DNA was isolated from four banana cultivars namely Grand naine, Berangan, Lemak Manis and Malaccensis. In this experiment, DNA sample was extracted from the

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leaf tissues of 2-month-old tissue culture plantlets maintained in Murashige and Skoog (1962) basal medium as described in Sayed Abdul Rahman *et al*. (2010). Briefly, banana leaf tissue was ground into fine powder using pre-cooled mortar and pestle in the presence of liquid N_2. Approximately 50 mg of the fine powder were added into a fresh 2 mL microcentrifuge tube containing the Extraction Buffer [4% CTAB, 100 mM of Tris-HCl (pH 8.0), 1.4 M of NaCl, 50 mM of Na-EDTA (pH 8.0) and 1% DTT]. The sample was then vortexed and incubated for 30 min at 55 ºC and later centrifuged at 2350 x *g* for 5 min. The resulting supernatant was transferred into a fresh 2 mL microcentrifuge tube and treated with 200 µg/mL RNase A. Following that, the sample was incubated for 2h at 37ºC and an equal volume (to the supernatant) of Phenol: Chloroform: Isoamyl alcohol (PCI; 25:24:1) solution was added to the sample. This mixture was later vortexed and centrifuged at 2350 x *g* for 5 min at room temperature. The resulting upper aqueous phase was transferred into a fresh 2 mL microcentrifuge tube and re-extracted with an equal volume of Chloroform: Isoamyl alcohol (CI; 24:1). Similar vortexing and centrifugation step was applied as described for PCI. DNA was precipitated with an equal volume of isopropanol for 30 min in -80 ºC. The resulting supernatant was discarded and the pellet was washed twice with 1 mL of 70% EtOH. Subsequently, the mixture was centrifuged at 2350 x *g* for 5 min and the EtOH was discarded each time. Finally, the pellet was left to dry and dissolved in 30 µL sdH_2O. DNA concentration and quality ($A_{260/280}$ and $A_{260/280}$) was estimated using Nanophotometer (Implen Gmbh, Germany) and agarose gel electrophoresis (AGE).

3.3.2.2 RNA isolation from nematode-inoculated banana root tissues

RNA sample was extracted from 1 g of galled Grand naine root fragment according to Kistner and Matamoros (2005) with an up scaled volume. Briefly, 7 mL Extraction Buffer [2% hexadecyltrimethylammonium bromide (CTAB), 2% PVP

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(polivinylpyrrolidone), 100 mM Tris-HCl pH8.0, 25 mM EDTA, 2 M NaCl and 2% v/v β-mercaptoethanol] was first prepared and incubated at 65 ºC. One gram of a snap-frozen root sample was ground into fine powder in the presence of liquid N_2 using a pair of pre-cooled mortar and pestle. The powder was then transferred to a 50 mL polypropylene tube containing 7 mL of extraction buffer. An equal volume of PCI (25:24:1) pH 8.0 was then added to the tube. The mixture was mixed by inverting the tube and incubated at 55 ºC for 10 min. The sample was later centrifuged at 15900 x *g* for 10 min at room temperature and the resulting upper phase was transferred into a fresh 50 mL polypropylene tube. The sample was re-extracted with PCI (25:24:1) pH 8.0 and similar steps as previously described were repeated. The resulting upper phase was then collected and transferred into a fresh 15 mL polypropylene tube. Lithium chloride (LiCl) stock solution was added to the collected solution to a final concentration of 2 M. The RNA was precipitated using 2 M LiCl overnight at -20 ºC. Following this, the sample was thawed on ice and RNA was collected by subjecting the sample to a centrifugation step at a maximum speed of 18400 x *g* for 10 min at 4 ºC. The resulting supernatant was discarded and the RNA pellet was washed with 5 mL cold 2 M LiCl and later centrifuged at 18400 x *g* for 5 min at 4 ºC. The pellet was again washed with 80% EtOH and centrifuged at 18400 x *g* for 5 min at 4 ºC. This step was repeated once again and the pellet was left to dry for approximately 10 min before being resuspended in 30 µL of DEPC dH_2O. RNA concentration and quality ($A_{260/280}$ and $A_{260/230}$) were determined using Nanophotometer (Implen Gmbh, Germany) and AGE. The sample was then stored in -20 ºC for future use.

3.3.3 Isolation of partial *NBS-LRR Resistance* (*R*) gene DNA via PCR

Partial *NBS-LRR Resistance* (*R*) gene fragment was isolated from banana leaf DNA using an in-house designed forward primer targeting Kinase-2 motif 5'-

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YTBMTWGTHYTNGATSAYGTBTGG-3' denoted as Kinase-2 UM that was paired with a reverse primer 5'-CGGCCAAGTCGTGCAYVAKRTCRTGCA-3' targeting the LRR motif obtained from Miller *et al*. (2008) and was denoted as 13-LRR (Figure 3.2). The partial *NBS-LRR R* gene was amplified in a 25 µL reaction mixture containing 1X PCR buffer A (EURx, Poland), 200 µM of each dNTPs, 1.5 mM $MgCl_2$, 0.4 µM each primer, 1 U *Taq* polymerase (EURx, Poland), 50-100 ng DNA template and sterile dH_2O in a 0.2 mL microcentrifuge tube. The PCR programme consisted of an initial denaturation step of 3 min at 94 °C followed by 38 cycles of 1 min at 94 °C, 1 min at 40 °C and 1 min at 72 °C with a final elongation step of 72 °C for 5 min in a thermocycler (peqSTAR, USA).

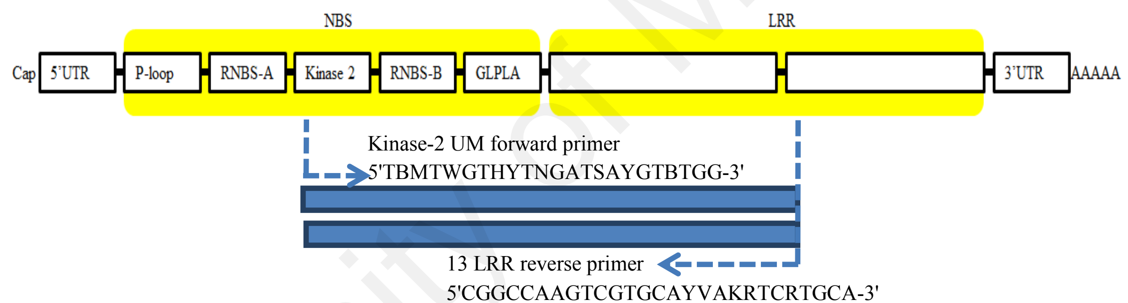

Figure 3.2: Schematic diagram of *NBS-LRR R* gene mRNA structure. *NBS-LRR R* gene mRNA structure adapted from Miller *et al*. (2008) and Peraza (2009) showing the amplification target site using Kinase-2 UM -13-LRR primer pair. Arrows indicate polymerisation direction of the reaction.

3.3.4 Isolation of *NBS-LRR Resistance* (*R*) gene transcript using Reverse-Transcription Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed on the isolated root RNA sample using SuperScript® One-Step RT-PCR System with Platinum® *Taq* DNA Polymerase (Invitrogen, USA) according to the manufacturer’s protocol. RT-PCR was carried out in a 25 µL reaction containing 12.5 µL of 2X Buffer, 8.5 µL of DEPC-treated sterile dH_2O, 300 – 1000 ng of root RNA, 0.4 µg of each primer and 1 µL of RT/ Platinum® Taq Mix in a 0.2 mL

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microcentrifuge tube. cDNA were synthesised at 50 ºC for 30 min and pre-denaturation step took place at 94 ºC for 2 min. This was then followed by PCR amplification step comprising 37 cycles of 15 sec at 94 ºC, 30 sec at 40 ºC and 1 min at 72 ºC with a final extension step at 72 ºC for 10 min using a thermocycler (peqSTAR, USA). The absence of genomic DNA in RNA preparation was verified by replacing RT/ Platinum® Taq Mix with 1 unit of *Taq* polymerase (EURx, Poland). Amplification product was subjected to AGE.

3.3.5 Agarose gel electrophoresis (AGE) for nucleic acid samples

3.3.5.1 DNA samples

Five microliter of DNA sample were electrophoresed on a 1% (w/v) agarose gel that was pre-stained with 1 μg ethidium bromide (EtBr) for 30 mL gel volume. Electrophoresis was carried out in Tris-borate EDTA (TBE) buffer at 120 V for 25 min. One microliter of 100 bp DNA ladder (Seegene, Korea) was used as molecular weight marker. The gel was then viewed under a UV light transilluminator (UV= 302 nm wavelength) using a gel documentation system (AlphaInnotech, USA).

3.3.5.2 RNA samples

All apparatus related to RNA sample analyses were first treated with 1% DEPCsolution. One microgram of RNA isolated from the root fragment was mixed with 1 μL of RNA loading dye (Thermo Scientific, USA). The mixture was heated at 65 ºC for 15 min to denature RNA secondary structures. Subsequently, RNA was electrophoresed in 1% (w/v) agarose gel stained with EtBr. Electrophoresis was carried out at 90 V for 45 min in a tank containing DEPC-treated 1 X Tris-Borate EDTA (TBE) buffer. The gel was then viewed under a UV light transilluminator (UV= 302 nm wavelength) using a gel documentation system (AlphaInnotech, USA).

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3.3.6 Purification of nucleic acid sample

The amplification product was purified using QIAquick Gel Extraction Kit (Qiagen, USA) according to the manufacturer's protocol. Briefly, the desired fragment was first excised from the agarose gel and weighed. Three volumes of Buffer QG were added to 1 volume of gel slice (100 mg = 100 µL). The gel slice was dissolved in QG Buffer for 10 min at 50ºC. During incubation, the gel was briefly vortexed every 2-3 min to assist efficient solubilisation. After the gel was completely dissolved, one gel volume of isopropanol was added and the sample was mixed. Subsequently, one QIAquick spin column was positioned in a 2 mL collecting tube and the dissolved gel was later pipetted onto the membrane of the spin column which possesses the maximum capacity of 750 µL. The sample was then centrifuged for 1 min at 17900 x *g* at room temperature. All centrifugation steps in this procedure were carried out using the above-mentioned parameters unless mentioned otherwise. The flow through was discarded and the QIAquick column was placed again in the same collection tube. Five hundred microliter of Buffer QG were added to the column and later centrifuged to remove the agarose gel traces. Next, the DNA was washed by adding 750 µL of Buffer PE and followed by centrifugation. The flow through were discarded and the sample was subjected to an additional 1 min centrifugation step to completely remove ethanol residues from Buffer PE. The QIAquick column was then placed into a fresh 1.5 mL microcentrifuge tube. Thirty microliters of sdH_2O were pipetted directly onto the column to elute the DNA. The column was left to stand for 1 min before being centrifuged. The eluate can either be used directly or stored at -20 ºC.

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3.3.7 T-A cloning

3.3.7.1 Ligation

The purified PCR product was ligated to pGEM-T Easy Vector (Promega, USA) according to manufacturer's protocol. Briefly, 1µL of dH_2O was pipetted into a 0.5 mL tube and later mixed with 5 µL of 2 X rapid ligation buffer. The vector was first centrifuged for 1 min at 15600 x *g* to collect the content at the bottom of the tube and 1 µL of the vector was added to the mixture containing the buffer. Finally, 2 µL of purified DNA and 1 µL of T4 DNA ligase (3 Weiss units/µL) were added into the mixture. The mixture was thoroughly mixed by gentle pipetting and incubated overnight at 4°C prior transformation.

3.3.7.2 Transformation

The ligation reaction was first centrifuged for 1 min at 15600 x *g* at room temperature prior to transformation procedure. Subsequently, 2 µL of the ligation products were pipetted into a fresh 1.5 mL microcentrifuge tube that was placed on ice. Competent cells strain JM109 (Promega, USA) were first thawed on ice for approximately 5 min. Next, 50 µL of the cells were pipetted into the tube containing the ligation product. The tube was gently flicked and incubated on ice for 20 min. Later, the cells were subjected to a 47 sec heat shock at 42ºC in water bath and directly subjected to ice-bath for 2 min. Subsequently, 950 µL of LB broth were added into the mixture and incubated at 37 ºC for 1.5 h with 220 rpm shaking. After the incubation, the transformation product was centrifuged for 2 min at 3000 x *g* to sediment the cells at the bottom whilst 800 µL of the supernatant were removed. The remaining 200 µL of transformation product were gently mixed and 100 µL of the product were plated onto an LB agar plate (37 mg/mL LB, 50 g/mL ampicillin, 0.5 mM IPTG, 0.08 mg/mL X-Gal). The plate was then incubated for 16 h at 37ºC.

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3.3.7.3 Selection of recombinant

Blue and white bacterial colonies resulting from the previous transformation experiment were screened for positive insertion. White colonies indicated the presence of an insert in *lacZ*, disrupting the formation of active β-galactosidase. The mutant β-galactosidase will not be able to cleave an organic compound known as X-gal, resulting in the development of a white colony. Unsuccessful transformations resulted in active β-galactosidase that cleaved X-gal into 5-bromo-4-chloro-indoxyl, which spontaneously dimerised and oxidised to form a bright blue insoluble pigment (5, 5'-dibromo-4, 4'-dichloro-indigo). The white single colony was selected using a sterile toothpick and transferred onto a 6 x 6 LB agar library plate. The remainder of the selected colony was resuspended into a 0.5 mL microcentrifuge tube containing 30 µL sdH_2O. The tube was then heated at 99ºC for 10 min prior to 15 sec centrifugation at 15600 x *g*. Finally, 2 µL of the DNA was used as a template in colony PCR to detect the presence of the desired insert using a vector-specific universal primer pair, M13.

3.3.7.4 Screening of bacterial colonies with positive inserts by PCR

Colony PCR was carried out in a 12.5 µL reaction mixture containing 1 X PCR Buffer A (EURx, Poland), 200 µM for each dNTP, 1.5 mM $MgCl_2$, 0.5 µM of each M13 primers (forward and reverse), 1 U *Taq* polymerase (EURx, Poland), and 50-100 ng of colony DNA sample in a 0.2 mL microcentrifuge tube. The PCR reaction profile included an initial denaturation step at 95°C for 3 min; 31 cycles of a denaturation step at 95°C for 27 sec, an annealing step at 60°C for 1 min and an extension step at 72°C for 1 min followed by a final extension step at 72°C for 5 min. The PCR product was then subjected to AGE.

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3.3.8 Plasmid DNA isolation by Alkaline Lysis with SDS

Plasmid DNA isolation was carried out following Sambrook and Russell (2001). A single colony from the mini-library was cultured in a universal bottle containing 10 mL LB broth medium with 50 μg/mL ampicillin. The colony culture was incubated overnight at 37°C with 220 rpm. Following this, 850 μL of the culture were transferred into a fresh 1.5 mL microcentrifuge tube containing 150 μL of glycerol and stored in -80°C while the remainder was transferred into a 15 mL Falcon tube. The sample in the Falcon tube was centrifuged at 11963 × *g* for 5 min and the resulting supernatant was discarded. The pellet was dissolved in 200 μL of Solution I (50 mM glucose, 10 mM EDTA, 25 mM Tris-Cl) at 0°C and transferred into a fresh 1.5 mL microcentrifuge tube. Solution I was added to destabilize the cell membrane and to prevent the shearing of DNA. Subsequently, 200 μL of Solution II (10 M NaCl, 10% SDS) were added to lyse the bacterial cells. The sample was gently mixed at room temperature for 4 min. Following this, 200 μL Solution III [3 M potassium acetate (pH 5.5)] were added and the sample was gently mixed at 0°C for 15 min. Solution III induced the formation of SDS-protein complex and the precipitation of high molecular weight RNA. These macromolecules could then be removed via centrifugation (Birnboim and Doly, 1979) for 10 min at 20217 × *g*. The supernatant was later transferred into a fresh 1.5 mL microcentrifuge tube containing 50 μg/mL RNase A. The sample was gently mixed and incubated at 37 ^{o}C for 3 h. Following this, one volume of phenol was added to the sample, vortexed and centrifuged for 5 min at room temperature. The supernatant was transferred into a fresh 1.5 mL microcentrifuge tube and one volume of chloroform was added. The sample was vortexed and centrifuged again as described in the previous step. The resulting upper layer was then transferred to a fresh 1.5 mL microcentrifuge tube. Subsequently, 50 μL of 5 M NaCl and 1250 μL of absolute isopropanol were added into the tube to precipitate the DNA for 20 min in ice. The sample was then

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centrifuged at 4 ^{o}C for 15 min at 20217 × *g*. Later, the resulting supernatant was discarded and the pellet was then rinsed with 1 mL of 70% EtOH and centrifuged for 5 min at 20217 × *g*. The supernatant was discarded and the pellet was air-dried. Later, the pellet was dissolved in 30 µL of sdH_2O and kept in 4°C for future use. The concentration and purity of the extracted plasmid DNA were determined by subjecting to AGE.

3.3.9 Restriction enzyme (RE) digestion

The isolated plasmid was digested with *Eco*RI enzyme that will cut at both ends of the vector arms releasing the insert of approximately 670 bp in size. Each isolated plasmid was subjected to RE digestion with final reaction volume of 10 µL containing 10 × Buffer 2 (NE BioLabs, England), 10 µg/mL of BSA (New England BioLabs, England), 1 U of *Eco*RI restriction enzyme (New England BioLabs, England) and 5 µg of DNA sample. Each reaction tube was incubated in a water bath at 37°C for 1 h. The enzymatic reaction was later terminated by heating the sample at 65°C for 10 min on a heat block. To confirm positive enzymatic reaction, 5µl of the product was subjected to AGE (Section 4.3.3). Plasmid with the right insert size was sequenced using 3130*xl* Genetic Analyzer from Applied Biosystem (ABI, USA).

3.3.10 Sequence and phylogenetic analyses

Forward and reverse chromatograms of each clone were analysed using Chromas version 2.4.3 (Technelysium Pty. Ltd) and later concatenated. The identity of all cloned sequences was confirmed by subjecting the sequences to similarity analysis using BLASTn algorithm in the GenBank.

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A total of 124 deduced amino acid sequences were subjected to multiple sequence alignment using ClustalX in MEGA 5.2 (Tamura *et al*., 2011). These sequences comprised of 37 genomic DNA sequences and 14 RNA transcripts along with 71 reference sequences and 2 outgroup sequences. The two outgroup sequences were the NBS-LRR type *R* gene isolated from *Manihot esculenta* (AY271884) and *Setaria italica* (GU930314). Since the sequences in the GenBank were not of the same length, all sequences subjected to alignment were edited to begin with Kinase-2 motifs and ended with hydrophobic domain (GLPL motif). Note that only sequences with un-interrupted reading frames were used for phylogenetic analysis The *NBS-LRR R* gene banana reference sequences were originated from eight *Musa* species namely *M. acuminata*, *M. balbisisana*, *M. banksii*, *M. ornata*, *M. schizocarpa*, *M. textilis*, *M. velutina* and a hybrid of *M. acuminata* and *M. balbisiana*. The best substitution model was first determined using *Find best DNA/Protein Models* function available in MEGA5. The evolutionary distance amongst the sequences was estimated by generating a Neighbour-Joining (NJ) that was computed using Jones-Taylor-Thornton (JTT) with gamma distribution value of 3.75 and a bootstrap value of 1000. All positions containing gaps and missing data were eliminated.

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CHAPTER 4: RESULTS

4.1 Conventional proteomics

Single inoculation experiment was conducted as a proof of concept to observe changes in protein abundance difference in susceptible banana cultivar, Grand naine when infected with 1000J2 *M. incognita.* Protein was extracted in all harvested root fragments and the concentration was quantified

4.1.1 Protein quantification

Protein sample was quantified based on BSA linear negative regression correlation standard curve with R^2 value of 0.9954 (Figure 4.1). Despite having a good protein yield, sample Grand naine 30 dai 0 J2c was discarded from further analysis due to technical error. In this study, the concentration of six protein samples isolated from control and inoculated root tissues were within the range of 1.24 to 2.58 µg/µL (Table 4.1).

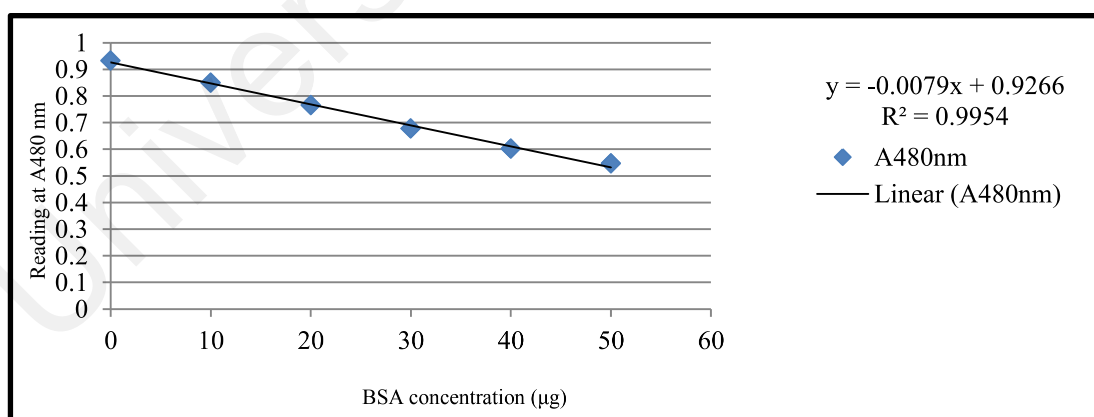

Figure 4.1: Negative linear regression correlation standard curve generated from BSA with concentrations ranged from 0 to 50 µg (x-axis) estimated at 480nm UV wavelength (y-axis). The R^2 value of this correlation graph was 0.9954 with y=-0.0079x + 0.9266. This standard curve was used to quantify 7µL protein samples.

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Table 4.1: The calculated Grand naine protein concentration in every sample based on a standard curve generated from BSA standard solution.

Sample	Protein concentration (µg/µL)	Gel number
Grand naine 30 dai 0 J2a	1.39	35
Grand naine 30 dai 0 J2b	1.24	36
Grand naine 30 dai 0 J2c	1.44	34 (discarded from further analyses)
Grand naine 30 dai 1000 J2a	2.58	37
Grand naine 30 dai 1000 J2b	1.73	38
Grand naine 30 dai 1000 J2c	1.49	39

4.1.2 Statistical analysis

4.1.2.1 Spot correlation analysis

Gel 39 was made as the reference gel for this analysis. The correlation values (R^2) obtained between each gel pair ranged from 0.4935 - 0.8733 (Figure 4.2). This analysis revealed that out of the four gels, only gel 36 showed lower correlation (R^2 = 0.4935) with the reference gel 39.

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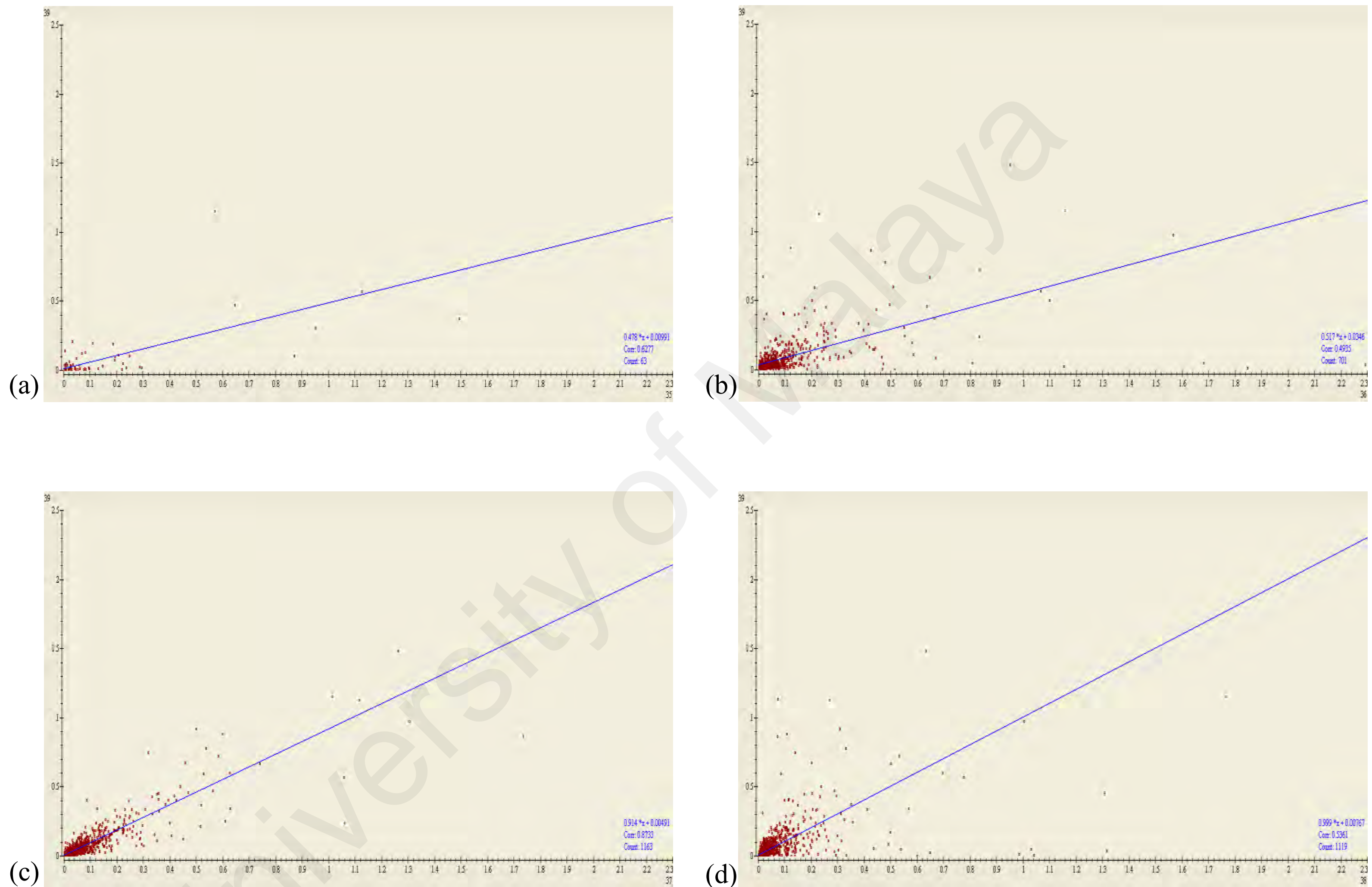

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Figure 4.2: Spot correlation analysis between gels with gel 39 serves as reference**.** Spot correlation analysis result between the reference gel 39 and gels (a) 35 with correlation value of 0.6277, (b) 36 with correlation value of 0.4935, (c) 37 with correlation value of 0.8733 and (d) 38 with correlation value of 0.5361. Gels (a) and (b) represent protein samples isolated from *M. incognita*-treated Grand naine root fragment while gels (c) and (d) represent protein samples isolated from the control Grand naine root fragments.

4.1.2.2 Factor projection plot

Factor projection plot on all five gels (35, 36, 37, 38 and 39) revealed that two gels for *M. incognita*-inoculated protein samples (37 and 39) were separated by Factor 1 from another two gels for control samples (35 and 36). However, one of the inoculated sample gels (Gel 38) was clustered together with the control group (Figure 4.3). This gel was included in subsequent analyses.

Figure 4.3: Factor projection plot analysis of control and inoculated sample gels. The analysis showed that control sample gels (Gels 35 and 36) were clustered into a group while gels for inoculated samples (Gels 37 and 39) were clustered into another group. However, one inoculated sample gel (Gel 38) showed similarity with control sample gels. Red dots represent protein spots obtained across five gels whilst the gel name was written in blue. The farther away the spot is from the origin, the more significant the abundance difference would be amongst the five gels.

4.1.2.3 Spot abundance analysis

In this study, a total of 1482 protein spots were recovered across five gels. Out of this, only 164 spots showed significant abundance changes (Kolmogorov-Smirnov value = 1.0) between control and inoculated samples. In this study, the Kolmogorov-Smirnov test will determine every protein if there is a significant difference between control and inoculated samples. Protein with Protein spot abundance difference was manually screened using spot analysis histogram (Figure 4.4) because of the non-definitive clustering obtained between control and inoculated gels in Factor Projection Plot analysis (section 4.1.2.2). Out of 164 significant protein spots with Kolmogorov-Smirnov value of 1.0, 51 protein spots showed significant

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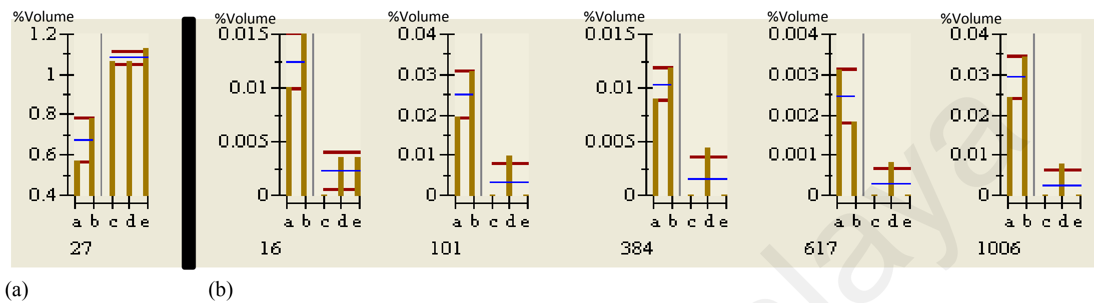
abundance changes between control and inoculated samples (ANOVA; $p \leq 0.05$) (Figure 4.5). Using ANOVA; $p \leq 0.05$, protein spots with 95% confident showing significant abundance changes were selected. Fifty protein spots were present at lower abundance level in inoculated samples and one was present at higher abundance level in inoculated samples when compared with control samples.



(a) (b)

Figure 4.4: Gel analysis histogram of control and inoculated sample gels. The analysis showing significant abundance difference (ANOVA $p \leq 0.05$) in inoculated samples (gels 'c', 'd' and 'e') when compared with control samples (gels 'a' and 'b'). Out of 51 spots showing significant abundance changes, only (a) spot 27 was present at higher abundance level in inoculated samples and the other 50 spots were present in decreased abundance level in inoculated samples as shown by spots 16, 101, 384, 617 and 1006 in (b). Blue horizontal bar indicates mean value while standard errors (SE) were represented with red horizontal bars. Gels 'a' and 'b' are control samples while 'c', 'd' and 'e' are inoculated samples.

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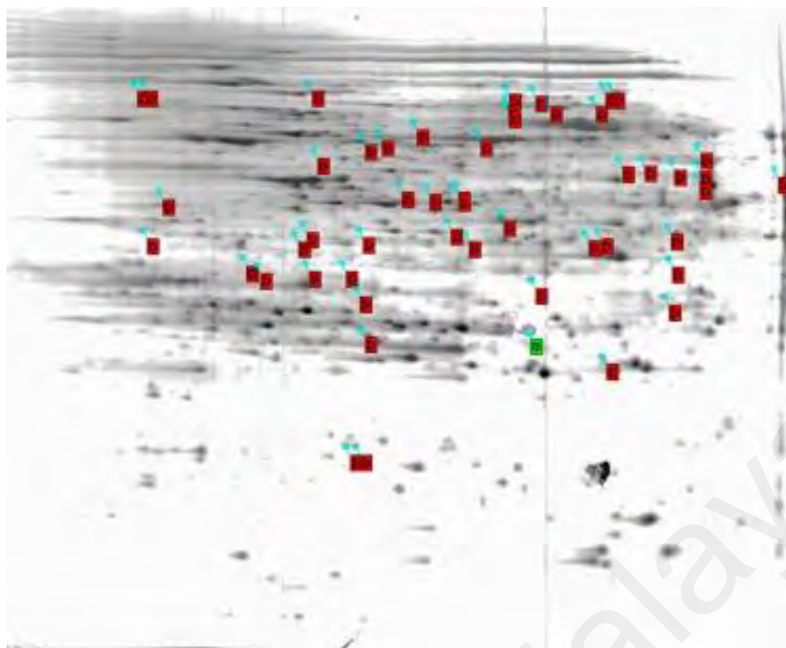

Figure 4.5: SDS-PAGE gel containing the protein spots. Grand naine root proteome map of 51 spots showing significant abundance difference (ANOVA $p \leq 0.05$) when inoculated with 1000J2 *M. incognita*. Fifty protein spots showed decreased abundance level in inoculated samples (marked in red) while only one spot showed increased abundance level in inoculated samples (marked in green) when compared with control samples.

4.2 LC-MS proteomics

4.2.1 Phenotypic assessment on *Meloidogyne incognita*-inoculated and control banana root tissues

Root tissues harvested at 30- and 60- dai were assessed for formation of galls. It was observed that galls were formed in all *M. incognita*-inoculated root fragments (Figure 4.6 d-f, j-l) and none was seen on control root fragments (Figure 4.6 a-c, g-i). It is noteworthy that galls formed on the root fragments harvested at 60-dai were more visible to the naked eye compared to that of harvested at 30-dai (Figure 4.6).

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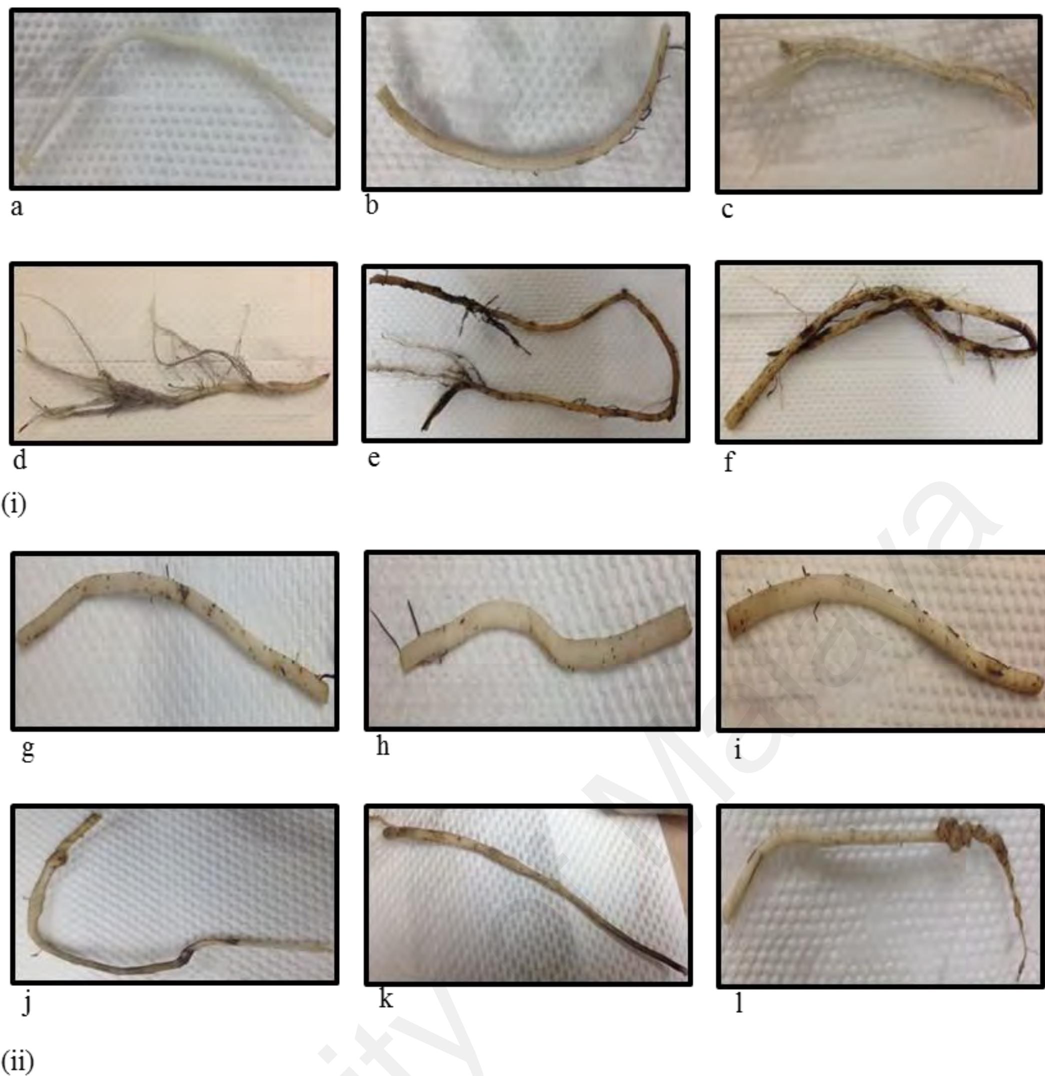

Figure 4.6: Post-inoculation harvested root fragments. Harvested root fragments after (i) 30-dai and (ii) 60-dai. Galls were not observed in all control root fragments (a, b, c, g, h, i) but present in all *M. incognita*-inoculated root fragments (d, e, f, j, k, l). Galls were more visible on root fragments harvested at 60-dai (j, k, l) compared to the ones harvested at 30-dai (d, e, f). Root fragments (50 mg) presented here were not to true scale.

4.2.2 Spectral analysis

A total of 9304 peptide spectra were successfully obtained in this experiment. Analysis using Progenesis software coupled to MASCOT database revealed 2065 proteins present in banana root proteome involving in this compatible interaction. Out of these only 159 proteins from the two time points (30- and 60-dai) showed

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significant abundance difference (ANOVA $p \leq 0.05$) between inoculated and control root fragments.

4.2.3 Principle Component Analysis (PCA)

Principle Component Analysis (PCA; Figure 4.7) conducted on the twelve samples revealed that sample from 30-dai and 60-dai were separated by principle component 2 (PC2). Significant clustering manner was obtained for control and inoculated samples at 60-dai. However, control and inoculated samples harvested at 30-dai did not show a definitive clustering manner. Therefore, further analyses on the significant protein abundance difference between control and inoculated samples were only conducted on samples harvested at 60-dai.

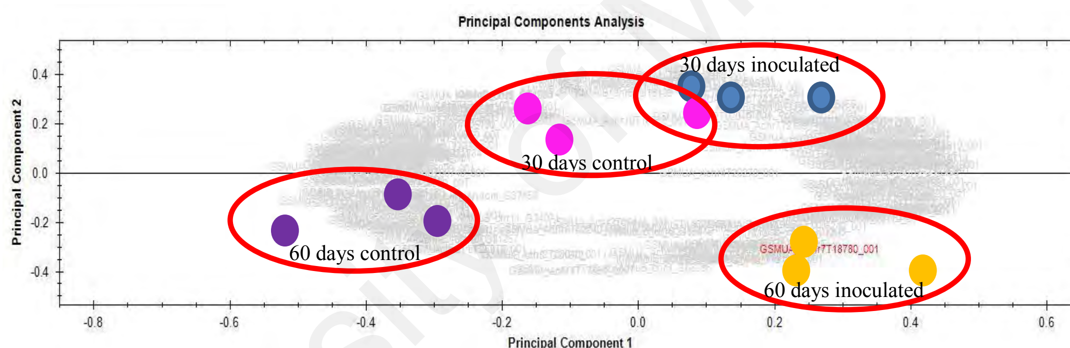

Figure 4.7: Principal component analysis (PCA) of banana root fragments harvested at 30- & 60- dai. Definitive clustering manner was obtained only for 60-dai samples while one of the control samples harvested at 30-dai was clustered together with inoculated samples harvested at the same time point.

4.2.4 Identification of proteins showing statistically significant abundance difference in inoculated root fragments when compared with control root fragments at 60-dai.

Among the 159 proteins showing significant abundance difference between inoculated and control root fragments (ANOVA $p \leq 0.05$) at 30- and 60-dai. However, only 112 proteins showed significant abundance difference at 60-dai.

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These proteins were selected for identification and biological function determination using Greenphyl and Uniprot databases. From the search, it was found that proteins involved in banana-nematode interaction can be grouped in 10 biological functions (Figure 4.8.) i.e. DNA replication, defence, energy-related, catalytic, structural component, carrier, stress response, metabolism, oxidation-reduction, biosynthesis. Six percent of 112 obtained proteins were not able to be characterised. Table 4.2 depicts the biological function and protein fold-change of the treated samples compared to control.

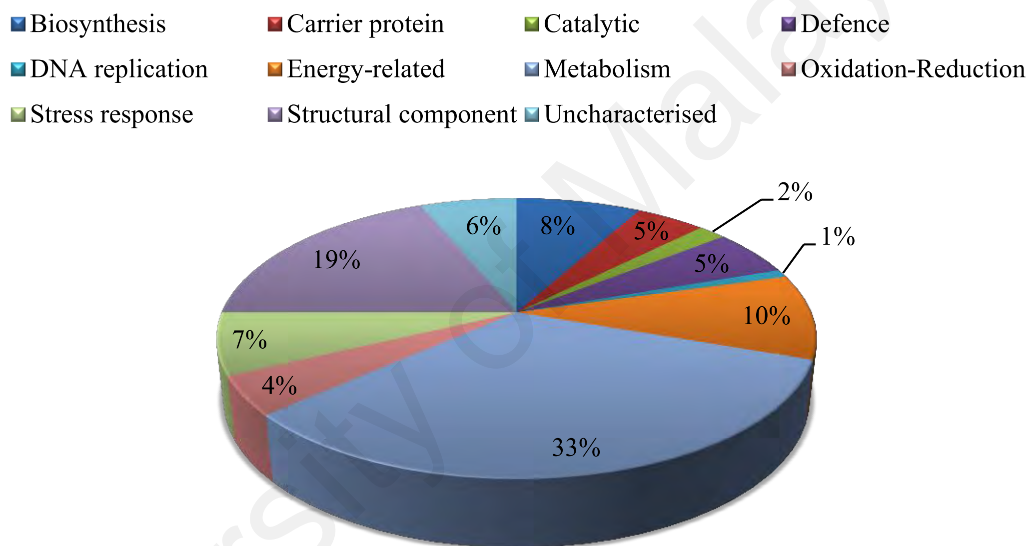

Figure 4.8: Pie chart showing percentage of protein functions identified from a total of 112 proteins recovered from LC-MS analysis. These proteins showed significant abundance changes in inoculated Grand naine root fragments when compared with control samples at 60-dai.

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Table 4.2: List of identified proteins showing significant abundance changes (p≤0.05) during banana-*M. incognita* interaction at 60-dai together with accession numbers grouped according to its biological function obtained from UniProt and GreenPhyl databases. The list was organised according to the fold-change level in nematode-inoculated samples with control samples as the baseline. Proteins with increased abundance level in inoculated samples were written in positive values while proteins with decreased abundance level were written in negative values.

Description	Accession no.	ANOVA P<0.05	Fold-change
	Biosynthesis		
Elongation factor 1	ITC1587_Bchr6_P15150	0.000	Present only in inoculated sample
Elongation factor 2	GSMUA_Achr4T01020_001	0.000	Present only in inoculated sample
O-methyltransferase	ITC1587_Bchr3_P07963	0.000	Present only in inoculated sample
Probable cinnamyl alcohol dehydrogenase	GSMUA_Achr4T06150_001	0.034	Present only in inoculated sample
Isoflavone reductase homolog	GSMUA_Achr2T14320_001	0.039	10.32
Alpha-1,3-glucan-protein synthase	ITC1587_Bchr4_P10810	0.000	3.72
Biotin carboxylase	ITC1587_Bchr8_P24200	0.04	2.96
Aspartate-semialdehyde dehydrogenase	GSMUA_Achr10T18110_001	0.022	-3.64
Putative Methylthioribose kinase	GSMUA_Achr7T05460_001	0.05	-4.4
	Carrier protein		
ATP-ADP translocator	ITC1587_Bchr8_P24300	0.01	Present only in inoculated sample
Putative SEC12-like protein 2	GSMUA_Achr6T10220_001	0.01	Present only in inoculated sample
Ras-related protein raba5d-like	ITC1587_Bchr11_P33367	0.00	Present only in inoculated sample
Cytochrome c oxidase subunit 6B	GSMUA_Achr7T11740_001	0.00	-2.23
Rab GDP dissociation inhibitor alpha	GSMUA_Achr6T18380_001	0.03	-2.89
	Catalytic		
Mitochondrial-processing peptidase subunit alpha	GSMUA_Achr7T13650_001	0.01	-2.32
Probable mitochondrial-processing peptidase subunit beta	GSMUA_Achr7T00560_001	0.01	-1.73

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Table 4.2, continued.

Description	Accession no.	ANOVA P<0.05	Fold-change
		Defence	
Germin-like protein 11-1	ITC1587_Bchr9_P27746	0.01	5.23
Putative Patatin group A-3	GSMUA_Achr6T05080_001	0.02	4.41
Germin-like protein 5-1	GSMUA_Achr1T25160_001	0.01	-2.66
Md-2-related lipid recognition domain-containing protein	ITC1587_Bchr5_P14231	0.03	-2.82
Germin-like protein 5-1	GSMUA_Achr5T18440_001	0.01	-7.48
Pathogenesis-related protein 1	ITC1587_Bchr9_P26466	0.02	-29.93
		DNA replication	
Seed specific protein Bn15D1B, putative, expressed	GSMUA_Achr8T13580_001	0.01	-5.18
		Energy-related	
A chain structure of banana lectin-methyl-alpha-mannose complex	ITC1587_Bchr9_P25965	0.02	73.63
V-type proton ATPase catalytic subunit A	GSMUA_Achr11T08060_001	0.03	3.53
ATP synthase subunit alpha, mitochondrial	GSMUA_AchrUn_randomT15230_001	0.03	-1.48
ATP synthase subunit d, mitochondrial	GSMUA_Achr10T27350_001	0.01	-1.64
Succinate dehydrogenase	ITC1587_Bchr7_P18621	0.01	-1.94
ATP synthase subunit beta, mitochondrial	GSMUA_Achr9T21710_001	0.01	-2.03
Probable ATP synthase 24 kda subunit, mitochondrial	GSMUA_Achr6T02850_001	0.00	-2.25
ATP-dependent Clp protease ATP-binding subunit clpc homolog, chloroplastic	GSMUA_AchrUn_randomT22440_001	0.04	-2.37
ATP synthase subunit mitochondrial-like	ITC1587_Bchr10_P31293	0.01	-2.6
Succinate dehydrogenase [ubiquinone] flavoprotein subunit 1, mitochondrial	GSMUA_Achr6T31640_001	0.01	-2.75

Table 4.2, continued.

Description	Accession no.	ANOVA P<0.05	Fold-change
	Energy-related		
Succinate dehydrogenase [ubiquinone] flavoprotein subunit 1, mitochondrial	GSMUA_Achr8T19050_001	0.00	-2.79
	Metabolism		
Probable fructokinase-2	GSMUA_Achr10T16420_001	0.02	Present only in inoculated sample
Adenosine kinase 2	GSMUA_Achr2T00250_001	0.00	Present only in inoculated sample
Aspartate aminotransferase, cytoplasmic	GSMUA_Achr4T08110_001	0.022	Present only in inoculated sample
Enoyl-[acyl-carrier-protein] reductase [NADH], chloroplastic	GSMUA_Achr1T19640_001	0.05	Present only in inoculated sample
Fructose-bisphosphate aldolase	ITC1587_Bchr8_P21572	0.02	Present only in inoculated sample
Isocitrate dehydrogenase [NADP]	GSMUA_Achr1T05110_001	0.01	Present only in inoculated sample
Rubisco subunit binding-protein alpha subunit	ITC1587_BchrUn_random_P35868	0.02	Present only in inoculated sample
Serine hydroxymethyltransferase	ITC1587_Bchr9_P25209	0.01	Present only in inoculated sample
Fructokinase-2	GSMUA_Achr11T11150_001	0.01	829.8
Glyceraldehyde-3-phosphate dehydrogenase, cytosolic 3	GSMUA_Achr5T25410_001	0.01	468.86
5-methyltetrahydropteroyltriglutamate-homocysteine expressed	ITC1587_Bchr5_P11892	0.00	19.84
S-adenosylmethionine synthetase	ITC1587_Bchr7_P18740	0.04	17.04
5-methyltetrahydropteroyltriglutamate-homocysteine expressed	ITC1587_Bchr4_P10741	0.01	16.26

Table 4.2, continued.

Description	**Accession no.**	**ANOVA P<0.05**	**Fold-change**
Metabolism			
Rubisco large subunit-binding protein subunit beta, chloroplastic	GSMUA_Achr9T23240_001	0.04	12.3
Sucrose synthase 2	GSMUA_Achr6T10890_001	0.03	7.29
5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase	GSMUA_Achr7T01530_001	0.04	5.36
Putative Pyruvate kinase, cytosolic isozyme	GSMUA_Achr10T15400_001	0.03	5.17
S-Adenosylmethionine synthetase	ITC1587_Bchr1_P01149	0.03	3.36
5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase	GSMUA_Achr4T22700_001	0.00	3.25
5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase	GSMUA_Achr4T21470_001	0.01	2.93
Pyruvate kinase	ITC1587_Bchr2_P03452	0.01	2.08
Dihydrolipoyllysine-residue acetyltransferase component 3 of pyruvate dehydrogenase complex, mitochondrial	GSMUA_Achr10T08050_001	0.02	-1.77
3-hydroxyisobutyryl-coa hydrolase-like protein 3	GSMUA_Achr6T00740_001	0.04	-2.01
CBS domain protein	ITC1587_Bchr3_P07894	0.01	-2.18
Malate dehydrogenase, mitochondrial	GSMUA_Achr4T08580_001	0.01	-2.21
Succinyl- ligase	ITC1587_Bchr2_P04196	0.02	-2.39
Aconitate hydratase 2, mitochondrial	GSMUA_Achr11T01170_001	0.03	-2.51

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Table 4.2, continued.

Description	**Accession no.**	**ANOVA P<0.05**	**Fold-change**
Metabolsim			
Phosphoenolpyruvate carboxylase, housekeeping isozyme	GSMUA_Achr6T26850_001	0.03	-2.57
PI-PLC X domain-containing protein At5g67130	GSMUA_Achr6T25660_001	0.01	-2.99
Methylmalonate-semialdehyde dehydrogenase [acylating], mitochondrial	GSMUA_Achr4T22360_001	0.01	-3.26
Pyruvate dehydrogenase E1 component subunit beta, mitochondrial	GSMUA_Achr5T25000_001	0.01	-3.66
Alpha-galactosidase	GSMUA_Achr6T15820_001	0.00	-4.29
Fructose-bisphosphate aldolase	ITC1587_Bchr5_P14394	0.04	-5.12
Cysteine synthase	ITC1587_Bchr4_P10620	0.01	-5.17
Delta-1-pyrroline-5-carboxylate dehydrogenase 12A1, mitochondrial	GSMUA_AchrUn_randomT11080_001	0.01	-5.76
Pi-plc x domain-containing protein at5g67130-like	ITC1587_Bchr6_P16564	0.02	-7.41
Oxidation-Reduction			
26s proteasome regulatory subunit 4 homolog a-like	ITC1587_Bchr4_P08913	0.01	24.17
26s protease regulatory subunit 6b homolog	ITC1587_Bchr7_P20965	0.02	9.57
Peroxiredoxin-2F, mitochondrial	GSMUA_Achr8T09520_001	0.04	-2.58
Oxidation-Reduction			
Peroxidase 5	GSMUA_Achr8T12370_001	0.03	-8.19
Monodehydroascorbate reductase, chloroplastic	GSMUA_Achr5T17510_001	0.03	-15.27
Stress response			
70 kda peptidyl-prolyl isomerase	GSMUA_AchrUn_randomT02470_001	0.01	Present only in inoculated sample

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Table 4.2, continued.

Description	Accession no.	ANOVA P<0.05	Fold-change
Stress response			
Heat shock cognate 70 kda protein	GSMUA_Achr9T03960_001	0.01	4.18
Heat shock 70 kda protein, mitochondrial	GSMUA_Achr3T12480_001	0.00	2.47
Flavoprotein wrba	GSMUA_Achr10T10080_001	0.04	-3.2
Lignin-forming anionic peroxidase	GSMUA_Achr4T05250_001	0.01	-4.14
Heat shock cognate 70 kda protein	GSMUA_Achr2T16250_001	0.00	-4.95
Osapx7 - Stromal Ascorbate Peroxidase encoding gene 5,8, expressed	GSMUA_Achr10T16040_001	0.02	-5.36
Flavoprotein wrba	GSMUA_Achr5T26440_001	0.02	-6.15
Structural component			
T-complex protein 1 subunit zeta	GSMUA_Achr1T14710_001	0.01	Present only in inoculated sample
40S ribosomal protein S2-4	GSMUA_AchrUn_randomT09450_001	0.01	Present only in inoculated sample
60s ribosomal protein l10a-1	ITC1587_Bchr3_P07546	0.00	Present only in inoculated sample
60S ribosomal protein L22-2	GSMUA_Achr3T00720_001	0.04	Present only in inoculated sample
Beta chain	ITC1587_BchrUn_random_P35428	0.01	Present only in inoculated sample
Eukaryotic translation initiation factor 5A	GSMUA_Achr3T18790_001	0.00	Present only in inoculated sample
60s ribosomal protein l9	ITC1587_Bchr5_P13916	0.04	469.69
Tubulin beta chain	ITC1587_Bchr6_P16601	0.01	200.81
40s ribosomal protein s20-2-like	ITC1587_Bchr3_P06192	0.01	54.97
40S ribosomal protein s15a-1	GSMUA_Achr1T17170_001	0.00	29.38
Structural Component			
40S ribosomal protein S14	GSMUA_Achr2T20380_001	0.01	22
Tubulin alpha-1 chain	ITC1587_Bchr6_P17875	0.02	14.21
60S ribosomal protein L4-1	GSMUA_Achr5T03060_001	0.03	6.69
40S ribosomal protein S27-2	GSMUA_Achr3T18600_001	0.00	3.7
40S ribosomal protein S4	GSMUA_Achr2T01640_001	0.00	3.38

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Table 4.2, continued.

Description	Accession no.	ANOVA P<0.05	Fold-change
Structural Component			
40S ribosomal protein Sa-2	GSMUA_Achr1T21820_001	0.02	2.44
Chaperonin CPN60-2, mitochondrial	GSMUA_Achr10T08040_001	0.03	-1.33
Actin-2	GSMUA_Achr10T03730_001	0.04	-1.83
Probable plastid-lipid-associated protein 2, chloroplastic	GSMUA_Achr4T20110_001	0.04	-5.92
Tubulin beta-1 chain	GSMUA_Achr6T04600_001	0.01	-201.48
Uncharacterised			
Uncharacterised protein	ITC1587_Bchr9_P28128	0.01	6.55
Hypothetical protein	GSMUA_Achr4T14260_001	0.01	-2.15
Uncharacterized protein At5g10860, mitochondrial; CBS domain-containing protein CBSX3, mitochondrial	GSMUA_Achr3T26630_001	0.00	-2.36
Uncharacterised protein	ITC1587_Bchr10_P31266	0.04	-2.51
Putative uncharacterized protein	GSMUA_Achr11T04110_001	0.01	-2.7
Putative Uncharacterized protein At4g06744	GSMUA_Achr8T15600_001	0.04	-3.05
Putative uncharacterized protein	GSMUA_Achr9T02000_001	0.00	-5.68

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4.3 *NBS-LRR Resistance* gene

4.3.1 Isolation of nucleic acid

4.3.1.1 DNA isolation from banana leaf tissues

The purity and quantity of the extracted DNA are referred in Table 4.3. The extracted DNA possessed good average purity ratio of A_{260}/A_{280} (1.824 - 2.000) whilst lower ratio of A_{260}/A_{230} (1.267-1.667) signifying carbohydrate and glycogen contaminations. Interestingly, the DNA concentration corroborated with the quality as evaluated through AGE in Figure 4.9 except for Malaccensis DNA 1 (M1) in which did not show any DNA band although the DNA concentration was similar to Grand Naine DNA 2 (GN2).

Table 4.3: Purity and concentration of the DNA extracted from *Musa* spp.

Sample	Purity (A_{260}/A_{280})	Purity (A_{260}/A_{230})	Concentration (µg/µL)
Berangan DNA 1 (B1)	2.000	1.600	0.056
Berangan DNA 2 (B2)	1.842	1.667	0.082
Grand Naine DNA 1 (GN1)	1.882	1.600	0.075
Grand Naine DNA 2 (GN2)	1.900	1.267	0.045
Malaccensis DNA 1 (M1)	1.824	1.257	0.045
Malaccensis DNA 2 (M2)	1.886	1.222	0.032
Lemak Manis 1 (LM1)	1.893	1.341	0.044
Lemak Manis 2 (LM2)	1.902	1.382	0.061

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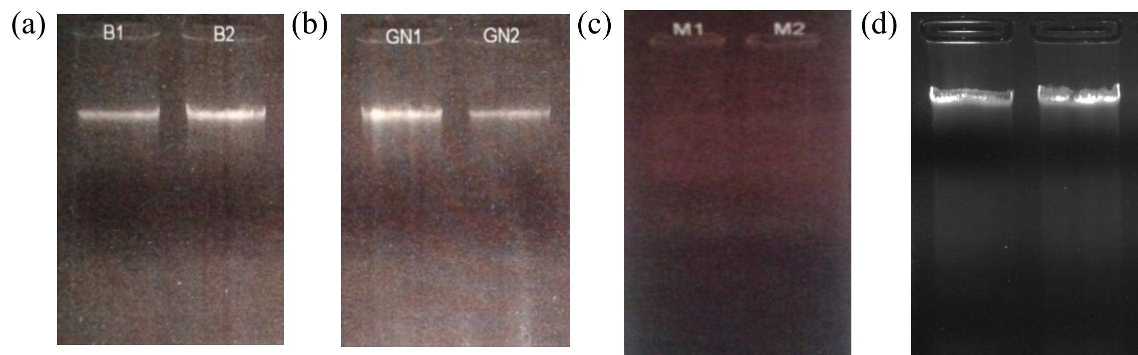

Figure 4.9: Nucleic acid quality evaluation. Agarose gel electrophoresis analysis showing DNA quality isolated from (a) Berangan (B1 and B2), (b) Grand Naine (GN1 and GN2), (c) Malaccensis (M1 and M2), and (d) Lemak Manis (LM1 and LM2) leaf tissues. Only DNA samples extracted from Malaccensis leaf tissues showed low DNA yield.

4.3.1.2 RNA extraction from the infected banana root fragments

Quantification of total RNA samples isolated from *M. incognita*-inoculated Grand naine root fragments revealed poor A_{260}/A_{280} and A_{260}/A_{230} values. The A_{260}/A_{280} values of both replicated Grand naine samples were 1.522 and 1.388 while the A_{260}/A_{230} values were 0.662 and 0.363 (Table 4.4). However, when AGE analysis was carried out, distinct *28S* (~3100 bases) and *18S* (~1900 bases) rRNA bands were obtained when 1µg of total RNA of the same samples were electrophoresed (Figure 4.10). This suggested that the quality of the isolated total RNA can be used for subsequent molecular analysis.

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Table 4.4: Purity and concentration of RNA samples isolated from Grand naine root fragments inoculated with *M. incognita*.

Sample	Purity (A_{260}/A_{280})	Purity (A_{260}/A_{230})	Concentration (µg/µL)
Grand naine RNA 1 (GNt1)	1.522	0.662	1.226
Grand naine RNA 2 (GNt2)	1.388	0.363	0.349

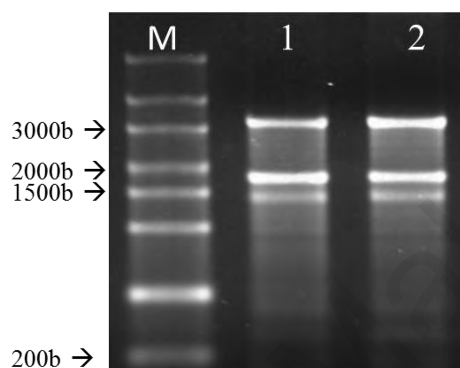

Figure 4.10: Agarose gel electrophoresis analysis showing the quality of total RNA isolated from Grand naine root tissues that were inoculated with *M. incognita*. Two distinct bands of high molecular weight rRNA bands of *28S* (~3 kb) and *18S* (~1.9 kb) were obtained from the two replicated Grand naine samples (Lanes 1 and 2) signifying successful isolation total RNA samples (Lane M: RNA ladder, Thermo Fischer Scientific, USA).

4.3.2 Isolation of NBS-LRR *R* gene from *Musa* sp.

4.3.2.1 Isolation of NBS-LRR *R* gene from banana genomic samples.

A ~650bp band was successfully amplified from the leaf tissues of four banana varieties namely Berangan (Figure 4.11a), Grand naine (Figure 4.11b), Malaccensis (Figure 4.11c), and Lemak manis (Figure 4.11d). However, the quality of amplification was observed to be lower in Lemak manis samples (Figure 4.11d) when fainter bands of ~650bp were obtained for the two sample replicates. The existence of a faint band at ~800 bp was noted in the two samples (Figure 4.11d) and regarded as a result of non-specific amplifications. All non-template reactions yielded no bands.

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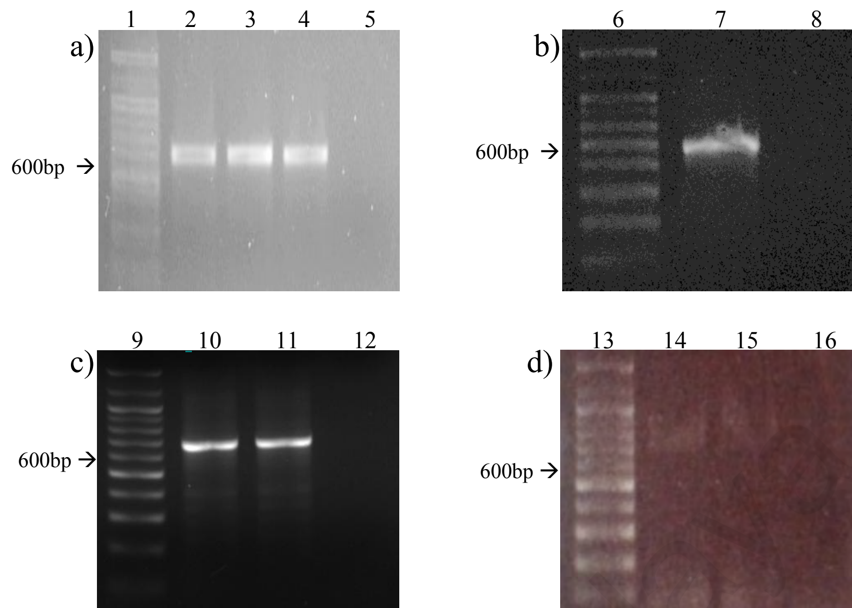

Figure 4.11: PCR and RT-PCR products of *NBS-LRR Resistance* gene subjected to AGE. Successful amplification of a 650 bp long target site for Berangan, Grand Naine, Malaccensis and Lemak Manis genomic DNA samples. (a) Lane 1: 100 bp DNA ladder; Lane 2: B1; Lane 3: B2; Lane 4: GN1; Lane 5: -ve control. (b) Lane 6: 100 bp DNA ladder; Lane 7: GN2; Lane 8: -ve control. (c) Lane 9: 100 bp DNA ladder; Lane 10: M1, Lane 11: M2, Lane 12: -ve control; d) Lemak Manis. Lane 13: 100 bp DNA ladder; Lane 14: LM1, Lane 15: LM2, Lane 16: -ve control.

4.3.2.2 Isolation of NBS-LRR *R* gene from *M. incognita*- inoculated Grand naine root samples.

One-step RT-PCR conducted on *M. incognita*- inoculated Grand naine root samples yielded no amplifications. However, when the RT-PCR products were subjected to PCR, a band of ~650 bp was obtained (Figure 4.11e) in one of the replicate samples. It is noteworthy that no DNA contamination was detected in reactions containing Taq DNA polymerase during One-step RT-PCR amplification. No bands were obtained in the non-template reaction.

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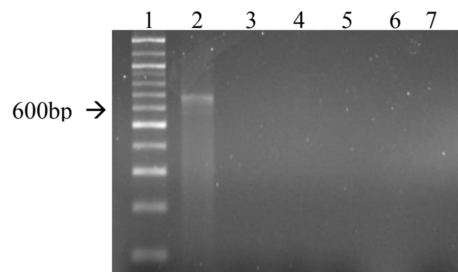

Figure 4.11, continued. e) PCR amplification of One-step RT-PCR product yielded a ~650 bp for only one of the replicate samples. Lane 1 is the 100bp ladder, Lane 2 and 3: Sample with RT, Lane 4 & 5: Samples with Taq, Lane 6: Empty lane & Lane 7: Negative control**.**

4.3.3 DNA purification using QIAquick Gel Extraction Kit

The amplification products of *NBS-LRR R* gene fragment from Berangan, Grand Naine, Malaccensis and Lemak Manis were purified using QIAquick Gel Extraction Kit (Qiagen, USA). Purification procedures yielded a band of 650bp long (Figure 4.12) for all banana varieties. However, the intensity of purified nucleic acid obtained for a replicate sample of Malaccensis (lane 24) and two replicate samples of Lemak manis (Lanes 26 and 27) decreased after the purification procedure (Figure 4.12).

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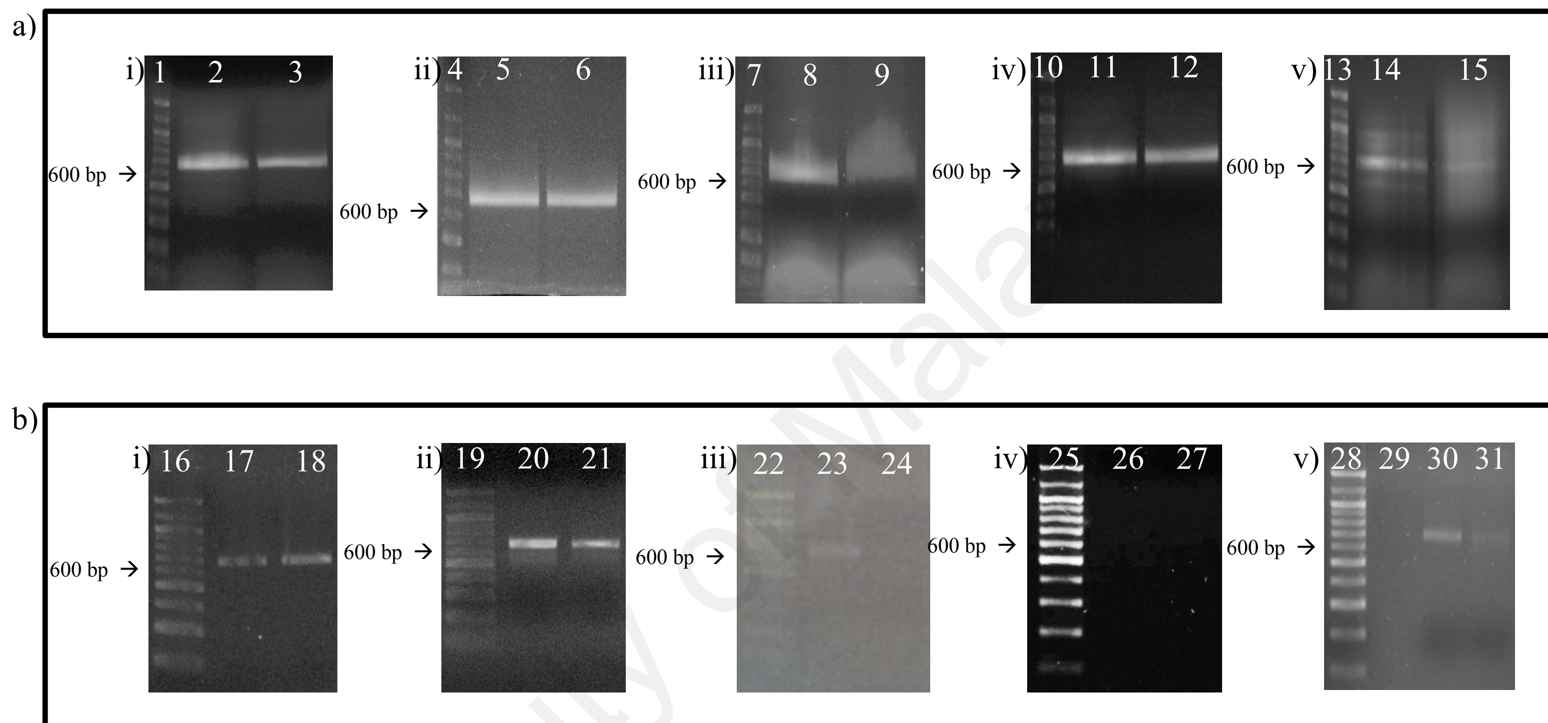

Figure 4.12: Agarose Gel Electrophoresis for Pre- and Post- gel extraction of the nucleic acid. Pictures of a) agarose gel prior gel extraction for samples i) Berangan, ii) Grand naine, iii) Malaccensis, iv) Lemak Manis and v) Grand naine for RNA. Panel b) is the result of purified nucleic acid for similar samples yielding a band of 650 bp long. Lane 1 & 16: 100 bp DNA ladder, Lane 2 &17: B1, Lane 3 & 18: B2, Lane 4 & 19: 100 bp DNA ladder, Lane 5 &20: GN1, Lane 6 & 21: GN2, Lane 7 & 22: 100 bp DNA ladder, Lane 8 & 23: M1, Lane 9 & 24: M2, Lane 10 & 25: 100bp DNA ladder, Lane 11 & 26: LM1, Lane 12 & 27: LM2, Lane 13 & 28: 100 bp DNA ladder, Lane 14 & 30: GNt1, Lane 15 & 31: GNt2. Lane 29: Empty lane.

4.3.4 T-A Cloning

4.3.4.1 Colony PCR & Restriction Enzyme Digestion

A total of 230 white colonies were screened for both of transcripts and genomic DNA studies. Agarose gel electrophoresis of colony PCR products amplified using a universal M13 primer pair showed positive insert of 900 bp band were obtained (Figure 4.13). PCR reactions with no DNA templates yielded no amplifications. When RE digestion assay was conducted on all the isolated plasmid, two bands of different sizes were obtained. The presence of a smaller band of 650 bp indicated the presence of insert while the bigger band of more than 1500bp signify the presence of linearised plasmids (Figure 4.14).

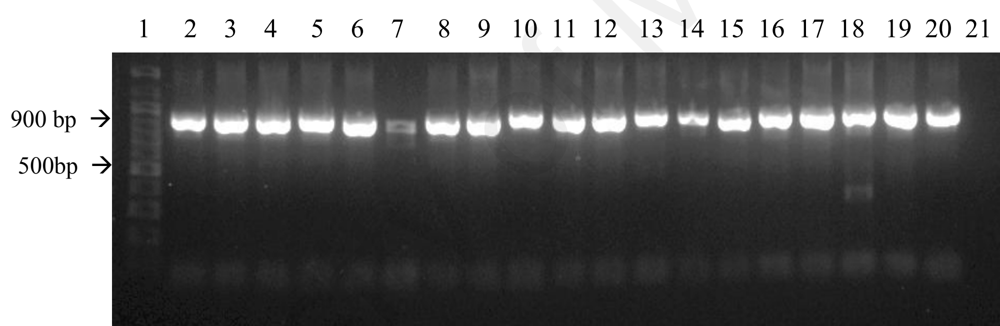

Figure 4.13: An example of colony PCR conducted on screened colonies. Lane 1: 100 bp DNA ladder; Lane 2-20: Clones of GN1 sample; Lane 21: -ve control.

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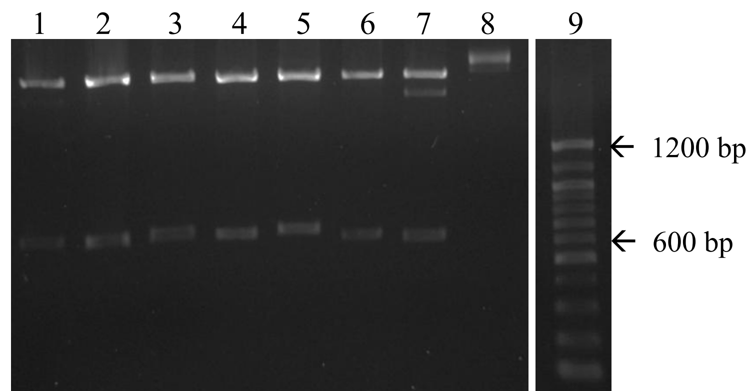

Figure 4.14: An example of AGE analysis on *Eco*RI-digested plasmids. Lane 1 to 4: Clones of Berangan 2 (B2) sample; Lane 5: Clone of Berangan 1 (B1) sample; Lane 8: -ve control; Lane 9: 100 bp DNA ladder.

4.3.5 Sequences analysis

A total of 73 *NBS-LRR Resistance* gene cloned sequences were successfully obtained in this study. BLASTn analysis tabulated in Table 4.5 and Table 4.6 confirmed the identity of the cloned sequences as *Musa Resistance* gene with E-value ranging from 0.00 to $2e^{-14}$. However, two out of the 73 clones namely GNtB2 and GNAB2 (isolated from Grand naine) showed similarity with RGA2-like (LOC105042261) disease protein of *Elaeis guineensis***.** Interestingly, the isolated clones showed high similarity with three Resistance Gene Analogues (RGA) present in Malaccensis; a Malaysian wild banana variety. The RGAs were RGA1 (LOC103988725, LOC103990112, LOC103996786), RGA3 (LOC103983322) and RGA4 (LOC103996787). When the cloned sequences were analysed, 73% of RNA transcripts (n=19) and 69% of genomic DNA (n=54) showed uninterrupted open reading frames (ORFs). These ORFs encode the expression of *NBS-LRR R* gene. On the other hand, the remainder five transcripts and 17 genomic DNA contained a premature stop codon that would putatively result in the occurrence of pseudogenes.

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The alignment of the putative pseudogenes and the occurrence of the premature stop codon were illustrated in Figure 4.15.

Table 4.5: BLASTn result of the isolated RNA transcripts showing the identity and similarity with sequences in the GenBank

Clone	Closest hit	Query cover (%)	E-value	Identity (%)
GNt1A6	*Musa acuminata* subsp. Siamea partial nbs pseudogene, cultivar Khae Phrae, clone N213_SM	100	$7e^{-137}$	98
GNt2D4	Predicted: *Musa acuminata* subsp. Malaccensis putative disease resistance protein RGA1 (LOC103988725)	95	$9e^{-144}$	99
GNt1B3	Predicted: *Musa acuminata* subsp. Malaccensis putative disease resistance protein RGA1 (LOC103988725)	97	$3e^{-147}$	99
GNt1E1	Predicted: *Musa acuminata* subsp. Malaccensis putative disease resistance protein RGA1 (LOC103988725)	95	$3e^{-140}$	99
GNt2F6	Predicted: *Musa acuminata* subsp. Malaccensis putative disease resistance protein RGA1 (LOC103988725)	95	$5e^{-142}$	99
GNt2C6	Predicted: *Musa acuminata* subsp. Malaccensis putative disease resistance protein RGA1 (LOC103988725)	98	$3e^{-143}$	98
GNt1B5	Predicted: *Musa acuminata* subsp. Malaccensis putative disease resistance protein RGA1 (LOC103990112), mRNA	97	$5e^{-142}$	99
GNt1C1	Predicted: *Musa acuminata* subsp. Malaccensis putative disease resistance protein RGA1 (LOC103990112), mRNA	95	$1e^{-138}$	99
GNt1D1	Predicted: *Musa acuminata* subsp. Malaccensis putative disease resistance protein RGA1 (LOC103990112), mRNA	99	$3e^{-143}$	99
GNtA3	Predicted: *Musa acuminata* subsp. Malaccensis putative disease resistance protein RGA1 (LOC103990112), mRNA	97	$8e^{-144}$	99
GNtB2	Elaeis guineensis disease protein RGA2-like (LOC105042261), mRNA	79	$2e^{-12}$	73
GNt2E1	Predicted: *Musa acuminata* subsp. Malaccensis putative disease resistance protein RGA1 (LOC103990112), mRNA	100	$1e^{-138}$	98
GNt1F1	Predicted: *Musa acuminata* subsp. Malaccensis uncharacterized LOC103979581 (LOC103979581), mRNA	94	$7e^{-137}$	100

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Table 4.5, continued.

Clone	Closest hit	Query cover (%)	E-value	Identity (%)
GNt2F3	Predicted: *Musa acuminata* subsp. Malaccensis putative disease resistance protein RGA4 (LOC103996787), transcript variant X2, mRNA	96	$3e^{-140}$	99

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Table 4.6: BLASTn result of the isolated genomic DNA showing the identity and similarity with sequences in the GenBank.

Clone	Closest hit	Query cover (%)	E-value	Identity (%)
GNA_D1	Predicted: *Musa acuminata* subsp. Malaccensis putative disease resistance protein RGA1 (LOC103988725)	95	$5e^{-142}$	99
GNA_C5	Predicted: *Musa acuminata* subsp. Malaccensis putative disease resistance protein RGA1 (LOC103990112), mRNA	100	$2e^{-145}$	99
GN1_E4	Predicted: *Musa acuminata* subsp. Malaccensis putative disease resistance protein RGA1 (LOC103996786), mRNA	95	$8e^{-137}$	98
GN1_B2	Predicted: *Musa acuminata* subsp. Malaccensis putative disease resistance protein RGA1 (LOC103990112), mRNA	99	$5e^{-138}$	98
GN2_C2	Predicted: *Musa acuminata* subsp. Malaccensis putative disease resistance protein RGA1 (LOC103996786), mRNA	95	$8e^{-137}$	98
GN2_A5	Predicted: *Musa acuminata* subsp. Malaccensis putative disease resistance protein RGA1 (LOC103996786), mRNA	95	$3e^{-140}$	99
B1_C1	Predicted: *Musa acuminata* subsp. Malaccensis putative disease resistance protein RGA1 (LOC103996786), mRNA	95	$1e^{-138}$	99
B1_D1	Predicted: *Musa acuminata* subsp. Malaccensis putative disease resistance protein RGA1 (LOC103996786), mRNA	95	$5e^{-142}$	99
B1_A2	Predicted: *Musa acuminata* subsp. Malaccensis putative disease resistance protein RGA1 (LOC103996786), mRNA	98	$3e^{-143}$	99
B2_E1	Predicted: *Musa acuminata* subsp. Malaccensis putative disease resistance protein RGA1 (LOC103990112), mRNA	95	$5e^{-142}$	100
B2_D5	*Musa acuminata* subsp. Siamea partial nbs pseudogene, cultivar Khae Phrae, clone N213_SM	93	$4e^{-135}$	100
B2_C2	Predicted: *Musa acuminata* subsp. Malaccensis putative disease resistance protein RGA4 (LOC103996787), transcript variant X2, mRNA	95	$9e^{-144}$	99
B2_F3	*Musa* AAB group nbs gene for NBS-LRR disease resistance protein, cultivar Pisang Ceylan, clone N321_MY	94	$3e^{-136}$	100

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Table 4.6, continued.

Clone	Closest hit	Query cover (%)	E-value	Identity (%)
B2_D2	Predicted: *Musa acuminata* subsp. Malaccensis putative disease resistance protein RGA4 (LOC103996787), transcript variant X2, mRNA	98	$3e^{-143}$	99
B2_D4	Predicted: *Musa acuminata* subsp. Malaccensis putative disease resistance protein RGA1 (LOC103988725)	98	$6e^{-145}$	99
M1_A2	Predicted: *Musa acuminata* subsp. Malaccensis putative disease resistance protein RGA4 (LOC103996787), transcript variant X2, mRNA	100	$5e^{-142}$	98
M1_A3	Predicted: *Musa acuminata* subsp. Malaccensis putative disease resistance protein RGA1 (LOC103990112), mRNA	99	$5e^{-138}$	98
GNA_A2	Predicted: *Musa acuminata* subsp. Malaccensis putative disease resistance protein RGA1 (LOC103996786), mRNA	95	$5e^{-142}$	99
GNA_A5	Predicted: *Musa acuminata* subsp. Malaccensis putative disease resistance protein RGA1 (LOC103990112), mRNA	98	$3e^{-140}$	99
GNA_B1	Predicted: *Musa acuminata* subsp. Malaccensis putative disease resistance protein RGA1 (LOC103988725)	98	$2e^{-145}$	99
GNA_B5	Predicted: *Musa acuminata* subsp. Malaccensis putative disease resistance protein RGA1 (LOC103988725)	99	$3e^{-143}$	98
GN_AC3	Predicted: *Musa acuminata* subsp. Malaccensis uncharacterized LOC103979581 (LOC103979581), mRNA	97	$1e^{-131}$	98
GNA_E5	*Musa* ABB group isolate YX-03 resistance protein gene, partial cds	100	$3e^{-140}$	98
GNA_A3	Predicted: *Musa acuminata* subsp. Malaccensis uncharacterized LOC103979581 (LOC103979581), mRNA	98	$7e^{-126}$	96
GNA_A6	Predicted: *Musa acuminata* subsp. Malaccensis uncharacterized LOC103979581 (LOC103979581), mRNA	93	$1e^{-116}$	96
GNA_B2	Predicted: Elaeis guineensis disease resistance protein RGA2-like (LOC105042261), mRNA	79	$4e^{-14}$	74

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Table 4.6, continued.

Clone	Closest hit	Query cover (%)	E-value	Identity (%)
GNA_C6	Predicted: *Musa acuminata* subsp. Malaccensis putative disease resistance protein RGA1 (LOC103996786), mRNA	96	$1e^{-138}$	99
GNA_D6	*Musa acuminata* subsp. Malaccensis partial nbs gene for NBS-LRR disease resistance protein, clone N272_ML	98	$5e^{-105}$	92
GNA_E1	Predicted: *Musa acuminata* subsp. Malaccensis putative disease resistance protein RGA3 (LOC103983322), mRNA	98	$3e^{-147}$	98
GNA_F1	Predicted: *Musa acuminata* subsp. Malaccensis putative disease resistance protein RGA3 (LOC103983322), mRNA	97	$2e^{-145}$	98

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```
#GNtB2   LLVLDDVWNE NGM---IWED LKVLLRCGKQ GSKIITTTRS ETVARIMGTV TLHKMPMLSF  [ 60]
#GNt1F1 LLVLDDVWNE DGL---KWER FCASLRYGEQ GSKILVTTRS KKIAEMVG-- KPIPLGGLDE  [ 60]
#GNt1A6 LLVFDHVWSE DSL---KWER FCAPLKYGEP GSKILVTTRS KKIAEMVG-- NPIPLGGLDE  [ 60]
#GNt1B3 FIVLDDVWSV DTHGLDEWQK LCTPLRFGAQ GSMVMVTTRD LRIASIVGTM KEILLDGLED  [ 60]
#GNt1C5 SLYWMMFGMK QEA---NGNN SATPWPPGPE EAPYW*
#GNt1E3 ----CLYWMT SGMT----RG IIGYLRWKDG ISF*
#GNtA6   CLYWMTCGAK TGM---TGKG CAHH*
#GNt1F6 PCFRSRVERK QG*
#GNt2F5 *
#GN_AC4 -------RPS RAP---GRAP CIRHPVYSER SACQLIRQKS GSQYPLNSFY PQIWIG*
#LM_C5   -SSR*
#GN1_B6 FIVLDDVWDE TGS---KWEQ LRDALASGAR GSTILVTTQS PLVAETMGTM EPIKLEVLGQ  [ 60]
#GN1_C3 FLVLDDVWSE DSL---KWER FCAPLRYGEP GSKILVTTRS KKIAEMVGNP IPLG----GL  [ 60]
#GN2_B6 LLVLDDVWDE TGS---KWKQ LRDALVSGAR GSTILVTTQS PLVAETMGTM EPIKLEVLGQ  [ 60]
#GN2_C3 LLVLDDVWDE TGS---KWKQ LRDALVSGAR GSTILVTTQS PLVAETMGTM EPIKLEVLGQ  [ 60]
#GN2_A4 FLVLDDVWDE TGS---KWEQ LRDALASGAR GSTILVTTQS PLVAEIMGTM EPIKLEVLGQ  [ 60]
#GN2_A6 FIVLDDVWDE TGS---KWEQ LRDALASGAR GSTILVTTQS PLVAETMGTM EPIKLEVLGQ  [ 60]
#GN2_B2 FIVLDHVWDE TGS---KWEQ LRDALASGAR GSTILVTTQS PLVAETMGTM EPIKLEVLGQ  [ 60]
#M1_A6   LLVLDDVWSE NRD---DWEK LCAPLRFAAR GSKVIVTTRD TKIASIIGTM KEISLDGLQD  [ 60]
#M1_B3   FLVLDHVWSV DTHGLDEWQK LCAPLRFGAQ GSMVMVTTRD LRIASIVGTM KEILLDGLED  [ 60]
#M1_A1   LIVLDDVWDE TGS---KWEQ LRDALASGAR GSTILVTTQS PLVAETMGTM EPIKLEVLGQ  [ 60]
#M1_B5   LLVLDDVWSV DTHGLDEWQK LCAPLRFGAQ GSMVMVTTRD LRIASIVGTM KEILLDGLED  [ 60]
#M1_C3   FIVFDDVWDE NSG---NWDR FCAPLRSGVP GSKILVTTRS GNIAEMVGNP IPLGVLDEA-  [ 60]
#M1_A4   LLVLDDVWSE NRD---DWEK LCAPLRFAAR GSKVIVTTRD TKIASIIGTM KEISLDGLQD  [ 60]

#GNtB2   EHCWLL---- FEQRAFR--L VREEEKPRFV EIGKQIVEKC GGLPLAAKTI GSLMGSKKKE  [120]
#GNt1F1 ASYWEF---- FKKCAFG--S EDAGEFPQLE AIAKKIAGRL KGLRLAARTV GGLLKAQMNE  [120]
#GNt1A6 TSYWKL---- FKKCAFG--S EDAGEFPHLE AIAKMIAGRL KGLPLAARTV GGLLKAQMNE  [120]
#GNt1B3 DDYWEL---- FKKCAFG--S LNPEEHPELE AIGRKIAGKL KGSPLAAKTI GSLLRSNANK  [120]
#GN1_B6 DDFWRL---- FERCAFGDKV PDPDLARKLE LIGREISGKL HGLPLAGKAM GSLLRRRLEE  [120]
#GN1_C3 DEASYC---- SRNVHLVPKT PVNFHI*
#GN2_B6 DDFWRL---- FERCAFGDKV LDPDLARKLE LIGREISGKL HGLPLAGKAM GSLLRRRLEE  [120]
#GN2_C3 DDFWRL---- FERCAFGDKV LDPDLARKLE LIGREISGKL HGLPLAGKAM GSLLRRRLEE  [120]
#GN2_A4 DDFWRL---- FERCAFGDKV PDPDLARKLE LIGREISGKL HGLPLAGKAM GSLLRRRLEE  [120]
#GN2_A6 DDFWRL---- FERCAFGDKV PDPDLARKLE LIGREISGKL HGLPLAGKRW EAC*
#GN2_B2 DDFWRL---- FERCAFGDKV LDPDLARKLE LIGREISGKL HGLPLAGKAM GSLLRRRLEE  [120]
#M1_A6   DAYWEL---- FKKCAFG--P VNPQEHLELE VIGRKIAGKL KG-SPLAENT RKLVAVGCEP  [120]
#M1_B3   DDYWEL---- FKKCAFG--S LNPEEHPELE AIGRKIAGKL KGSPLAAKTI GSLLRSNANK  [120]
#M1_A1   DDFWRL---- FERCAFGDKV SDPDLARKLE LIGREISGKL HGLPLAGRRW EAC*
#M1_B5   DDYWEL---- FKKCAFG--S LNPEEHPELE AIGRKIAGKL KGSPLAAKTI GSLLRSKRTR  [120]
#M1_C3   -SYWKL---- FKKCAFG--S EYAGECPQLE DIAKKIVSRL KGLPLAARML GGLLKEGIEG  [120]
#M1_A4   DAYWEL---- FKKCAFGPVN PQEHLELEVI G--RKIAGKL KGSPLAAKTL GSLLRSM*

#GNtB2   VDQWLAISES ELWRLPEDEN G----VLPAL MLSYNHLPSY LKSCFAYCSI FPKDYEIERM  [180]
#GNt1F1 K-HWRNIAGS EIWQLQHDEN G----VLPVL QLSYQCLPPH LKRCFVFCSL FPKDNRFDGE  [180]
#GNt1A6 K-HWRNIAGS EIWQLPQDEK G----VLPVL QLSYQCLPSH LKRCFVFCSM FPKDHPFNKR  [180]
#GNt1B3 G-YWRTTMES EVWELPQDEN G----VLSVL RLSYRYLPGH LKQCFTFCSL FPKAHEFYQD  [180]
#GN1_B6 Q-FWTTISES EWWEDDFVVE N----ILPSL GLSYQHLSTN LKQCFAYTSI FPKGHVFDKE  [180]
#GN2_B6 Q-FWTTISES EWWEDDFAVE N----ILPSL GLSYQHLSTN LKQCFAYTSY SRRAMCSIKN  [180]
#GN2_C3 Q-FWTTSRKA SGGRTTSPWK T----SFHLW V*
#GN2_A4 Q-FWTTISES EWWEDDFAVE N----ILPSL GLSYQHLSTN LKQCFAYTSI FPKGHVFEKN  [180]
#GN2_B2 Q-FWTTISES EWWEDDFAVE N----ILPSL GRVIST*
#M1_A6   RTLENYNGK*
#M1_B3   G-YWRTTMES EVWELPQDEN G----VLSVL RLSYRYLPGH LKQCFTFCSL FPKAHEFYQD  [180]
#M1_B5   D-TGELLWRV KYG?YHKMRM A----FCLSY G*
#M1_C3   --LEKHRRK*
```

Figure 4.15: Alignment of the truncated *Resistance* gene sequences. Highlighted in red is the premature stop codon on the sequence whereas highlighted in yellow were reference clones with uninterrupted open reading frame sequences.

```
#GNtB2   ILIQLWNAEG FIEKND---C SMLAEAVGNQ YFNDLVWRSL FEVTEKDEYD NIVKCKMHDI  [240]
#GNt1F1 HLVQLWMAEG YVDQDNMK-D NMTLEAVGSD YFHELVNRSF FQEAPWG--- --STYVMHDL  [240]
#GNt1A6 ELSWLWMAEG YVAQD----N NMTTEDTGSR YFLELVNRSF FQEAPWG--- --SQYVMHDM  [240]
#GNt1B3 QLIQIWMAEG YITPEE---- NKTVEEVGRS YVCELVNHSF FQASADG--- --DYYVMHDI  [240]
#GN1_B6 PISPMWIAQG FIHPKS---- ---EGKNETG GLGESDV*
#GN2_B6 D*
#GN2_A4 D*
#M1_B3  QLIQIWMAEG YITPEE---- NKTVET*

#GNtB2  VHDLA [245]
#GNt1F1 VHDLA [245]
#GNt1A6 VHDLA [245]
#GNt1B3 VHDLA [245]
```

Figure 4.15, continued.

4.3.6 Multiple sequence alignment of the isolated *R* genes and other published *R* genes

The analysis of Kinase-2 motif C-terminal end that ends with tryptophan (W) confirmed the grouping of the transcripts to be non-TIR whereas the end amino acid for TIR is aspartic acid (D). The alignment of the uninterrupted ORFs has identified three conserved motifs (Figure 4.16) pictured as grey boxes in the sequence alignment (P-loop, Kinase 2 and RNBS-B). Interestingly, two clones namely GN_AE1 and GN_AF1 have extra amino acid (NNRLSEM) at position 23-29 similar with RGA3 reference sequence.

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```
#MEGA
!Title fasta file;
!Format
   DataType=Protein
   NSeqs=123 NSites=104
   Identical=. Missing=? Indel=-;


!Domain=Data;
#GNt1A6                    LLVFDHVWSE DS-------L KWERFC-APL KYGEPGSKIL VTTRSKKIAE MVG--NPIPL GGLDETSYWK LF-KKCAFGS  [ 80]
#GNt2D4                    LLVLDDVWSV DTHG----LD EWQKLC-TPL RFGAQGSMVM VTTRDLRIAS IVGTMKEILL DGLEDDDYWE LF-KKCAFGS  [ 80]
#GNt1B3                    FIVLDDVWSV DTHG----LD EWQKLC-TPL RFGAQGSMVM VTTRDLRIAS IVGTMKEILL DGLEDDDYWE LF-KKCAFGS  [ 80]
#GNt1E1                    LLVFDDVWSV DTHG----LD EWQKLC-TPL RFGAQGSMVM VTTRDLRIAS IVGTMKEILL DGLEDDDYWE LF-KKCAFGS  [ 80]
#GNt2F6                    LIVLDDVWSV DTHG----LD EWQKLC-TPL RFGAQGSMVM VTTRDLRIAS IVGTMKEILL DGLEDDDYWE LF-KKCAFGS  [ 80]
#GNt2C6                    LIVLDDVWSV DTHG----LD EWQKLC-TPL RFGAQGSMVM VTTRDLRIAS IVGTMKEILL DGLEDDDYWE LF-KKCAFGS  [ 80]
#GNt1B5                    LLVLDDVWSE NR-------D DWERLC-APL RFAARGSKVI VTTRDTKIAS IIGTMKEISL DGLQDDAYWE LF-KKCAFGS  [ 80]
#GNt1C1                    LIVLDHVWSE NR-------D DWERLC-APL RFAARGSKVI VTTRDTKIAS IIGTMKEISL DGLQDDAYWE LF-KKCAFGS  [ 80]
#GNt1D1                    LLVLDDVWSE NR-------D DWERLC-APL RFAARGSKVI VTTRDTKIAS IIGTMKEISL DGLQDDAYWE LF-KKCAFGS  [ 80]
#GNtA3                     LLVLDDVWSE NR-------D DWERLC-APL RFAARGSKVI VTTRDTKIAS IIGTMKEISL DGLQDDAYWE LF-KKCAFGS  [ 80]
#GNtB2                     LLVLDDVWNE NG-------M IWEDLK-VLL RCGKQGSKII TTTRSETVAR IMGTVTLHKM PMLSFEHCWL LF-EQRAFR-  [ 80]
#GNt2E1                    LLVLDDVWSE NK-------D DWERLC-APL RFAARGSKVI VTTRDTKIAS IIGTMKEISL DGLQDDAYWE LF-KKCAFGS  [ 80]
#GNt1F1                    LLVLDDVWNE DG-------L KWERFC-ASL RYGEQGSKIL VTTRSKKIAE MVG--KPIPL GGLDEASYWE FF-KKCAFGS  [ 80]
#GNt2F3                    LLVLDHVWDE TG-------T TWKELR-SAL TFGAKGSTIL LTTQSPKVAE IMGTMNPIHL EPLEEHDFRR LF-ELCAFGD  [ 80]
#GN_AD1                    FIVFDDVWSV DTHG----LD EWQKLC-TPL RFGAQGSMVM VTTRDLRIAS IVGTMKEILL DGLEDDDYWE LF-KKCAFGS  [ 80]
#GN_AC5                    LLVFDDVWSE NR-------D DWERLC-APL RFAARGSKVI VTTRDTKIAS IIGTMKEISL DGLQDDAYWE LF-KKCAFGS  [ 80]
#GN1_E4                    LLVLDDVWDE TG-------S KWKQLR-DAL VSGARGSTIL VTTQSPLVAE TMGTMEPIKL EVLGQDDFWR LF-ERCAFGD  [ 80]
#GN1_B2                    LLVFDHVWSE NR-------D DWERLC-APL RFAARGSKVI VTTRDTKIAS IIGTMKEISL DGLQDDAYWE LF-KKCAFGS  [ 80]
#GN2_C2                    FLVLDDVWDE TG-------S KWEQLR-DAL ASGARGSTIL VTTRSPLVAE IMGTMEPIKL EVLGQDDFWR LF-ERCAFGD  [ 80]
#GN2_A5                    FLVLDDVWDE TG-------S KWEQLR-DAL ASGARGSTIL VTTQSPLVAE TMGTMEPIKL EVLGQDDFWR LF-ERCAFGD  [ 80]
#B1_C1                     LIVFDDVWDE TG-------S KWKQLR-DAL VSGARGSTIL VTTQSPLVAE TMGTMEPIKL EVLGQDDFWR LF-ERCAFGD  [ 80]
#B1_D1                     LLVLDDVWDE TG-------S KWEQLR-DAL ASGARGSTIL VTTQSPLVAE TMGTMEPIKL EVLGQDDFWR LF-ERCAFGD  [ 80]
#B1_A2                     FLVLDDVWDE TG-------S KWEQLR-DAL ASGARGSTIL VTTQSPLVAE TMGTMEPIKL EVLGQDDFWR LF-ERCAFGD  [ 80]
#B2_E1                     FIVLDDVWSE NR-------D DWERLC-APL RFAARGSKVI VTTRDTKIAS IIGTMKEISL DGLQDDAYWE LF-KKCAFGS  [ 80]
#B2_D5                     LLVFDDVWSE DS-------L KWERFC-APL KYGEPGSKIL VTTRSKKIAE MVG--NPIPL GGLDETSYWK LF-KKCAFGS  [ 80]
#B2_C2                     FLVFDDVWDE TG-------T TWKELR-SAL TFGAKGSTIL LTTQSPKVAE IMGTMNPIHL EPLEEHDFRR LF-ELCAFGD  [ 80]
#B2_F3                     LIVLDHVWDE NR-------Q NWDRFR-APL GYGVPGSKIL VTTRSEKIAE MVG--NRIHL GGLDEASYWK LF-KKCAFGS  [ 80]
#B2_D2                     FLVLDHVWDE TG-------T TWKELR-SAL TFGAKGSTIL LTTQSPKVAE IMGTMNPIHL EPLEEHDFRR LF-ELCAFGD  [ 80]
#B2_D4                     FIVFDDVWSV DTHG----LD EWPKLC-APL RFGAQGSMVM VTTRDLRIAS IGGTMKEILL DGLEDDDYWE LF-KKCAFGS  [ 80]
#M1_A2                     LLVLDHVWDE TG-------T TWKELR-SAL TFGAKGSTIL LTTQSPKVAE IMGTMNPIHL EPLEEHDFRR LF-ELCAFGD  [ 80]
#M1_A3                     LLVLDDVWSE NR-------D DWEKLC-APL RFAARGSKVI VTTRDTKIAS IIGTMKEISL DGLQDDAYWE LF-KKCAFGP  [ 80]
#GN_AA2                    LLVLDDVWDE TG-------S KWEQLR-DAL ASGARGSTIL VTTQSPLVAE TMGTMEPIKL EVLGQDDFWR LF-ERCAFGD  [ 80]
```

Figure 4.16: Amino acid alignment of the non-truncated *Resistance* gene sequences. The grey boxes represent the conserved motifs according to appearance (P-loop, kinase 2 and RNBS-B) according to Peraza *et al*. (2009) present along the *NBS-LRR Resistance* sequence.

```
#GN_AA5                    FLVLDDVWSE NR-------D DWERPC-APL RFAARGSKVI VTTRDTKIAS IIGTMKEISH DGLQDDAYWE LF-KKCAFGS  [ 80]
#GN_AB1                    LLVLDDVWSV DTHG----LD EWQKLC-APL GFGAQGSMVM VTTRDLRIAS IVGTMKEILL DGLEDDDYWE LF-KKCAFGS  [ 80]
#GN_AB5                    LLVLDDVWSA DTHG----LD EWQKLC-TPL RFGAQGSMVM VTTRDLRIAS IVGTMKEILL DGLEDDDYWE LF-KKCAFGS  [ 80]
#GN_AC3                    -IVLDDVWDE NS-------G NWDRFC-APL RSGVPGSKIL VTTRSGNIAE MVG--NPIPL GVLDEASYWK LF-KKCAFGS  [ 80]
#GN_AE3                    FLVLDDVWDE TG-------S KWKRLR-DAL VSGARGSTIL VTTQSPLVAE TMGTMEPIKL EVLGQDDFWR LF-ERCAFGD  [ 80]
#GN_AE5                    FLVLDDVWDA QI-------- -WDDLLRNPL QGGAAGSRVL VTTRNAGIAR QMKAAHVHEM KLLPPEDGWS LLCKKATMNA  [ 80]
#GN_AA3                    LIVLDDVWDE NR-------Q NWDRFR-APL NSGVLGSKIL VTTRSRKIAE MVG--NPIPL GVLDDASYWE FF-KQCAFSS  [ 80]
#GN_AA6                    FIVLDHVWDE NR-------Q NWDRFR-APL NSGVLGSKIL VTTRSRKIAE MVG--NPIPL GVLDDASYWE FF-KQCAFSS  [ 80]
#GN_AB2                    LLVLDDVWNE NE-------M IWEDLK-VLL RCGKQGSKII TTTRSETVAR IMGTVTLHKM PMLSFEHCWL LF-EQRAFR-  [ 80]
#GN_AC6                    -IVLDDVWDE TG-------S KWKQLR-DAL VSGARGSTIL VTTQSPLVAE TMGTMEPIKL EVLGQDDFWR LF-ERCAFGD  [ 80]
#GN_AD6                    LIVLDDVWNE DS-------K KWTTFR-APL WYGVSGSKIL VTTRSKNIAD MVG--NPIPL GVLDEASYWK LF-KKYAFGS  [ 80]
#GN_AE1                    LIVLDDVWND ERNNRLSEME RWDKLL-APL KAGKSGSKIL VTTRSGTVSE TLGTMHSIDL KGLRDQDCWS LI-KEHTFRD  [ 80]
#GN_AF1                    LLVLDDVWND ERNNRLSEME RWDKLL-APL KAGKSGSKIL VTTRSGTVSE TLGTMHSIDL KGLRDQDCWS LI-KEHTFRD  [ 80]
#LM_A1                     LLVLDHVWNE KP-------S LWELLK-VPL LDAGVG-KVI VTTRNECVAR IMQTMEPLSL NILSFDKCWM LF-EKLALLE  [ 80]
#LM_A2                     LLVLDDVWSE NR-------D DWEKLC-APL RFAARGSKVI VTTRDTKIAS IIGTMKEISL DGLQDDAYWE LF-KKCAFGP  [ 80]
#LM_A3                     LLVLDDVWSV DTHG----LD EWQKLC-APL RFGAQGSMVM VTTRDLRIAS IVGTMKEILL DGLEDDDYWE LF-KKCAFGS  [ 80]
#LM_A5                     LIVLDDVWSE NR-------D DWERLC-APL RFAARGSKVI VTTRDTKIAS IIGTMKEISL DGLQDDAYWE LF-KKCAFGS  [ 80]
#LM_B1                     LIVLYDVWDE TG-------S KWEQLR-DAL ASGARGSTIL VTTQSPLVAE TMGTMEPIKL EVLGQDDFWR LF-ERCAFGD  [ 80]
#LM_B2                     LIVLDDVWSV DTHG----LD EWQKLC-APL RFGAQGSMVM VTTRDLRIAS IVGTMKEILL DGLEDDDYWE LF-KKCAFGS  [ 80]
#Malaccensis_RGA1          LFVLDDVWDE TG-------S KWEQLR-DAL ASGARGSTIL VTTQSPLVAE TMGTMEPIKL EVLGQDDFWR LF-ERCAFGD  [ 80]
#Musa_Acuminata(BR-4)      LLVLDDVWDE NR-------Q NWDRFR-APL GYGEPGSKIL VTTRSRKIAE MVG--NPFPL GVLDDASYWE FF-KQCAFSS  [ 80]
#Musa_Acuminata(BR-19)     LLVLDDVWNE DS-------L KWERFC-APL RSGVPGSKIL VTTRSRKIAE MVG--NPIPL YGLDNASYWE FF-KTCAFGS  [ 80]
#Musa_ABB_Pisang_Awak      LLVLDDVWNE DS-------L KWERFC-APL RYGVPGSKIL VTTRSEKIAE MVG--NRIHL GGLDEASYWK LF-KKCAFGS  [ 80]
#MA_Pisang_Berangan        LLVLDDVWNE DG-------L KWERFC-ASL RYGEQGSKIL VTTRSKKIAE MVG--KPIPL GGLDEASYWE FF-KKCAFGS  [ 80]
#MA_Giant_Cavendish        LLVLDDVWNE DS-------L KWERFC-APL RYGEPGSKIL VTTRSKKIAK MVG--NPIPL GGLAEASYWK LF-KKCAFGS  [ 80]
#MA_Pisang_Jari_Buaya      LLVLDDVWNE DS-------L KWERFC-APL RYGEPGSKIL VTTRSKKIAE MVG--NPIPL GGLDEASYWK LF-KKCAFGS  [ 80]
#MB_Klutuk_Wulung          LLVLDDVWNE DG-------L KWERFC-ASL RYGEQGSKIL VTTRSKKIAE MVG--KPIPL GGLDEASYWE FF-KQCAFGS  [ 80]
#MA_Pisang_Nangka          LLVLDDVWNE DS-------L KWERFC-APL RYGEPGSKIL VTTRSKKIAE MVG--NPIPL GGLDEASYWK LF-KKCAFGS  [ 80]
#Musa_AAB_Obino_lEwai      LLVLDDVWNE DS-------L KWERFC-APF RYGEPGSKIL VTTRSKKIAE MVG--NPIPL GGLDEASYWK LF-KKCAFGS  [ 80]
#MT                        LLVLDDVWNE ES-------L KWERFC-APL RSGVPGSKIL VTTRSRKIAD MVG--NPIPL DGLDEASCWK LF-KKCAFGS  [ 80]
#Musa_velutina             LLVLDDVWNE DS-------L KWGRFC-APL RYGEPGSKIL ITTRSKMIAE MVG--NPIPL GGLDETSYWK LF-KKCAFGS  [ 80]
#Pisang_Rastali            LLVLDDVWNE DS-------L KWERFC-APL RYGEPGSKIL VTTRSEKIAE MVG--NPIPL GGLDEASYWK LF-KKCAFGS  [ 80]
#Pisang_Batu               LLVLDDVWNE DS-------Q KWERFC-APL RYGEPGSKIL ITTRYKMIAE MVG--NPIPL GGLDEASYWK LF-KTCAFGS  [ 80]
#Pisang_Batu_1             LLVLDDVWNE DS-------Q KWERFC-APL RYGEPGSKIL ITTRYKMIAE MVG--NPIPL GGLDEASYWK LF-KTCAFGS  [ 80]
#Bluggoe                   LLVLDDVWNE DS-------L KWERFC-APL RYGVPGSKIL VTTRSEKIAE MVG--NRIHL GGLDEASYWK LF-KKCAFGS  [ 80]
#Agutay_clone_N202         LLVLDDVWNE DS-------L KWGRFC-APL RYGEPGGKIL VTTRSKKIAE MVG--NPFPL GGLDEASYWE FF-KKCAFGS  [ 80]
#Agutay_clone_N205         LLVLDDVWNE DS-------L KWERFC-APL RYGEPGSKIL VTTRSKKIAE MVG--NPIPL GGLDEASYWK LF-KECAFGS  [ 80]
#Khae_Phrae_clone_N212     LLVLDDVWNE DS-------L KWERFC-APL RYGVPGSKIL VTTRSEKIAE MVG--NRIHL GGLDEASYWK LF-KKCAFGS  [ 80]
#Paliama_clone_N224        LLVLDDVWDE DG-------L KWERFC-ASL RYGEQGSKIL VTTRSKKIAE MVG--KPIPL GGLDEASYWE FF-KKCAFGS  [ 80]
#Honduras_clone_N243       LLVLDDVWNE DG-------L KWERFC-ASL RYGEQGSKIL VTTRSKKIAE MVG--KPIPL GGLDEASYWE FF-KKCAFGS  [ 80]
#Honduras_clone_N244       LLVLDDVRNE DS-------R NWERFC-APL RYGEPGSKIL VTTRSKKIAE MVG--NPIPL RGLDETSYWK LF-KKCAFGS  [ 80]
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Figure 4.16, continued.

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#Saba_clone_N254              LLVLDDVWNE DS-------L KWERFC-APL RYGEPGSKIL VTTRSKKIAE MVG--NPIPL GGLDEASYWK LF-KKCAFGS  [ 80]
#Yawa_2_clone_N261            LLVLDDVWNE DS-------L KWERFC-APL RYGVPGSKIL VTTRSEKIAE MVG--NRIHL GGLDEASYWK LF-KKCAFGS  [ 80]
#Yawa_2_clone_N263            LLVLDDVWNE DS-------L KWERFC-APL RYGVPGSKIL VTTRSEKIAE MVG--NRIHL GGLDEASYWK LF-KKCAFGS  [ 80]
#Malaccensis_clone_N271       LLVLDDVWNE DS-------L KWERFC-APL RYGEPGSKIL ITTRSKMIAE MVG--NPIPL GGLDETSYWK LF-KKCAFGS  [ 80]
#Malaccensis_clone_N273       LLVLDDVWNE DS-------L KWERFC-APL RYGEPGSKIL VTTRSKKIAK MVG--NPIPL GGLDEASYWK LF-KKCAFGS  [ 80]
#Tiparot_clone_N291           LLVLDDVWNE DS-------L KWERFC-APL RYGEPGSKIL VTTRSKKITK MVG--NPIPL GGLDEASYWK LF-KKCAFGS  [ 80]
#Tiparot_clone_N295           LLVLDDVWNE NS-------R DWDRFC-APL RSGVPGSKIL VTTRPRKIAE MVG--NPIPL GVLDEASYWK LF-KKYAFGS  [ 80]
#Pisang_Bakar_clone_N311      LLVLDDVWNE DS-------L KWERFC-APL RYGGPGSKIL VTTRSKKIAE MVG--NPIPL GGLDEASYWK LF-KKCAFGS  [ 80]
#Pisang_Bakar_clone_N314      LLVLDDVWNE DS-------L KWERFC-APL RYGEPGSKIL VTTRSEKIAK MVG--NPVPL GGLAEASYWK LF-KKCAFGS  [ 80]
#Pisang_Ceylan_clone_N321     LLVLDDVWDE NR-------Q NWDRFR-APL GYGVPGSKIL VTTRSEKIAE MVG--NRIHL GGLDEASYWK LF-KKCAFGS  [ 80]
#Pisang_Ceylan_clone_N323     LLVLDDVWDE NR-------Q NWDRFR-APL GYGVPGSKIL VTTRSEKIAE MVG--NRIHL GGLDEASYWK LF-KKCAFGS  [ 80]
#Gaba-gaba_clone_N342         LLVLDDVWNE DS-------Q KWERFC-APL RYGEPGSKIL ITTRYKMIAE MVG--NPIPL GGLDEASYWK LF-KTCAFGS  [ 80]
#Gaba-gaba_clone_N343         LLVLDDVWNE DS-------Q KWERFC-APL RYGEPGSKIL ITTRYKMIAE MVG--NPIPL GGLDEASYWK LF-KTCAFGS  [ 80]
#Musa_schizocarpa_1           LLVLDDVWNE DS-------Q KWERFC-APL RYGEPGSKIL ITTRYKMIAE MVG--NPIPL GGLDEASYWK LF-KTCAFGS  [ 80]
#Musa_schizocarpa_2           LLVLDDVWNE DS-------Q KWERFC-APL RYGEPGSKIL TTTRYKMIAE MVG--NPIPL GGLDEASYWK LF-KTCAFGS  [ 80]
#Malaccensis_1                LLVLDDVWNE DS-------Q KWERFC-APL RYGEPGSKIL ITTRYKMIAE MVG--NPIPL GGLDEASYWK LF-KTCAFGS  [ 80]
#Malaccensis_2                LLVLDDVWNE DS-------Q KWERFC-APL RYGEPGSKIL ITTRYKMIAE MVG--NPIPL GGLDEASYWK LF-KTCAFGS  [ 80]
#Malaccensis_3                LLVLDDVWNE DS-------Q KWERFC-APL RYGEPGSKIL ITTRYKMIAE MVG--NPIPL GGLDEASYWK LF-KTCAFGS  [ 80]
#Malaccensis_4                LLVLDDVWNE DS-------L KWERFC-APL RYGEPGSKIL VTTRSKKIAE MVG--NPIPL GGLDEASYWK LF-KKCAFGS  [ 80]
#Malaccensis_5                LLVLDDVWNE DS-------L KWERFC-APL RYGEPGSKIL VTTRSKKIAE MVG--NPIPL GGLDEASYWK LF-KKCAFGS  [ 80]
#Malaccensis_6                LLVLDDVWNE DS-------L KWERFC-APL RYGEPGSKIL VTTRSKKIAE MVG--NPIPL GGLDEASYWK LF-KKCAFGS  [ 80]
#Malaccensis_7                LLVLDDVWNE DS-------L KWERFC-APL RYGEPGSKIL VTTRSKKIAE MVG--NPIPL GGLAEASYWK LF-KKCAFGS  [ 80]
#Malaccensis_8                LLVLDDVWNE DS-------L KWERFC-APL RYGVPGSKIL VTTRSEKIAE MVG--NRIHL GGLDEASYWK LF-KKCAFGS  [ 80]
#Malaccensis_9                LLVLDDVWNE DS-------L KWERFC-APL RYGEPGSKIL VTTRSKKIAE MVG--NPIPL GGLDEASYWK LF-KKCAFGS  [ 80]
#Microcarpa_1                 LLVLDDVWNE DS-------L KWERFC-APL RSVVPGSKIL VTTRSRKIAD MVG--NPIPL GVLDEASYWK LF-KKCAFGS  [ 80]
#Microcarpa_2                 LLVLDDVWNE DS-------L KWERFC-APL RYGVPGSKIL VTTRSEKIAE MVG--NRIHL GGLDEASYWK LF-KKCAFGS  [ 80]
#Pisang_Lilin_1               LLVLDDVWNE DS-------L KWERFC-APL RYGVPGSKIL VTTRSEKIAE MVG--NRIHL GGLDEASYWK LF-KKCAFGS  [ 80]
#Pisang_Lilin_2               LLVLDDVWNE DG-------L KWERFC-ASL RYGEQGSKIL VTTRSKKIAE MVG--KPIPL GGLDEASYWE FF-KKCAFGS  [ 80]
#Musa_schizocarpa_3           LLVLDDVWNE DS-------Q KWERFC-APL RYGEPGSKIL ITTRYKMIAE MVG--NPIPL GDLDEASYWE LF-KTCAFGS  [ 80]
#Musa_schizocarpa_4           LLVLDDVWNE DS-------L KWERFC-APL RYGEPGSKIL VTTRSKKIAE MVG--NPIPL GGMAGASYWK LF-KKCAFGS  [ 80]
#Musa_balbisiana_1            LLVLDDVWNE DS-------L KWERFC-APL RYGEQGSEIL VTTRSRKIAD MVG--NPIPL DGLDEASYWK LF-KKCAFGS  [ 80]
#Musa_balbisiana_2            LLVLDDVWNE DS-------L KWERFC-APL RYGEQGSEIL VTTRSRKIAD MVG--NPIPL DGLDEASYWK LF-KKCAFGS  [ 80]
#Musa_AAB                     LLVLDDVWNE DS-------L KWGRFC-APL RYGEPGSKIL ITTRSKMIAE MVG--NPIPL GGLDETSYWK LF-KKCAFGS  [ 80]
#Musa_ABB_Karthombiumtham_1   LLVLDDVWNE DS-------L KWERFC-APL RYGEQGSKIL VTTRSRKIAD MVG--NPIPL DGLDEASYWK LL-KKCAFGS  [ 80]
#Musa_ABB_Karthombiumtham_2   LLVLDDVWNE DS-------L KWERFC-APL RYGEQGSKIL VTTRSRKIAD MVG--NPIPL DGLDEASYWK LL-KKCAFGS  [ 80]
#Changpui                     LLVLDDVWNE DS-------L DWERFC-APL RYGEPGSKIL VTTRSKKIAE MVG--NPIPL GGLDEASYWK LF-KKCAFGS  [ 80]
#Changpawl                    LLVLDDVWNE DS-------L KWERFC-APL RYGEPGSKIL VTTRSKKIAE MVG--NPIPL GGLDEACYWK LF-KKCAFGS  [ 80]
#Banpawl                      LLVLDDVWNE DS-------L KWERFC-APL RYGEPGSKIL VTTRSKKIAE MVG--NPIPL GGLDDASYWK LF-KKCAFGS  [ 80]
#Balhlasen                    LLVLDDVWNE DS-------L KWERFC-APL RYGEPGSKIL VTTRSKKIAE MVG--NPIPL GGVDEASYWK LF-KKCAFGS  [ 80]
#Pisang_Mas                   LLVLDDVWSE DS-------L KWERFC-APL KYGEPGSKIL VTTRSKKIAE MVG--NPIPL GGLDETSYWK LF-KKCAFGS  [ 80]
#Pisang_Rastali_1             LLVLDDVWNE DS-------L KWERFC-APL RYGEPGGKIL ITTRYKMIAE MVG--NPIPL GGLDEASYWK LF-KKCAFGS  [ 80]
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Figure 4.16, continued

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#Musa_schizocarpa_5      LLVLDDVWNE DS-------L KWERFC-APL RYGEPGSKIL VTTRSKKIAE MVG--NPIPL GGMAEASYWK LF-KKCAFGS  [ 80]
#Musa_ABB                LLVLDDVWDA QI-------- -WDDLLRNPL QGGAAGSRVL VTTRNTGIAR QMKAGLVHEM KLLPPEDGWS LLCKKATMNA  [ 80]
#Musa_textilis           LIVLDDVWCA DV-------- -WENLLRKPV MNGVGSSKIV VTTRDAGIAR SMN-ACIYHV EQIDEESGWE LL-RKMALAD  [ 80]
#RGC3                    VVLLDDVWKK FQ-------- -LADVG-IPT PSSDNGWKLI LASRSNQVCV EMGDKEPMEM PCLGDNESLR LF-RSNLMAE  [ 80]
#RGA3                    LLVLDDVWND ERNNRLSEME RWDKLL-APL KAGKSGSKIL VTTRSGTVSE TLGTMHSIDL KGLRDQDCWS LI-KEHAFRD  [ 80]
#RGC5                    LLVLDDVWSE NR-------D DWERLC-APL RFAARGSKVI VTTRDTKIAS IIGTMKEISL DGLQDDAYWE LF-KKCAFGS  [ 80]
#Manihot_esculenta       LLVLDDVWNE NR-------E KWQNLK-RLL VGGSSGSKIL ITTRSKKVAD ISSTMAPHVL EGLSPDESWS LF-LHVALE-  [ 80]
#Setaria_italica         LLVLDDVWNE DR-------D KWLSYR-AAL LSGGFGSKIV VTSRNENVGR IMGGIEPYRL QQLSDDDSWS VF-KSHAFRD  [ 80]

#GNt1A6                  E--DAGEFPH LEAIAKMIAG RLKG [104]  #GN_AA5                     V--NPQEHLE LEVIGRKIAG KLKG [104]
#GNt2D4                  L--NPEEHPE LEAIGRKIAG KLKG [104]  #GN_AB1                     L--NPEEHPE LEAIGRKIAG KLKG [104]
#GNt1B3                  L--NPEEHPE LEAIGRKIAG KLKG [104]  #GN_AB5                     L--NPEEHPE LEAIGRKIAG KLKG [104]
#GNt1E1                  L--NPEEHPE LEAIGRKIAG KLKG [104]  #GN_AC3                     E--YAGECPQ LEDIAKKIVS RLKG [104]
#GNt2F6                  L--NPEEHPE LEAIGRKIAG KLKG [104]  #GN_AE3                     KVLDPDLARK LELIGREISG KLHG [104]
#GNt2C6                  L--NPEEHPE LEAIGRKIAG KLKG [104]  #GN_AE5                     D--EERDAQD LKDTGMKIVD KCGG [104]
#GNt1B5                  V--NPQEHLE LEVIGRKIAG KLKG [104]  #GN_AA3                     K--YNGEYPQ LEAIAKKIVS RLKG [104]
#GNt1C1                  V--NPQEHLE LEVIGRKIAG KLKG [104]  #GN_AA6                     K--YNGEYPQ LEAIAKKIVS RLKG [104]
#GNt1D1                  V--NPQEHLE LEVIGRKIAG KLKG [104]  #GN_AB2                     -LVREEEKPR FVEIGKQIVE KCGG [104]
#GNtA3                   V--NPQEHLE LEVIGRKIAG KLKG [104]  #GN_AC6                     KVLDPDLARK LELIGREISG KLHG [104]
#GNtB2                   -LVREEEKPR FVEIGKQIVE KCGG [104]  #GN_AD6                     E--YAGGCPQ LEDIAKKIVS RLKG [104]
#GNt2E1                  V--NPQEHLE LEVIGRKIAG KLKG [104]  #GN_AE1                     A--NHEEQLK LERIGSEVAQ QLKG [104]
#GNt1F1                  E--DAGEFPQ LEAIAKKIAG RLKG [104]  #GN_AF1                     A--NHEELLK LERIGSEVAQ QLKG [104]
#GNt2F3                  EELKPDLKAK LQLIGHKILQ KLHG [104]  #LM_A1                      GLDSSSRHND LVEIGRKIVE KCKG [104]
#GN_AD1                  L--NPEEHPE LEAIGRKIAG KLKG [104]  #LM_A2                      V--NPQEHLE LEVIGRKIAG KLKG [104]
#GN_AC5                  V--NPQEHLE LEVIGRKIAG KLKG [104]  #LM_A3                      L--NPEEHPE LEAIGRKIAG KLKG [104]
#GN1_E4                  KVLDPDLARK LELIGREISG KLHG [104]  #LM_A5                      V--NPQEHLE LEVIGRKIAG KLKG [104]
#GN1_B2                  V--NPQEHLE LEVIGRKIAG KLKG [104]  #LM_B1                      KVLDPDLARK LELIGRVISG KLHG [104]
#GN2_C2                  KVPDPDLARK LELIGREISG KLHG [104]  #LM_B2                      L--SPEEHPE LEAIGRKIAG KLKG [104]
#GN2_A5                  KVPDPDLARK LELIGREISG KLHG [104]  #Malaccensis_RGA1           KVLDPDLARK LELIGREISG KLHG [104]
#B1_C1                   KVLDPDLARK LELIGREISG KLHG [104]  #Musa_Acuminata(BR-4)       K--YNGEYPQ LEAIAKKIVS RLKG [104]
#B1_D1                   KVPDPDLARK LELIGREISG KLHG [104]  #Musa_Acuminata(BR-19)      E--YAGECPQ LEDIAKKIVY RLNG [104]
#B1_A2                   KVLDPDLARK LELIGREISG KLHG [104]  #Musa_ABB_Pisang_Awak       E--DAGEFPQ LEAIAKKIVG RLKG [104]
#B2_E1                   V--NPQEHLE LEVIGRKIAG KLKG [104]  #MA_Pisang_Berangan         E--DAGEFPQ LEAIAKKIAG RLKG [104]
#B2_D5                   E--DAGEFPH LEAIAKMIAG RLKG [104]  #MA_Giant_Cavendish         E--DAGEFPH LEAIAKKIAG RLKG [104]
#B2_C2                   EELKPDLKAK LQLIGQQILQ KLHG [104]  #MA_Pisang_Jari_Buaya       E--DAGEFPH LEAIAKKIAG RLKG [104]
#B2_F3                   E--DAGEFPQ LEAIAKKIVG RLKG [104]  #MB_Klutuk_Wulung           E--DAGEFPQ LEAIAKKIAG RLKG [104]
#B2_D2                   EELKPDLKAK LQLIGQQILQ KLHG [104]  #MA_Pisang_Nangka           E--DAGEFPQ LEAIAKKIAG RLKG [104]
#B2_D4                   L--NPEEHPE LEAIGRKIAG KLKG [104]  #Musa_AAB_Obino_lEwai       E--DAGEFPQ LEAIAKKIAG RLKG [104]
#M1_A2                   EELKPDLKAK LQLIGQQILQ KLHG [104]  #MT                         E--DAGEYPQ LEAIAKKIVG RLKG [104]
#M1_A3                   V--NPQEHLE LEVIGRKIAG KLKG [104]  #Musa_velutina              E--DAGEFPQ LEAIAKKIAG RLKA [104]
#GN_AA2                  KVPDPDLARK LELIGREISG KLHG [104]  #Pisang_Rastali             E--DAGEFPQ LEAIAKKIAG RLKG [104]
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Figure 4.16, continued.

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#Pisang_Batu                 E--DAGEFPH LEAIAKKIAG RLKA [104]
#Pisang_Batu_1               E--DAGEFPH LEAIAKKIAG RLKG [104]
#Bluggoe                     E--DAGEFPQ LEAIAKKIVG RLKG [104]
#Agutay_clone_N202           Q--DAGEFPQ LEAIAKKIAG RLKG [104]
#Agutay_clone_N205           E--DAGEFPQ LEAIAKKIAG RLKG [104]
#Khae_Phrae_clone_N212       E--DAGEFPQ LEAIAKKVVG RLKA [104]
#Paliama_clone_N224          E--DAGEFPQ LEAIAKKIAG RLKG [104]
#Honduras_clone_N243         E--DAGEFPQ LEAIAKKIAG RLKG [104]
#Honduras_clone_N244         E--DAGEFPQ LEAIAKKIAG RLKA [104]
#Saba_clone_N254             E--DAGEFPH LEAIAKKIAG RLKG [104]
#Yawa_2_clone_N261           E--DAGEFPQ LEAIAKKIVG RLKG [104]
#Yawa_2_clone_N263           E--DAGEFPQ LEAIAKKIVG RLKG [104]
#Malaccensis_clone_N271      E--DEGEFPQ LEAKAKKIAG RLKG [104]
#Malaccensis_clone_N273      E--DAGEFPH LEAIAKKIAG RLKG [104]
#Tiparot_clone_N291          E--DAGEFPH LEAIAKKIAG RLKG [104]
#Tiparot_clone_N295          E--DACEFPQ LEAIAKKIVG RLKG [104]
#Pisang_Bakar_clone_N311     K--DAGEFPQ LEAIAKKIAG RLEG [104]
#Pisang_Bakar_clone_N314     E--DAGEFPH LEAIAKKIAG RLKG [104]
#Pisang_Ceylan_clone_N321    E--DAGEFPQ LEAIAKKIVG RLKG [104]
#Pisang_Ceylan_clone_N323    E--DAGEFPQ LEAIAKKIVG RLKG [104]
#Gaba-gaba_clone_N342        E--DAGEFPH LEAIAKKIAG RLKA [104]
#Gaba-gaba_clone_N343        E--DAGEFPH LEAIAKKIAG GLKA [104]
#Musa_schizocarpa_1          E--DAGEFPH LEAIAKKIAG RLKV [104]
#Musa_schizocarpa_2          E--DAGEFPH LEAIAKKIAG RLRG [104]
#Malaccensis_1               E--DAGEFPH LEAIAKKIAG RLKG [104]
#Malaccensis_2               E--DAGEFPH LEAIAKKIAG RLKG [104]
#Malaccensis_3               E--DAGEFPH LEAIAKKIAG RLKG [104]
#Malaccensis_4               E--DAGEFPH LEAIAKKIAG RLKG [104]
#Malaccensis_5               E--DAGEFPH LEAIAKKIAG RLKG [104]
#Malaccensis_6               E--DAGEFPH LEAIAKKIAG RLKG [104]
#Malaccensis_7               E--DAGEFPQ LEAIAKKIVG RLKG [104]
#Malaccensis_8               E--DAGESPQ LEAIAKKIVG RLKG [104]
#Malaccensis_9               E--DAGEFPH LEAIAKKIAG RFKG [104]
#Microcarpa_1                E--YAGEFPQ LEAIAGMIVG RLKG [104]
#Microcarpa_2                E--DAGEFPQ LEAIAKKIVG RLKG [104]
#Pisang_Lilin_1              E--DAGEFPQ LEAIAKKIVG RLKG [104]
#Pisang_Lilin_2              E--DAGEFPQ LEAIAKKIAG RLKG [104]
#Musa_schizocarpa_3          E--DAGEFPH LEAIAKKIAG RLKG [104]
#Musa_schizocarpa_4          E--DAGEFPH LEAIAKKIAG RLKG [104]
#Musa_balbisiana_1           E--DVGEFPQ LEAIAGMIVG RLKG [104]
#Musa_balbisiana_2           E--DVGEFPQ LEAIAGMIVG RLKG [104]
#Musa_AAB                    E--DEGEFPQ LEAIAKKIAG RLKG [104]
#Musa_ABB_Karthombiumtham_1  E--DVGEFPQ LEAIAGMIVG RLKG [104]
#Musa_ABB_Karthombiumtham_2  E--DVGEFPQ LEAIAGMIVG RLKG [104]
#Changpui                    E--YAGEFPQ LEAIAKKIAG RLKG [104]
#Changpawl                   E--DAGEFPQ LEAIAEKIVG RLKG [104]
#Banpawl                     E--DAGEFPQ LEAIAKKIAG RLKG [104]
#Balhlasen                   E--DAGEFPQ LEAIAKKIAG RLKG [104]
#Pisang_Mas                  E--DAGEFPH LEAIAKMIAG RLKG [104]
#Pisang_Rastali_1            E--DAGEFPQ LEAIAKKIAG RLKG [104]
```

Figure 4.16, continued.

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4.3.7 Phylogenetic analysis of *NBS-LRR Resistance* gene transcripts and the genomic sequences

A phylogenetic Neighbour-Joining tree (Figure 4.17) constructed based on the aligned deduced *NBS-LRR Resistance* amino acid sequences of eight *Musa* species together with the isolated cloned sequences revealed that there were six types of *NBS-LRR Resistance* gene according to clades they are grouped in (Figure 4.18-4.21). From this analysis, it was found that RGAs from all eight *Musa* species were clustered into Clade 1 with 1000 bootstrap values. Eight of the isolated clones (GNt1A6, B2_D5, GNt1F1, B2_F3, GN_AC3, GN_AD6, GN_AA3 and GN_AA6) were also clustered into this clade which also included *Musa acuminata* AAA Group NBS-LRR class resistance protein sequence which showed resistance towards Blight (accession no. EF515836 and EU123885). On the other hand, the rest of the transcript and genomic clones were in Clade 2 to Clade 7. Interestingly, clones in Clade 2 clustered together with one of the reference sequences, *Musa acuminata* (RGC5). Clade 8 served as an outgroup of the tree.

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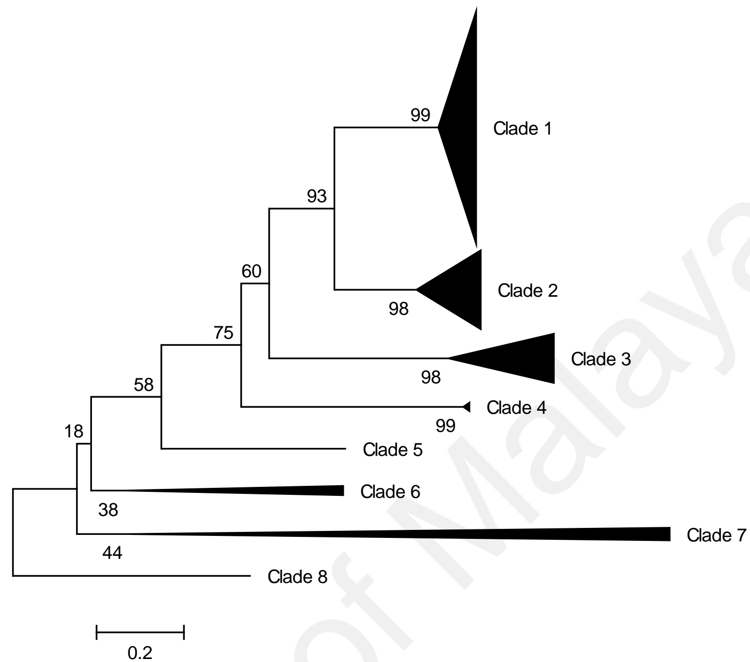

Figure 4.17: A Neighbour-Joining tree with simplified branches of deduced amino acids of RNA transcripts, genomic DNA, reference sequences and outgroup. The numbers on the branches indicate bootstrap values (1000 replications). Branches corresponding to distinct reference sequences are labelled as Clade 1, 2, 3, 4, 5, 6, 7 and 8.

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Legends:

■: Berangan genomic

●: Grand naine genomic

▼: Lemak Manis genomic

▲: Malaccensis genomic

○: Grand naine transcripts

Figure 4.18: A condensed Neighbour-Joining tree with elaborated Clade 1 of deduced amino acids of RNA transcripts, genomic DNA and reference sequences. The numbers on the branches indicate bootstrap values (1000 replications).

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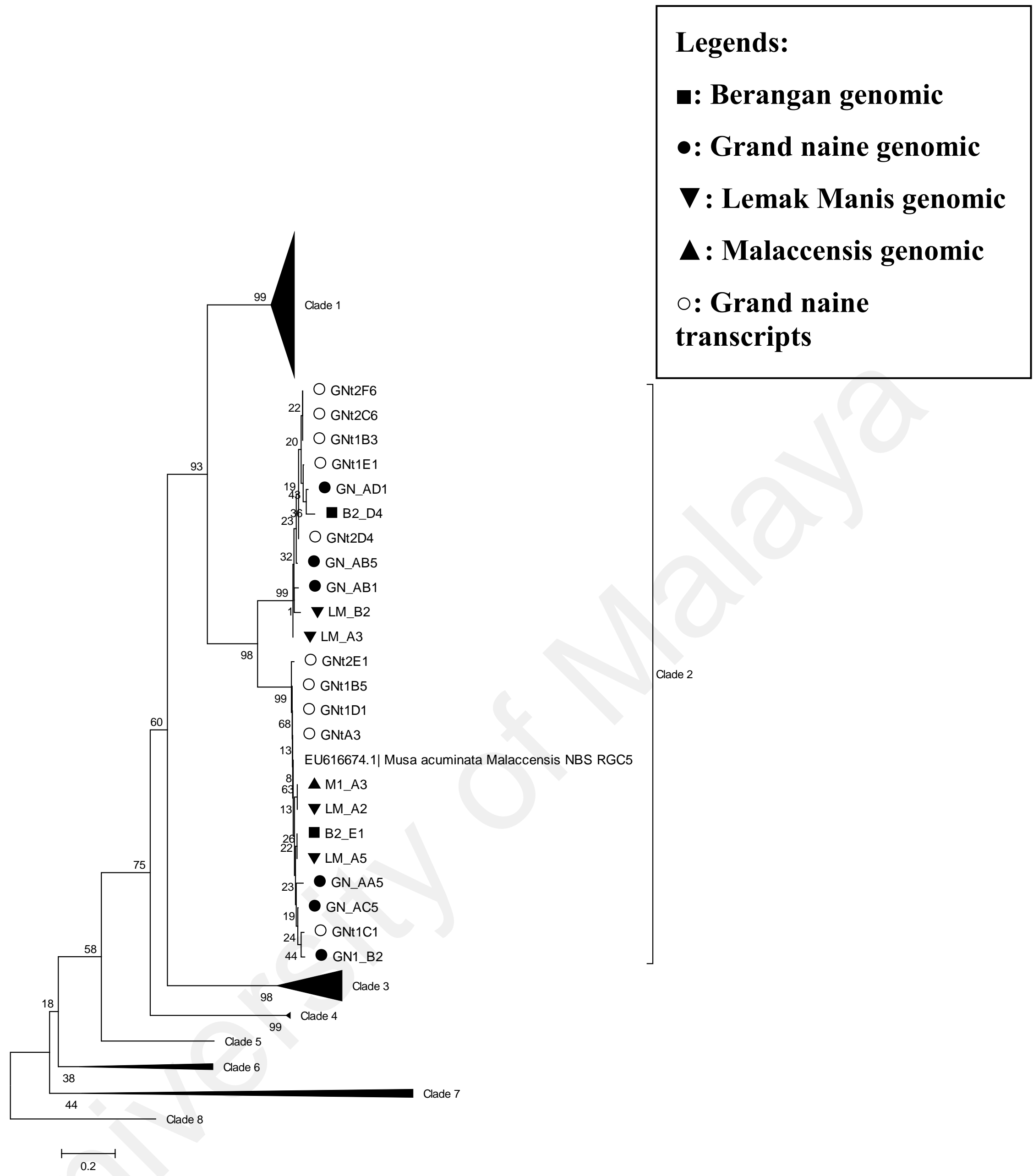

Figure 4.19: A condensed Neighbour-Joining tree with elaborated Clade 2 of deduced amino acids genomic DNA, RNA transcripts and reference sequences. The numbers on the branches indicate bootstrap values (1000 replications).

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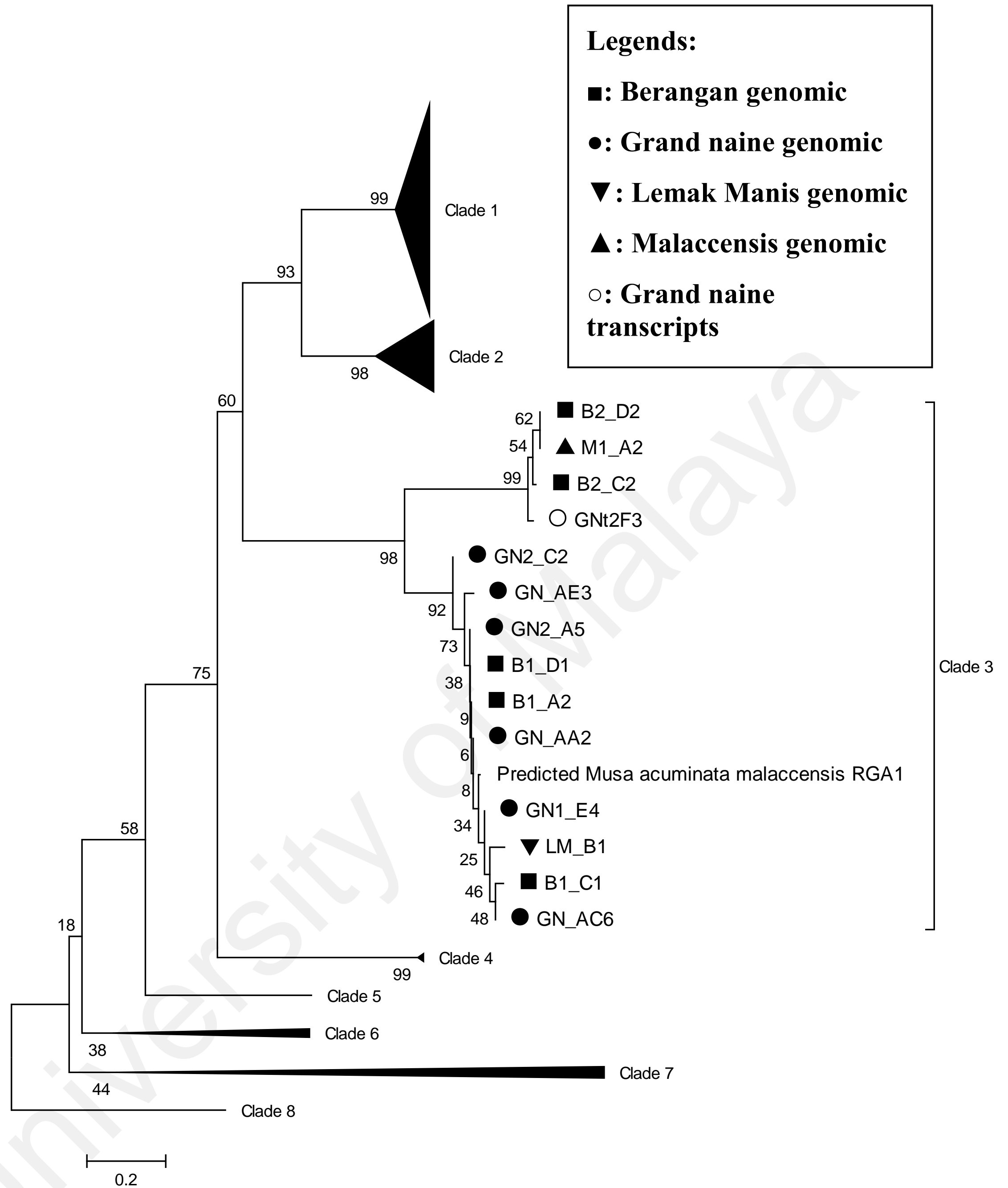

Figure 4.20: A condensed Neighbour-Joining tree with elaborated Clade 3 of deduced amino acids of RNA transcripts, genomic DNA and a single reference sequence. The numbers on the branches indicate bootstrap values (1000 replications).

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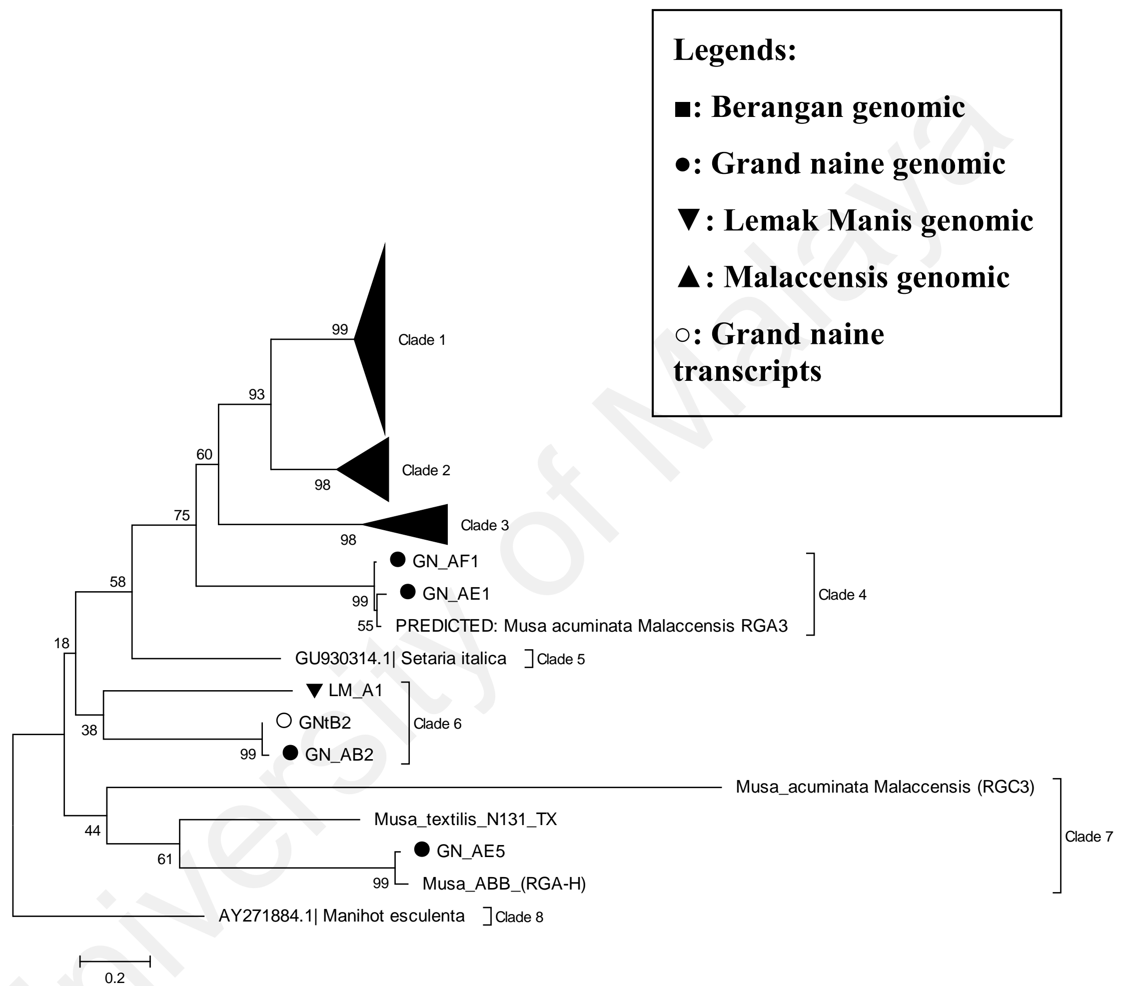

Figure 4.21: A condensed Neighbour-Joining tree with elaborated Clade 4, 5, 6, 7 and 8 of deduced amino acids genomic DNA, RNA transcripts, reference sequences and outgroup. The numbers on the branches indicate bootstrap values (1000 replications).

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CHAPTER 5: DISCUSSION

5.1 Understanding a compatible plant-nematode interaction via Proteomics approaches

A challenge experiment at one time point (30-dai) was first carried out on a nematode-susceptible *Musa acuminata* variety namely Grand Naine using a gel-based Proteomics approach. This experiment served as a proof of principle to observe changes in protein abundance difference when the susceptible cultivar, Grand naine was inoculated with 1000 juvenile stage 2 (J2) *Meloidogyne incognita*. Thirty-dai was chosen as the time point for this experiment due to the fact that root-knot nematodes (RKN) complete their life cycle within 25 days (Shurtleff and Averre, 2000). Therefore, harvesting the roots at 30-dai was hypothesised to reveal significant protein abundance changes between control and nematode-inoculated root tissues.

Indeed, galls were visible on *M. incognita*-inoculated root fragments and none were formed on control root fragments (Figure 4.6). The formation of these galls may suggest that re-structuring of cellular and molecular components of root tissues had occurred in *M. incognita*-infected root tissues. This notion is supported by Goverse *et al*. (2000) postulating that RKN infection in plant roots would induce the formation of feeding cells surrounding the nematode's head. These feeding cells will get enlarged and eventually formed multinucleated giant cells due to acytokinetic nuclear division occurring in the infested cells (Caillaud *et al*., 2007). This phenomenon served as the ground of the hypothesis that proteins isolated from nematode-inoculated root samples will cluster separately from proteins isolated from control root samples in a Factor Projection Plot analysis due to differing molecular changes occurring in the two sample types. However, Factor Projection Plot analysis result obtained yielded no specific

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clustering between control and nematode-inoculated samples (Figure 4.3). Concurring with Monteoliva & Albar (2004), such may be the result of the inability of this system to detect low abundance proteins. Despite its robustness, this platform was not able to detect lower abundant proteins that are normally masked by the presence of higher abundant ones. Therefore, in this study, each protein spot was manually analysed using ImageMaster 2D Platinum 7.0 software. From this analysis, 51 protein spots were found to show significant abundance difference (ANOVA $p<0.05$) between the two sample types (Figure 4.4), hence confirming the hypothesis that significant protein abundance changes can be observed in nematode-infected banana root tissues when compared with control root tissues. Because of the disadvantages of the gel-based Proteomics system, further experiments were conducted using Liquid Chromatography-Mass Spectrometry (LC-MS) platform using single inoculation strategy (Figure 3.1).

In LC-MS proteomics-based experiment, two time points were chosen i.e. 30-dai and 60-dai in order to identify proteins involved in banana- *M. incognita* compatible interaction. Principle Component Analysis (PCA) revealed that at 60-dai, protein abundance changes obtained from nematode-inoculated root tissues significantly differ from protein abundance changes obtained in control root tissues (Figure 4.7). However, as opposed to 60-dai, PCA could not separate protein abundance profile between nematode-inoculated root tissues and control root tissues at 30-dai. Such a difference may be the result of nematode population build-up at 60-dai root tissues that translates into an increased number of infected cells per 50 mg root fragment compared to those harvested at 30-dai. This postulation was corroborated by Hussain *et al*. (2011) in their assessment of Okra damages caused by *M. incognita*. They reported that increased nematode inoculum level will increase the number of galls and egg masses, hence contributing to nematode population build up. The current study had profiled 112

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proteins that showed significant abundance changes between control and nematode-infected root tissues at 60-dai. These proteins can be predicted into 10 different biological functions (Figure 4.8). Out of the ten protein group, 5 protein groups involved in DNA replication, defence, oxidation-reduction, carrier and stress response were further discussed to manifest interesting roles in plant-pathogen interactions based on the findings of other researchers.

The first is DNA-replication related protein group. Seed-specific protein Bn15D1B (GSMUA_Achr8T13580_001) was found to be differentially abundant in nematode-inoculated root tissues. This protein is grouped in gyrase/isomerase II protein family and was isolated from *Arabidopsis thaliana* (Xie & Lam, 1994). Interestingly, since the banana cultivar used in this experiment is sterile, seed-specific protein was not expected to be expressed in its genome. This result suggests that although the protein is seed-specific in Arabidopsis, it may acquire a different function in a sterile plant. This protein was found to be present at ~5-fold lower in nematode-inoculated tissues suggesting a reduced gyrase function. Note that gyrase is an enzyme that functions to uncoil double-stranded DNA structure to allow DNA replication to take place (Manjunatha *et al*., 2002). A reduced gyrase function signalled for the presence of DNA damage. Willis and Rhind (2009) reported that the cells will slow their replication in response to DNA damage by entering into checkpoints during the S- and M-phase, hence halting the cell cycle. Such a mechanism is required in order to allow some time for the cell to repair the damaged DNA, ensuring the daughter cells to only receive undamaged DNA (Hartwell & Weinert, 1989). DNA damage may result from either endogenous sources such as reactive oxygen species (ROS) produced by cellular metabolism, spontaneous depurination of DNA and when replication forks collapse at various replication fork barriers; or from exogenous sources including ionizing and

ultraviolet radiation (Kastan & Bartek, 2004). Majority of cells are able to produce, detoxify and be protected from their own ROS. However, the cells will fail to protect themselves from their own ROS if a rapid ROS production via oxidative burst is triggered. Oxidative burst will result as one of the signalling mechanisms and a reaction to pathogen invasion in a given cell (Wojtaszek, 1997; Zurbriggen *et al*., 2010). This reaction will then trigger a cascade of hypersensitive response (HR) in the infected cells in order to defend the plant from pathogen colonisation (Tenhaken *et al*., 1995; Dangl & Jones, 2001; Jones & Dangl, 2006).

Next, a total of 6 defence proteins namely garmin-like protein 11-1 (ITC1587_Bchr9_P27746), putative patatin group A-3 (GSMUA_Achr6T05080_001), garmin-like protein 5-1 (GSMUA_Achr1T25160_001), md-2-related lipid recognition domain-containing protein (ITC1587_Bchr5_P14231), garmin-like protein 5-1 (GSMUA_Achr5T18440_001) and pathogenesis-related protein 1 (ITC1587_Bchr9_P26466) showed significant abundance changes in *M. incognita*-inoculated root tissues when compared with the control root tissues. It was found that pathogenesis-related protein 1 (PR-1) was present at 29.93-fold lower in abundance in inoculated samples compared to control samples. Kitajima and Sato (1999) reported that plant will express PR gene in response to pathogen infection or stresses. In addition, Ding *et al*. (2002) had shown in their study that the overexpression of *PR* genes in transgenic plants resulted in increased plant resistance towards several pathogens. This suggests that the decrease in abundance of PR protein in the inoculated root samples obtained in the current study was due to nematode manipulation of the plant's defence mechanism, rendering susceptibility status to the plant towards *M. incognita* infection. This suggestion was corroborated by Taheri and Tarighi (2011) in their study on expression analysis of *PR* gene in susceptible and partially resistant tomato cultivars.

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They found that the expression of *PR* gene was significantly lower in susceptible cultivar compared to the partially resistant cultivar.

The third interesting protein group is related with oxidation-reduction processes. Photosynthesis and respiration are examples of cellular metabolism processes occurring in plants. This metabolism process involves the activation and reduction of oxygen that gives rise to reactive oxygen species (ROS) such as singlet oxygen (O_2), superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (HO●) (Tripathy & Oelmüller, 2012). Various environmental stresses may also lead to excessive secretion of ROS (Sharma *et al*., 2012) through the process of oxidative burst. Recent studies have found that ROS plays a significant role in various biological processes regulation in plants such as growth and development. Besides that, ROS also served as plant's signalling response to biotic and abiotic stresses as well as programmed cell death (Bailey-Serres & Mitler, 2006). Normally, basic protection mechanism from ROS is via the ascorbate-glutathione cycle of which an antioxidant namely the ascorbate (vitamin C) is employed to convert harmful hydrogen peroxide (H_2O_2) to water (H_2O). On the other hand, the reduced form of the ascorbate is regulated by monodehydroascorbate reductase (MDHAR) by recycling the oxidised ascorbate (Yoon *et al*., 2004). Interestingly in this study, MDHAR protein (GSMUA_Achr5T17510_001) was found to be lower in abundance in nematode-inoculated root samples suggesting that the regulation of ROS is turned down and thus, may contribute to the susceptibility of this cultivar to nematode infestation.

Next is the transport protein that transfers a solute molecule across the lipid bilayer (Alberts *et al*., 2002). In this experiment, five transport proteins namely ATP_ADP translocator (ITC1587_Bchr8_P24300), putative SEC12-like protein 2 (GSMUA_Achr6T10220_001), ras-related protein raba 5d-like

(ITC1587_Bchr11_P33367), cytochrome c oxidase subunit 6B (GSMUA_Achr7T11740_001), rab GDP dissociation inhibitor alpha (GSMUA_Achr6T18380_001) showed significant abundance changes upon infection. One protein that was of interest was the putative SEC12-like protein 2. This protein was only detected in the inoculated samples. SEC12 protein was reported to be involved in the transport of phosphorus in plants. Phosphorus in the form of phosphate is an essential macronutrient where it constitutes the nucleic acid, phospholipids and cellular metabolites (Gonzalez *et al*., 2005). However, the phosphorus is not evenly distributed in soils and not readily available to roots (Raghothama, 1999). Therefore, plants employ the SEC12, a high-affinity phosphate transport protein that enhances phosphate acquisition (Rausch & Bucher, 2002). The relationship between plant phosphorus uptake and pathogen interaction was reported in 1979 by Jasper *et al*. (1979). They found that ryegrass roots were more susceptible to vesicular-arbuscular mycorrhizal (vam) infection in unfertilised, low-phosphorus virgin soil compared to fertilised agricultural soil. However, when phosphorus was applied to the unfertilised soil, mycorrhiza infestation on the ryegrass was inhibited. This report illustrated the importance of phosphorus in plant defence against pathogen infestation and the detection of SEC12-like protein in this nematode-inoculated cultivar suggested an increased phosphorus uptake of the infected host perhaps as an effort to fight off the nematodes. At this point however, no literature has been reported on direct mechanism of how this gene helps in preventing pathogen infection.

Lastly, stress response proteins constitute 7% of banana proteins that showed significant abundance change upon *M. incognita* inoculation. Cellular stress response is defined as a reaction to changes or fluctuations of extracellular conditions that damage the structure and function of macromolecules in a given cell (Kültz, 2003). When an

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organism is facing an emergency situation, its cells will produce stress proteins that will repair the damages that occurred in it (Welch, 1993). In this study, one of the stress response proteins that showed significant abundance change in inoculated tissues was the 70kda peptidyl-prolyl isomerase. Interestingly, this protein was detected only in *M. incognita*-inoculated banana root samples. Dwivedi *et al*. (2003) reported that 70kda peptidyl-prolyl isomerase protein (GSMUA_Achr9T03960_001) was expressed in the heat-treated wheat root tips and involved in the formation of cytoplasmic chaperone complex with other heat-shock proteins namely Hsp104, Hsp90, Hsp70, Hsp60/GroEL, and small Hsps. This molecular chaperone complex was found to be able to recognise and selectively bind to non-native proteins under physiological and stress conditions (Buchner, 1996) and prevent the proteins to be irreversibly misfolded or aggregated and thus, functioning well. This current study also found lignification protein namely lignin-forming anionic peroxidase (GSMUA_Achr4T05250_001) to be implicated in *M. incognita*-Grand naine interaction. This protein was found to be present in significantly lesser in abundance in *M. incognita*- inoculated root tissues. Generally, the biosynthetic pathway leading to disease resistance involves lignification of injured host cells. Robb *et al*. (1987) demonstrated that a tomato cultivar with an ability to coat its xylem vessel through lignification was more resistant towards *Verticilium albo-atrum* infection compared to susceptible cultivars. Besides, Zacheo *et al*. (1993) postulated that lignification assists plant defence mechanism by forming a mechanical barrier to barricade pathogen infection. In this study therefore, the lesser lignin-forming anionic peroxidase protein found in the treated samples explained why this cultivar is susceptible towards nematode infection.

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5.2 Partial *NBS-LRR Resistance* (R) gene isolation from *Musa acuminata* spp.

Plants acquire specific mechanisms to cope with pathogen infections. In an incompatible interaction, a pathogen contact with a host will trigger the host's systemic and localised responses. These responses can be in the form of physical or/and chemical reaction(s) which include the involvement of the *NBS-LRR Resistance* (*R*) gene. *R* gene was reported to confer resistance to *Nicotiana tabacum* and *Nicotiana benthamiana* (Zhang *et al*., 2015) and tomato (Xiuhong *et al*., 2012) against *Meloidogyne incognita* infection. Since there is as yet no reports on the isolation of the *NBS-LRR R* gene against *M. incognita* in banana, the current study is, to the author's knowledge, the first to report the types of *NBS-LRR R* gene that were expressed during *M. incognita* infestation. The diversity of *NBS-LRR R* gene analogues (RGA) was also assessed in this study by comparing isolated genomic RGA clones from A genome banana varieties namely Berangan, Grand Naine, Malaccensis and Lemak Manis.

A total of 19 RNA transcripts from *M. incognita*-inoculated Grand naine root samples and 45 genomic DNA clones were isolated with the size of ~ 650 bp. When aligned, the analysis of Kinase-2 motif C-terminal end that ends with tryptophan (W) confirmed the grouping of the *NBS-LRR* transcripts to be non-Toll interleukin-1 receptor (non-TIR) whereas the end amino acid for TIR is aspartic acid (D) (Meyers, 1999).

The alignment of the deduced amino acid sequences revealed that 20 out of 64 isolated RGA clones contain a premature stop codon within the isolated cds stretch and resulted in truncated proteins (Figure 4.14). Vanin (1985) described pseudogenes as genomic sequences that resemble the functional RNA or protein-coding genes but could not be translated into functional proteins. However, Pink *et al*. (2011) also reported that

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many of these 'junk artefacts' were actually transcribed into RNA, regulating specific gene expression. Indel or nucleotide substitutions that interfere with the reading frame are among the possible causes of the presence of pseudogenes in a genome (Chandrasekaran & Betrán, 2008). As reported by Marone *et al*. (2013), some *Resistance* pseudogenes existed in various plant species but in different amount such as *Arabidopsis thaliana* (8.05%), *Medicago truncatula* (14.7%), polyploidy cotton (24.6%), Nipponbare (47.6%) and 93-11 genotype rice (55.7%). At present, there is no published report on the number of *NBS-LRR R* pseudogenes present in banana genomes despite the work published by Pei *et al*. (2007) and Mohamed and Heslop-Harrison (2008). Both groups only reported on the diversity of *Resistance* gene in *Musa* spp. at the genomic level. However, the current study was able to isolate 5 pseudogene clones out of 19 RGA transcripts. Although Harper *et al*. (2003) motioned that most pseudogenes were not transcribed, there were however, examples of successfully transcribed pseudogenes such as those found for tumour suppressor *PTEN* (Fujii *et al*., 1999), adrenal steroid hydroxylase P450c21A (Bristow *et al*., 1993), and human leukocyte interferon (Pink *et al*., 2011). The expression of these pseudogenes was reported to be tissue-specific and can be influenced by the physiological conditions, including disease (Pink *et al*., 2011). Specifically, these non-coding RNA sequences can also become the antisense RNAs in a research conducted on human, mouse and pufferfish (Dahary *et al*., 2005) and human miRNAs (Zhang, 2008). It is noteworthy that in contrast to multicellular organisms that will conserve their pseudogenes, various unicellular organisms opted to remove the pseudogenes from their system by rapid deletional process (eg. *Salmonella* genome) (Kuo & Ochman, 2010). This may be due to another potential benefit of these non-coding sequences in which they act as a source of genetic for antibody production towards antigenic variation through gene conversion or recombination with functional genes (Balakirev & Ayala, 2003). In plants however, no

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direct evidence was reported relating the presence of pseudo *R* gene contributing to the plant defence against the nematode. However, Kohler *et al*. (2008) and Lozano *et al*. (2012) in their research on *Populus* and potato respectively suggested the function of pseudogenes as adaptor molecule in which they can interact with other NBS-LRR proteins. Besides that, Mastrangelo *et al*. (2012) also reported that truncated Resistance proteins could also be originated from alternative splicing and demonstrated a role in promoting disease resistance in plants (Marone *et al*., 2013).

The diversity of the RGAs in the *Musa* genus was also analysed in this study. In the constructed Maximum Parsimony Neighbour-joining tree, eight clones that were grouped together with the reference sequences in Clade 1 showed a different degree of amino acid variation compared to the remaining isolated cloned in other clades (Figure 4.16 - 4.24). The reference sequences in Clade 1 were isolated from banana cultivars that were distributed mainly in South East Asia region namely Malaysia, Indonesia, Thailand, The Philippines, India as well as other region such as Cameroon and Papua New Guinea. Despite the difference, they were still grouped with other *NBS-LRR Resistance* gene in the *Musa* genus that consists of the cultivated genomes of *acuminata* (A), *balbisiana* (B) and hybrids of A and B genomes (AAB, ABB, AB) whereas the wild species consisted of the *schizocarpa*, *velutina*, *banksii* and *ornata*. Two reference sequences in Clade 1 also consisted of two *NBS-LRR Resistance* genes (EF515836 and EU123885) that showed resistance against blight in plants as reported by Xu *et al*. (2007) in the Genbank (unpublished journal). It is perhaps essential to state that the current study had 10 transcripts namely GNt2F6, GNt2C6, GNt1B3, GNt1E1, GNt2D4, GNt2E1, GNt1B5, GNt1D1, GNtA3 and GNt1C1 that showed close relationship with *Musa acuminata* (RGC 5). Note that RGC 5 is an NBS-LRR *R* gene isolated by Peraza-Echeverria *et al.* (2007) that was demonstrated to confer banana resistance against

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Fusarium oxysporum f. sp. cubense (FOC). However, the fact that the isolated clones were only partial fragments of NBS-LRR *R* gene, further investigation inclusive of full length isolation of these fragments is required.

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CHAPTER 6: CONCLUSION

In this thesis, the interaction between *Musa* cv. Grand naine and *Meloidogyne incognita* was first studied using a conventional gel-based Proteomics approach. Despite deviations from the expected result (as demonstrated in factor projection analysis; Figure 4.3), this study proved that molecular changes occurred in nematode-infested cells. Results obtained revealed that 51 banana root proteins showed significant abundance changes in nematode-inoculated root fragments when compared with the control root fragments (Figure 4.4). Despite the robustness of this approach, the platform used was not able to detect lower abundant proteins that are masked by the presence of higher abundant ones. Therefore, Orbitrap LC-MS Proteomics platform was opted. This non-gel-based Proteomics experiment had successfully profiled 112 banana root proteins that showed significant abundance changes in *Meloidogyne incognita*-inoculated root tissues at 60- dai. From these 112 proteins, peptide identification analysis revealed that five proteins were found to be grouped in stress response biological function [70 kda peptidyl-prolyl isomerase (GSMUA_Achr Un_randomT02470_001), heat shock cognate 70 kda proteins (GSMUA_Achr9 T03960_001, GSMUA_Achr3T12480_001, GSMUA_Achr2 T16250_001), flavoprotein wrba derivatives (GSMUA_Achr10T10080_001, GSMUA_Achr5 T26440_001), lignin-forming anionic peroxidase (GSMUA_Achr4 T05250_001) and osapx7 - stromal ascorbate peroxidase encoding gene 5, 8 (GSMUA_Achr10 T16040_001) whereas five proteins in plant defence mechanism [germin-like protein 11-1 (ITC1587_Bchr9_P27746), putative patatin group A-3 (GSMUA_Achr6 T05080_001), germin-like protein 5-1 (GSMUA_Achr1T25160_001 and GSMUA_Achr5T18440_001), md-2-related lipid recognition domain-containing protein (ITC1587_Bchr5_P14231) and pathogenesis-related protein 1

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(ITC1587_Bchr9_P26466)]. However, no NBS-LRR Resistance (R) peptides were detected to show significant abundance difference between the two sample types (inoculated vs. control) using this platform. Therefore, potential NBS-LRR Resistance RNA transcripts and DNA fragments were isolated from *M. incognita*-infected banana root tissues via PCR using degenerate primers. Seventy-three clones were successfully isolated with 71 of them showing high sequence similarity (E-value: 0; Tables 4.5 - 4.6) with banana *R* gene sequences in the GenBank. From these clones, 22 sequences contained a premature stop codon, potentially leading to the production of truncated peptides (Figure 4.15). It is hypothesised that these pseudogenes may function as an adaptor molecule that interact with other NBS-LRR R proteins, hence play a role in plant defence. However, further analysis should be carried out to justify this claim. Phylogenetic analysis revealed that the isolated clones can be grouped into six banana *R* gene clades with Clade 2 containing an *R* gene that confers resistance to *Fusarium oxysporum* namely *RGC 5*.

It is noteworthy that, to the author's knowledge, the discovery of proteins involved in a compatible interaction between *Musa acuminata* - *M. incognita* is novel especially with regards to non-model crops (Al-Idrus *et al*., 2017). The LC-MS Proteomics result obtained in this study corroborated that of found by Castaňeda *et al*. (2017) at the transcript level and serves as a foundation to researches carried-out towards the production of an elite banana variety with resistance/tolerance mechanism against *M. incognita* infestation.

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RESEARCH ARTICLE

Elucidation of the compatible interaction between banana and *Meloidogyne incognita* via high-throughput proteome profiling

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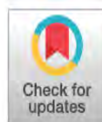

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Abstract

With a diverse host range, *Meloidogyne incognita* (root-knot nematode) is listed as one of the most economically important obligate parasites of agriculture. This nematode species establishes permanent feeding sites in plant root systems soon after infestation. A compatible host-nematode interaction triggers a cascade of morphological and physiological process disruptions of the host, leading to pathogenesis. Such disruption is reflected by altered gene expression in affected cells, detectable using molecular approaches. We employed a high-throughput proteomics approach to elucidate the events involved in a compatible banana- *M. incognita* interaction. This study serves as the first crucial step in developing natural banana resistance for the purpose of biological-based nematode management programme. We successfully profiled 114 Grand naine root proteins involved in the interaction with *M. incognita* at the 30th- and 60th- day after inoculation (dai). The abundance of proteins involved in fundamental biological processes, cellular component organisation and stress responses were significantly altered in inoculated root samples. In addition, the abundance of proteins in pathways associated with defence and giant cell maintenance in plants such as phenylpropanoid biosynthesis, glycolysis and citrate cycle were also implicated by the infestation.

Introduction

Plants are constantly exposed to a range of pathogenic organisms inhabiting the soil. Amongst these, plant-parasitic nematodes (PPN) are documented as soil pathogens of economic importance incurring approximately US$100 billion worth annual crop losses [1]. Amongst the PPN, sedentary root-knot nematodes (RKN; *Meloidogyne* spp.) are one of nature's most successful obligate parasites. *Meloidogyne incognita* was reported to be the most widely distributed

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APPENDIX A

Reagents and solutions

PCR

Sterile distilled water (sdH2O)	Up to 25µL
Reaction buffer (1X)	2.5µL of 10X
$MgCl_2$ (1.5mM)	1.5µL of 25mM
dNTPs (10mM)	4.0µL
Forward primer (0.4pmole/µL)	1.0µL of 10pmole
Reverse primer (0.4pmole/µL)	1.0µL of 10pmole
Taq DNA polymerase (1U)	0.2µL of 5U/µL

Agarose gel electrophoresis

5X Tris Borate EDTA (TBE)	54.0g Tris-base (445mM) 27.5g Borate (445mM) 20mL of 5.0M EDTA (10mm, pH 8.0)
6X loading dye	30% glycerol, 0.25% bromophenol blue 0.25% xylene cyanol FF

Cloning into *Escherichia coli*

Luria Bertani (LB) Broth	20g/L LB broth powder
Luria Bertani (LB) agar with Ampicillin	35g/L LB broth powder 50mg/mL ampicillin 0.08mg/mL X-Gal 0.5mM IPTG

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