DEVELOPMENT OF TRANSGENIC *Musa acuminata* cv. 'BERANGAN' WITH PATHOGENESIS-RELATED (PR10) GENE CONFERRING TOLERANCE TO *Fusarium oxysporum* f. sp. *cubense* TROPICAL RACE 4

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Field of Study: Plant biotechnology

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ABSTRACT

Fusarium wilt is caused by a type of soil-borne fungus, *Fusarium oxysporum* f. sp. *cubense* (Foc) that colonizes banana roots and causes blockage in the transportation of the necessary nutrients through xylems. The disease greatly hinders the production of banana worldwide. Although various measures have been executed, such as introduction of new breeding programs and disease control, none was found to be effective in combating this disease. Hence, the development of disease-resistant banana cultivars is vital. In this study, an anti-fungal gene called pathogenesis-related (PR) 10 was isolated from wild-type banana, *Musa acuminata* ssp. malaccensis (*MaPR-10*). Using rapid amplification of cDNA ends (RACE) technique, the full-length DNA and cDNA sequences of the gene were revealed to be 949 bp and 773 bp, respectively. *MaPR-10* has a 176-bp-intron which is spanned in between two exons with 5‘ untranslated region (UTR) and 3‘UTR of 62 bp and 228 bp, respectively. *MaPR-10* encodes a putative protein of 160 amino acids with a predicted molecular mass of 17.46 kDa and an isoelectric point of 5.42. The predicted MaPR-10 protein contains a functional domain, called Betv1-like, and a prominent conserved glycine-rich, P-loop motif of GxGxxG. *MaPR-10* was successfully introduced into a commercial banana cultivar ‗Berangan‘ via co-cultivation of *Agrobacterium tumefaciens* and embryogenic cells suspension. A total of 17 putative transgenic lines were recovered on the hygromycin-selection medium and 11 of them tested positive for transgene integration following PCR analysis. Plantlets of line labeled 19 (Line-19) demonstrated the most rapid *in-vitro* propagation and thus were selected for further analysis. Line-19 plantlets possibly contain a single copy of *MaPR-10* and successfully over-express the transgene relative to the untransformed controls. To investigate the effect of *MaPR-10* in enhancing tolerance against Foc race 4 (FocR4) C1 HIR, the baseline disease response was first established using wild-type ‗Berangan‘ plants. Using root-dipping technique, it was found that inoculation of two-month-old plants in C1 HIR suspension culture containing 1 x 10^6 spores/ml for two hours was able to produce consistent disease symptoms. Wild-type ‗Berangan‘ plants exhibited wilting and yellowing leaf symptoms as early as one week after inoculation. Scoring of leaf symptoms index (LSI) and rhizome discoloration index (RDI) was performed in the 5^{th} week post-inoculation to evaluate the disease severity of the infected plants. Wild-type ‗Berangan‘ was found to be highly susceptible to C1 HIR isolate with LSI and RDI scores of 4.3 and 6.0, respectively. In the bioassay evaluation of Line-19 plants, about 30% showed a delay in wilting and yellowing leaf symptoms, which were observed two weeks after inoculation. In addition, Line-19 plants harboring *MaPR-10* were found to be highly susceptible to C1 HIR isolate with LSI and RDI scores of 3.4 and 6.1, respectively. Based on this observation, Line-19 plants may have acquired tolerance against the infection as shown by the delayed disease progression. For future studies, disease evaluation of other transgenic lines as well as introduction of more than one anti-fungal gene can be considered to achieve a better resistance trait.

ABSTRAK

Penyakit layu *Fusarium* yang disebabkan oleh sejenis kulat dalam tanah yang dinamakan *Fusarium oxysporum* f. sp. *cubense* (Foc) menyerang melalui akar pokok pisang mengakibatkan penyaluran nutrien ke bahagian lain pokok terhalang. Penyakit ini telah memberi kesan kepada pengeluaran buah pisang di seluruh dunia. Walaupun pelbagai usaha telah dilakukan, terutamanya dalam kawalan penyakit dan pembiakbakaan tumbuhan, masih belum ada cara yang berkesan untuk menanganinya. Penghasilan tanaman terubahsuai genetik yang mempunyai daya ketahanan penyakit yang tinggi dilihat sebagai satu langkah yang berpotensi untuk mengatasi masalah ini. Dalam kajian ini, satu gen anti-kulat yang dinamakan _pathogenesis-related (PR) 10' telah dipencilkan daripada sejenis pisang liar, *Musa acuminata* ssp. malaccensis (*MaPR-10*). Melalui teknik _*rapid amplification of cDNA ends*' (RACE), jujukan lengkap DNA dan cDNA untuk *MaPR-10* telah diperolehi dengan masing-masing dianggarkan sepanjang 949 bp dan 773 bp. Gen ini turut mempunyai dua exon yang dipisahkan oleh satu intron sepanjang 176 bp serta diapit oleh kawasan yang tidak ditranslasi di hujung 5' dan 3' masing-masing sepanjang 62 bp dan 228 bp. Protin yang terhasil pula dianggarkan sepanjang 160 asid amino dengan anggaran berat molekul 17.46 kDa dan titik isoeletrik 5.42. Protin ini turut mempunyai beberapa ciri lain termasuk domain yang dipanggil _Betv-1 like' serta motif P-loop, GxGxxG, yang kaya dengan glisin. *MaPR-10* telah berjaya diintegrasikan ke dalam genom satu kultivar pisang komersial iaitu _Berangan' melalui kaedah kultivasi antara *Agrobacterium tumefaciens* dan ampaian sel embriogenik. Sebanyak 17 jaluran (_line') pokok transgenik terhasil di mana 11 daripadanya disahkan positif bagi integrasi transgen melalui analisis PCR. Jaluran berlabel 19 (Jaluran-19) menunjukkan kadar propagasi _*in-vitro'* paling cepat dan dipilih untuk analisis seterusnya. Jaluran-19 berkemungkinan mengandungi satu salinan transgen serta menunjukkan ekspresi gen yang lebih tinggi berbanding pokok kawalan yang tidak ditransform. Untuk menyelidik keberkesanan *MaPR-10* dalam meningkatkan daya tahan pokok terhadap jangkitan Foc _race' 4 (FocR4) C1 HIR, pokok Berangan yang tidak ditransform pada mulanya digunakan untuk melihat tindak balas asas terhadap jangkitan FocR4 C1 HIR. Melalui teknik rendaman akar, keadaan jangkitan yang konsisten boleh diperolehi dengan menggunakan pokok yang berusia dua bulan yang diinokulasi selama dua jam di dalam ampaian C1 HIR dengan kepekatan 1 x 10^6 spores/ml. Pokok _Berangan' tidak ditransformasi menunjukkan tanda-tanda penyakit seperti daun layu dan kekuningan seawal minggu pertama selepas inokulasi. Pengiraan skor _*leaf symptoms index*' (LSI) dan _*rhizome discoloration index*' (RDI) dijalankan pada minggu ke-5 selepas inokulasi untuk menilai tahap jangkitan penyakit pada pokok. Pokok _Berangan' tidak ditransformasi didapati sangat sensitif terhadap jangkitan C1 HIR dengan skor LSI dan RDI masing-masing 4.3 and 6.0. Dalam penilaian Jaluran-19, 30% daripada anak pokok menunjukkan kadar penularan penyakit yang lebih lambat di mana tanda-tanda penyakit yang sama dikesan dua minggu selepas inokulasi. Jaluran-19 didapati terdedah kepada jangkitan C1 HIR dengan skor LSI dan RDI masing-masing 3.4 and 6.1. Jaluran-19 berpotensi mempunyai daya ketahanan yang lebih baik terhadap jangkitan C1 HIR yang ditunjukkan oleh kelewatan penyebaran tanda-tanda penyakit. Untuk menghasilkan pokok yang mempunyai daya tahan penyakit yang lebih tinggi, kajian akan datang boleh dilakukan dengan menilai tindakbalas jaluran lain terhadap jangkitan C1 HIR di samping melakukan transformasi dengan lebih daripada satu gen anti-kulat.

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°C	:	degree Celcius
%	:	percent
µg	:	microgram
µl	:	microlitre
µm	:	micrometer
µM	:	micromolar
α	:	alpha
β	:	beta
a.a.	:	amino acid
ABA	:	abscisic acid
ATP	:	adenosine triphosphate
bp	:	basepair
BTH	:	benzothiadiazole
cDNA	:	complimentary deoxyribonucleic acid
cds	:	coding sequences
CEBiP	:	chitin elicitor binding protein
CERK1	:	chitin elicitor receptor kinase 1
CIP	:	calf intestinal alkaline phosphatase
cm	:	centimeter
C_T	:	threshold cycle
cv.	:	cultivar
Da	:	Dalton
DEPC	:	diethylpyrocarbonate
DMSO	:	dimethyl sulfoxide

DNA	:	deoxyribonucleic acid
DNase	:	deoxyribonuclease
dNTP	:	deoxyribonucleoside triphosphates
DTT	:	dithiothreitol
E. coli	:	*Escherichia coli*
EDTA	:	ethylenediaminetetraacetic acid
eg	:	for example (exempli gratia in Latin)
EtBr	:	ethidium bromide
F	:	forward
g	:	gram
g	:	relative centrifugal force or G-force
GA3	:	gibberellic acid
GTP	:	guanosine triphosphate
H_2O	:	water
H_2O_2	:	hydrogen peroxide
ha	:	hectare
HEPES	:	2-[4-(2-hydroxyethyl)piperazin-1-y] ethanesulfonic acid
ITS	:	internal transcribed spacer
JA	:	jasmonic acid
kb	:	kilo basepair
kDa	:	kilo Dalton
L	:	litre
LB	:	Luria-Bertani
M	:	molar
m	:	meter
Mbp	:	mega basepair

mg	:	miligram
$MgCl_2$	:	magnesium chloride
MeJa	:	methyl jasmonate
min	:	minute
ml	:	mililitre
mM	:	milimolar
mm	:	milimeter
$MnCl_2$	:	manganase (II) chloride
mRNA	:	messenger ribonucleic acid
MW	:	molecular weight
n	:	haploid number
NaCl	:	sodium chloride
NaOH	:	sodium hydroxide
NBS-LRR	:	nucleotide-binding leucine-rich repeat
NCBI	:	National Center for Biotechnology Information
ng	:	nanogram
OD	:	optical density
PCR	:	polymerase chain reaction
psi	:	pounds per square inch
qPCR	:	quantitative polymerase chain reaction
R	:	reverse
RE	:	restriction endonuclease
RM	:	Ringgit Malaysia
RNA	:	ribonucleic acid
RNase	:	ribonuclease

rpm	:	revolutions per minute
RT	:	reverse transcriptase
SA	:	salicylic acid
sdH_2O	:	sterile distilled water
SDS	:	sodium dodecyl sulphate
sec	:	second
SOC	:	super optimal broth with catabolite repression
TAP	:	tobacco acid phyrophosphatase
TBE	:	tris-borate-EDTA
T-DNA	:	transfer-DNA
U	:	unit
USD	:	United States dollar
UTR	:	untranslated region
UV	:	ultraviolet
v/v	:	volume/volume
w/v	:	weight/voume
YT	:	yeast-triptone
$ZnCl_2$	:	zinc chloride

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 General introduction

Banana is the most exported fresh fruit worldwide with annual trade values of more than USD 7 billion (Gondolini, 2014). With respect to Malaysia, it is listed among one of the most valuable non-seasonal tropical fruits under Agriculture National Key Economic Area (Agriculture NKEA) program (ETP, 2013). Regarded as the second most important fruit after durian, bananas were grown on more than 15% of the total fruit plantation areas in Malaysia with approximate sales revenue of RM 595 million in 2013 (DOA, 2013).

Despite being a key player for global agriculture sector, the production of this crop has unfortunately been hampered by various serious diseases including *Fusarium* wilt. *Fusarium* wilt or also known as Panama disease is caused by a type of soil-borne fungus, *Fusarium oxysporum* f. sp. *cubense* (Foc). Foc initiates the infection through banana roots and occludes the vascular tissues thus obstructing the distribution of essential nutrients to other plant parts (Moore et al., 1995). One of the most significant *Fusarium* epidemics took place in Latin America and Caribbean around 1940s-1950s which had caused complete replacement of _Gros Michel‘ (AAA) bananas with _Cavendish‘ (AAA) as the main cultivar traded (Stover, 1962). Recent outbreaks of Foc tropical race 4 (TR4), a subclass of Foc, in Mozambique and Jordan, signify the never-ending threat of this disease (García-Bastidas et al., 2014).

In the past, various conventional breeding approaches had been executed to circumvent this problem. However, none was shown to be completely effective probably due to high sterility and polyploidy nature of banana plants (Hu et al., 2013). Nowadays, the strategies have been shifted towards the production of commercial cultivars that could confer enhanced tolerance against *Fusarium* wilt via genetic

modification. Aided by wide gene selection and diverse transformation parameters, transgenic approach offers huge potential for tackling this long-running issue.

In this study, transgenic banana (*Musa acuminata*) cv. _Berangan' clone harboring a defense-related gene called pathogenesis-related (PR) 10 isolated from *M. acuminata* ssp. malaccensis *(MaPR-10)* has been produced. The study of *MaPR-10* sequences elucidates certain domains and conserved sequences that may have contributed to the gene's function, particularly as an anti-fungal protein. The regenerated plants are expected to confer increased tolerance against *Fusarium* wilt.

1.2 Objectives

The objectives of this study are:

1. To isolate and analyze the full-length DNA and cDNA sequences of pathogenesis-related (PR) 10 from *Musa acuminata* ssp. malaccensis (*MaPR-10*)
2. To produce transgenic *M. acuminata* cv. _Berangan' clones harboring *MaPR-10* via co-cultivation of *Agrobacterium tumefaciens* and embryogenic cells suspension
3. To establish the baseline disease response of *M. acuminata* cv. _Berangan' plants infected with *Fusarium oxysporum* f. sp. *cubense* (Foc) race 4 (FocR4) C1 HIR isolate
4. To analyze the disease response of transgenic *MaPR-10* plants against FocR4 C1 HIR isolate

CHAPTER 2: LITERATURE REVIEW

2.1 Bananas and plantains

Bananas and plantains are classified under the *Musa* genus in the Zingiberales order and the family of Musaceae (Stover & Simmonds, 1987). The term plantains, which refer to a specific type of cooking banana (AAB subgroup), are widely used in Africa and Latin America but are not distinctly differentiated in the native languages of the Southeast Asian (SEA) regions (Valmayor et al., 1999). In general, all plantains are bananas but not all bananas are plantains (Valmayor et al., 1999). While plantains are found to be cultivated in the humid lowlands of West and Central Africa (Kole, 2011), the origin of bananas could probably be mapped back into the wild of Southeast Asian regions, precisely in Malaysia, Indonesia and the Philippines (Simmonds, 1959).

2.1.1 General background

Bananas and plantains, henceforth referred to as bananas, are giant herbaceous plants that can grow up to 1.5-1.9 m in height (Pillay & Tripathi, 2007). Suitably grown on well-irrigated soil, banana plants are currently cultivated across the humid tropical and subtropical areas in the altitude range of 0-920 m. It is estimated that a banana plant needs approximately 25-75 mm (L/m^2) water per week, though the more accurate amount is highly dependent on different factors such as type of soil and cultivars, cultivated area and sun exposure (Nelson, Ploetz & Kepler, 2006; Pillay & Tripathi, 2007). Despite being highly prone to wind breakage, banana plants can endure a fair range of temperature swing from as low as -2 to 30°C (mean of the coldest month) to as high as 35-37°C (mean of the hottest month). The optimum temperatures for shoot formation and fruit production are around 26-28°C and 29-30°C, respectively (Nelson, Ploetz & Kepler, 2006).

2.1.2 The botany of bananas and plantains

The *Musa* genus is comprised of five different sections with over 50 species that are grouped based on the number of basic chromosomes as well as the organization of the flower buds. Cultivars that fall under the Callimusa and Australimusa sections have a basic chromosome number of n=10 while those classified under Eumusa and Rhodochlamys possess a genome make up of n=11. On the other hand, Ingentimusa consists of only one species with n=7. Callimusa and Rhodochlamys are quite similar as such both sections are made of non-parthenocarpic species that are only significant as ornamental crops. While cultivar Fe'i is among the rare edible species that belongs to Australimusa section, most of the other bananas under this section are utilized for their fibers and pseudostems. *M. textilis Vees* (Abaca) is an instance of notable species under Australimusa section known for its Manila hemp product. With well-varied cultivars, Eumusa is undoubtedly the largest section among all with about 13-14 species classified under it. It is also the oldest and the most significant section in which most edible bananas were derived from at least two wild species, namely *M. acuminata* Colla (A genome) and *M. balbisiana* Colla (B genome) (Pillay & Tripathi, 2007; Talengara, 2007). Dessert bananas are mostly derived from *M. acuminata* species while cooking bananas and plantains are derivation from *M. balbisiana* species or hybridization of both (Valmayor et al., 1999) (Figure 2.1).

The principal factor that determined the edibility of a particular banana/plantain is its parthenocarpic trait (Cheesman, 1948). This is commonly associated with seedless fruit being the result of female sterility. Possibly through crosses of compatible cultivars and chromosome arrangement between edible diploid and wild species, sterile triploid cultivars were produced (Cheesman, 1947; Pillay & Tripathi, 2007). Triploid clones have high commercial value and are well-received by the consumers (Pillay & Tripathi, 2007).

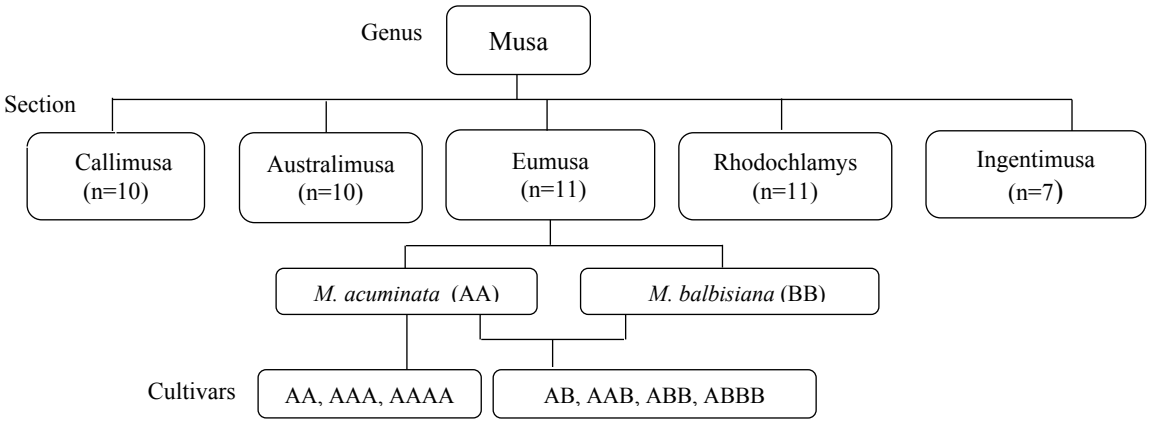


Figure 2.1: Summary of *Musa* genus

2.1.3 Global production and challenges

Grown in about 135 countries worldwide, banana is the world's most exported fresh fruit with trade values of more than USD 7 billion/year (Gondolini, 2014). It is also the 4th most significant fruit crop in the least developed countries in terms of production and total consumption (CGIAR, 2012). From 2006-2010, total export from Asia had been severely affected as a result of production decline in the Philippines due to natural diseasters including typhoon. The country made a successful comeback as being the second largest banana exporter after Ecuador following a healthy export leap to 2.6 million tonnes in 2012, which contributed to about 94% of total Asia's export (FAO, 2014). Despite the positive outlook, the fruit production continuously faces challenges impacted by economic, social, environmental and biological factors (Gondolini, 2014).

Banana is also exposed to a vast number of diseases caused by bacteria, fungus, nematodes, insects and other pathogens (Table 2.1). Of these, *Fusarium* wilt, caused by *Fusarium oxysporum* f. sp. *cubense* (Foc), is considered as one of the most threatening banana diseases and has been associated with the declining banana cultivation areas in many regions including China, Taiwan, Australia, Malaysia and Indonesia (Pérez-Vicente, 2014).

Table 2.1: Common pathogens and diseases associated with banana (adapted from Nelson, Ploetz & Kepler, 2006)

Category	Pathogen	Disease caused	Damage
Insects	*Aphis gossypii* (melon aphid) *Pentalonia nigronervosa* (banana aphid)		• Capable of transmitting banana bunchy top virus (BBTV)
	Cosmopolites sordidus (banana weevil)		• Damage on roots • Effect on growth and fruit production
	Bactrocera dorsalis (Oriental fruit fly) *Ceratitis capitata* (Mediterranean fruit fly)		• Effect on mature bananas
	Aleurodicus dispersus (spiraling whitefly)		• _Honeydew' production promotes sooty mold formation on the organ surface • Causes tissue destruction and discoloration
Bacteria	*Ralstonia solanacearum* race 2 (Biovar 1)	Moko disease	• Causes leaf wilting and affects fruits • Bacteria can be spread by insects
Virus	Banana bunchy top virus (BBTV)	Bunchy top	• Transmitted by *Pentalonia nigronervosa* • Causes —sticking up" or bunching up/rosetting of leaves
	Banana streak virus (BSV)	Banana streak	• Causes leaf necrosis and streak • May retard plant growth and fruit • Vector: mealybugs
Nematodes	*Radopholus similis* (burrowing nematode)		• Lesion on roots and rhizome • Affects fruit production, plants are more susceptible to wind • Plants collapsing • Causes poor fertilizer absorbance
	Meloidogyne sp., *Meloidogyne incognita* (root-knot nematodes)		• Causes galling of banana roots • Plants collapsing
Fungus	*Pseudocercospora fijiensis* (syn. *Mycosphaerella fijiensis*)	Black sigatoka, black leaf streak	• A leaf disease • Severe lesion that leads to browning and wilting of leaves • Affects fruit production
	Pseudocercospora musaea (syn. *Mycosphaerella musicola*)	Yellow sigatoka	• A leaf disease • Leaves turn brown or grey and eventually fall off (defoliate) • Affects fruit production
	Fusarium oxysporum f. sp. *cubense*	*Fusarium* wilt (Panama disease)	• A lethal disease caused by races of a soil-borne fungus • Causes leaf wilting and corm discoloration • Affects fruit production

2.2 *Fusarium oxysporum* f. sp. *cubense* (Foc)

2.2.1 General background

Fusarium genus was first introduced by Link in 1809. The members were recognized by distinct canoe- or banana-shaped conidia and were capable of causing diseases in humans (Short et al., 2013), animals (Cortinovis, Pizzo, Spicer & Caloni, 2013) as well as plants (Brown & Proctor, 2013). In plants, the pathogens were widely dispersed even in seemingly healthy individuals as in the saying _if it is green, there's some *Fusarium* that can grow on it, in it or with it' (Leslie, Summerell & Bullock, 2006). Over 1000 *Fusarium* species were claimed to be associated with numerous diseases due to poor identification and lack of characterization methods. Wollenweber and Reinking (1935) managed to reduce the number of species to 65 that fall under 16 sections with 77 subspecific varieties in the *Fusarium* taxonomy.

An isolate was assigned to a particular species based on their unique morphological features in a two-step process. Firstly, the isolate was grouped to a section and within that particular section, it was then allocated to a certain species. Snyder and Hansen (1940) further simplified the genus classification into nine species and introduced the single spore isolation technique prior to identification. Among the nine *Fusarium* species named, two of them are still extensively accepted which are *F. solani* and *F. oxysporum*.

The naming system of *F. oxysporum* was usually followed by forma speciales (f. sp.) to designate the host of the pathogens. For instance, *F. oxysporum* f. sp. *cubense* refers to the pathogens that cause infection on banana plants while *F. oxysporum* f. sp. *lycopersici* was associated with tomatoes (Inami et al., 2014). Within the specific host, further subdivision was applied, such as races or vegetative compatibility groups (VCG) to differentiate narrower host ranges affected by certain group of isolates (Smith, 2007). Thus, the common naming order of a *Fusarium* isolate can be summarized as follows:

Fusarium (genus) followed by species, forma speciales (f. sp.) then lastly by race or VCG or both if available (Example 1).

Fusarium oxysporum f. sp. *cubense* race 4 (VCG 01213)

↓ (genus) ↓ (species) ↓ (forma speciales) ↓ (race) ↓ (VCG)

Example 1: Example of common naming order of *Fusarium oxysporum* f. sp. *cubense* (Foc) which is responsible for *Fusarium* wilt in banana

2.2.2 Morphological characteristics of Foc

Morphological description is often most frequently chosen for species characterization, especially in fungi (Leslie, Summerell & Bullock, 2006). Even though the characterization based on sizes and distinct features of the spores can be influenced by the geographical distribution and environmental factors (Das et al., 2013), reliable amount of information can still be gathered for species differentiation. Secondary characteristics in terms of pigmentation, growth rate and secondary metabolites serve as supplementary support to better elucidate the species (Leslie, Summerell & Bullock, 2006). *F. oxysporum*, which is a common soil saprophyte, by far has no known sexual stage. It produces three types of asexual spores namely macroconidia, microconidia and chlamydospores (Table 2.2 and Figure 2.2). Pigmentation of the mycelia grown on potato dextrose agar (PDA) varies from white to pale violet.

2.2.3 Evolutionary theory of Foc

Evolutionary theory and the origin of Foc have been a subject of interest for many scientists. One of the most widely speculated theory is that Foc originated from South East Asia (SEA) and has co-evolved with its banana hosts ever since (Fourie et al.,

2011). Movement of planting materials infected with Foc has spread the disease to other parts of the world (Pegg, Moore & Bentley, 1996). Using electrophoretic karyotype, Boehm et al. (1994) divided Foc into two main groups. The first group consists of isolates that commonly attack cultivars that have at least one B chromosome inherited from *M. balbisiana*; for example _Lady Finger' (AAB) and _Bluggoe' (ABB). The other group, on the other hand, is linked to pure A genome from *M. acuminata* such as _Cavendish' (AAA) and _Gros Michel' (AAA). Besides co-evolutionary with hosts, diversity in Foc isolates is predicted to be influenced by a few reasons that include mutation (Ploetz, 1992), parasexual recombination (Buxton, 1962; Kuhn et al., 1995) and horizontal gene transfer.

Table 2.2: Characteristics of different types of Foc spores as described by Leslie, Summerell & Bullock (2006)

Type of spore	**Description**
Macroconidia	• Short to medium-length, straight to slightly curved, relatively slender and thin walled • Apical cell morphology: Tapered and curved, sometimes with a slightly hook • Basal cell morphology: Foot shaped to pointed • Number of septa: Usually 3-septate • Abundance: Sparse in some strains, usually abundant in sporodochia, occasionally from hyphae growing on the agar surface
Microconidia	• Shape/septation: Oval, elliptical or kidney-shaped, usually 0-septate • Aerial mycelium presentation: False head • Conidiogenous cells: Short monophialides • Abundance: In the aerial mycelia
Chlamydopsores	• Abundance/Speed of formation: Depends on isolates. Can be 2-4 weeks for some of them or even longer • Location: Usually formed singly or in pairs, maybe found in clusters/short chains as well • Either terminal/intercalary in aerial, submerged or surface hyphae • Appearance: Smooth/roughly walled

Table 2.3: Detection and characterization methods of Foc

Approach	Description	References
DNA amplification fingerprinting (DAF)	• PCR amplification of DNA polymorphism by short-length primers (8-10 bp) • Visualized as banding patterns of amplicons resulted by different combinations of primers and templates	Bentley & Bassam, 1996; Bentley et al., 1998
Random amplified polymorphic DNA (RAPD)	• A PCR application using arbitrary, short primers (8-12 bp) to amplify random target sequences • No primary knowledge on the sequences of the target template required	Bentley, Pegg & Dale, 1995; Javed , Chai & Othman, 2004; Lin et al., 2009; LinFeng et al., 2009; Wang et al., 2012
Restriction fragment length polymorphism (RFLP)	• Detecting variation in the homologous DNA by digestion using specific restriction endonuclease (RE) that results in different fragment length (if variation does occur)	Koenig, Ploetz & Kistler, 1997; Leong, Latiffah & Baharuddin, 2010; Thangavelu & Jayanthi, 2009
Amplified fragment length polymorphism (AFLP)	• A PCR-based application • Genomic DNA is first digested using restriction endonucleases before adaptors were ligated to the sticky end of the restricted fragment • Re-amplification of partial restricted fragment is performed using primers that anneal to the adaptor sequences	Groenewald et al., 2006; Sutherland et al., 2013
Inter simple sequence repeats (ISSR) analysis	• A PCR-based application • Primers are designed based on the microsatellite sequences (16-25 bp) • Amplifies fragment between two identical microsatellite repeat sequences placed in opposite direction	Thangavelu et al., 2012
Quantitative-PCR (qPCR) based methods	• Detecting specific Foc regions using qPCR	Lin et al., 2013; Yang et al., 2015
Enterobacterial repetitive intergenic	• Amplification of enterobacterial repetitive intergenic consensus (ERIC)	Leong, Latiffah & Baharuddin, 2009; Leong, Latiffah & Baharuddin,

consensus PCR (ERIC-PCR)	regions of Foc isolates using ERIC-specific primers	2010
Random amplified microsatellites (RAMS)	• A PCR-based application • Primers contain microsatellite sequences and degenerate anchor at 5‘ end	Leong, Latifah & Baharuddin, 2010
Restriction analysis of the internal transcribed spacer and 5.8S regions	• Amplification of specific ITS+5.8S regions of Foc isolates followed by restriction digestion of Foc	Leong, Latifah & Baharuddin, 2009
Sequencing of translation elongation factor 1α (TEF-1α) and intergenic spacer region (IGS)	• Amplification of translation elongation factor 1α (TEF-1α) and intergenic spacer region (IGS) using specific primers	Dita et al., 2010
Loop-mediated isothermal amplification (LAMP)	• Typically uses four primer pairs to amplify six different regions of target gene • Amplification can be performed under constant temperature without using thermal cycler	Li et al., 2013a; Peng et al., 2014; Zhang et al., 2013

Table 2.3 continued

2.3 *Fusarium* wilt

2.3.1 Background

Fusarium wilt is caused by a type of soil-borne fungus, *Fusarium oxysporum* f. sp. *cubense* (Foc). The pathogen penetrates through banana roots and dominates the vascular tissues which impedes the dissemination of the necessary nutrients (Moore et al., 1995). The disease was first discovered in Australia in 1874 (Ploetz & Pegg, 1997). However, it was not widely recognized until the epidemic occured in Panama in 1890. Ever since, *Fusarium* wilt gradually became a threat in many banana-growing regions. Severity of the disease was well-demonstrated by the Foc outbreak on *M. acuminata* cv. ‗Gros Michel' (AAA) in Central American and Caribbean in 1940s-1950s. Prior to 1940s, *M. acuminata* cv. ‗Gros Michel' was an economically important cultivar in the regions and was exported to many other countries. Devastated by the decline in the production and export pattern of *M. acuminata* cv. ‗Gros Michel' around 1950s-1960s, the cultivar was replaced by *M. acuminata* cv. Cavendish (AAA) (Ploetz, 2006a; Stover, 1962). The estimated loss in the ‗Gros Michel' cultivar trade due to the *Fusarium* wilt cases up to 1960 was worth USD 400,000 (Ploetz, 2005). However, if one were to convert the figure to 2016 dollars, it would total up to about USD 3 billion. The history of *Fusarium* wilt has come a long way with the recent outbreaks in Oman, Jordan, Mozambique, Pakistan and Lebanon indicating at the persistency of the disease (Table 2.4).

2.3.2 Geographical distribution

The occurrences of *Fusarium* wilt have been documented from various parts of the world. These include South East Asian countries such as Malaysia (Jamaluddin et al, 2001), Indonesia (Muharam, 1991), Thailand (Moore et al., 1995; Ploetz et al., 1996)

and Vietnam (Nhi, 1997). It is also prevalent in Southern Asian countries including India (Chandra, 1991; Thangavelu & Mustaffa, 2010) and Pakistan (Ordoñez et al., 2015), Eastern Asia (China and Taiwan), Western Asia (Jordan and Lebanon) (Butler, 2013; García-Bastidas et al., 2014; Ordoñez et al., 2015), South Africa and South America (Moore et al., 1995; Ploetz & Pegg, 1997).

Table 2.4: Significant events that took place in the history of *Fusarium* wilt (Bancroft, 1876; History of Panama disease, n.d.; Ordoñez et al., 2015; Pérez-Vicente, 2014; Ploetz, 2005; Snyder & Hansen, 1940; Stover, 1962)

Year	Description
1874	First disease discovery by Dr. Joseph Bancroft in Australia. He speculated a type of fungus might have caused the disease based on microscopic examinations
1890	First case reported in Panama and Costa Rica
1940	Snyder and Hansen suggested the pathogen be renamed as *Fusarium oxysporum* f. sp. *cubense*
1890-mid 1950s	*Fusarium* epidemic (race 1) in Central and Southern America wiped out more than 50,000 ha of 'Gros Michel' plantations. 'Gros Michel' was replaced with Cavendish as the major cultivar for trade
1911	First report in India
1916	First report in Java, Indonesia
1920	First report in the Philippines
1953	First report in Malaysia
1967	First report in Taiwan
1990-present	Spread of Foc tropical race 4 (FocTR4) severely affected the banana production worldwide
1994	First FocTR4 case on Cavendish
Mid 1990s	Severe epidemics in Malaysia and Indonesia
1996	First report on infected Cavendish in Panyu, Guangdong, China
1997-1999	Three FocTR4 outbreaks in sites near Darwin (Northern Territories), Australia
2000	Epidemic in Southern China severely affected Cavendish plantation
2002	FocTR4 destroyed about 60,000 ha plantation in China
2013	Reported FocTR4 cases in Oman, Jordan and Mozambique indicated that the pathogen has spread outside the common Asian skirt
2015	Reported FocTR4 cases in Pakistan and Lebanon

2.3.3 Race classification

To date, there are no standardized groupings of Foc isolates based on their genetic makeup. Race, by far, is the most widely used system for Foc classification. Races of *Fusarium oxysporum* isolates are determined by the ability of the strains to cause infection on specific host cultivars (Fourie et al., 2011; Waite & Stover, 1960) (Table 2.5). Prior to 1980s, only races 1, 2 and 3 of *Fusarium oxysporum* f. sp. *cubense* were reported until race 4 was discovered in Australia (Leong, Latiffah & Baharuddin, 2010; Mayers, 1983), Taiwan (Su, Hwang & Ko, 1986) and Africa (Leong, Latiffah & Baharuddin, 2010).

Table 2.5: List of banana cultivars/plantains affected by different Foc races

Race	Infected cultivars	Reference
1	• _Gros Michel‘ (AAA)	Stover, 1962
	• _Silk‘ & _Pome‘ (AAB) • _Awak‘ (ABB) • _Maqueño‘ (AAB) • Hybrid I.C.2 (AAA)	Ploetz, 2000
2	• _Bluggoe‘ (ABB), _Monthan‘ (ABB) and other cooking bananas and plantains • Some hybrid tetraploids (eg: _Bodles Altafort‘)	Thangavelu et al., 2012
3	• *Heliconia* species	Waite, 1963
4	• Cavendish (AAA) and race 1 and 2-affected cultivars	Daly & Walduck, 2006
	• _Mas‘ (AA)	Thangavelu et al., 2012
	• _Lilin‘ (AA)	Ploetz, 2006b

Race 4 of Foc is recognized as the most virulent and can be further divided into tropical (TR4) and sub-tropical (SR4) variants (Leong, Latiffah & Baharuddin, 2010; Ploetz, 2006a). Subtropical race 4 isolates cause infection on plants that are grown in imperfect environment such as cool temperature, poor soil and under stress conditions. Tropical race 4 of Foc, on the other hand, is able to infect banana plants regardless of the pre-disposing conditions (Thangavelu et al., 2012).

TR4 infestations were predominantly observed in Taiwan, Indonesia, Malaysia, China, the Philippines and northern Australia (Butler, 2013). Ploetz (2015a) reported that only Indonesia, Malaysia and Taiwan have the estimated figures for loss suffered due to TR4 cases which were USD 121 million and USD 253 million (respectively described by Hermanto et al. 2011 and Peng et al. 2013, as cited in Aquino et al., 2013). He also added that a more significant number could have been approximated in Cavendish-producing countries like China and the Philippines. The recent outbreaks of TR4 in Mozambique and Jordan were claimed as the first confirmed reports of TR4 cases outside those regions (García-Bastidas et al., 2014). Now that the disease has spread outside the common regions, without any proper control, it is no longer a surprise if the disease paved its way to Latin America, infamously known as the largest banana exporter in the world (Krivonos, 2012).

2.3.4 Symptoms

Symptoms of infected plants can be observed both externally and internally, though the former are more obvious and are easier to be spotted. Physically, wilting symptoms and discoloration will appear in older leaves that grow at the lower part of the stalk, closer to pseudostem before moving to younger leaves. Initially appear as light yellowish, the color of the infected leaves will turn bright yellow. As the disease

progresses, the leaves will completely wilt out (Ploetz, 2000). At this stage, wilting leaves that tumble at their petioles will form _skirt' appearance of dead leaves (Figure 2.3). In contrast to this _skirt' formation, the unaffected, healthy leaves will remain erect upwards forming a _spiky' appearance. The discoloration may spread to the pseudostem but to date, there is no report on disease progressing to fruits. Other symptoms include wrinkles and deformation in the lamina of the leaves. Interestingly, as much as the leaves and pseudostem are affected, the plants will continue growing for some time before they eventually die (Moore et al., 1995).

Classic internal symptoms involve discoloration in the vascular tissues (Figure 2.4), starting at the initial infection site, which is the root. Pathogen invasion in the xylem vessels of the roots triggers the vascular occluding response of the plants. In the case when the host is unable to curb the spread of the fungus, the infection will move to the rhizome (Ploetz & Pegg, 2000; Stover, 1962).

Figure 2.3: External leaf symptoms of Foc-infected banana plants. The symptoms vary from mild yellowing to severe wilting that eventually cause the stalks to collapse (Pictures by Prof. Dr. Baharuddin Salleh)

Figure 2.4: Internal symptoms of Foc-infected banana plants. Internal Foc infection is primarily described by the discoloration of the pseudostem (Picture by Prof. Dr. Baharuddin Salleh)

2.3.5 *Fusarium*-host interactions

Without any stimulates from suitable plant hosts, Foc spores can reside on plant surfaces as macroconidia or remain latent in the soil in the form of chlamydospores (Ravishankar et al., 2011). However, when triggered by exudates produced by banana hosts, the resting spores germinated and started to penetrate through the host's root tips. Germinated spores, or hyphae, initially infect the lateral roots before they advance into larger roots and arrive at the xylem tissue via the cortex. The infection eventually progresses from roots towards rhizome and later pseudostem (Daly & Walduck, 2006; Rishbeth, 1955; Swarupa, Ravishankar & Rekha, 2014). Germinated hyphae pass through the perforated end wall of one xylem vessel into another and continue the disease cycle by producing more spores. The host's responses upon interaction with the pathogen can be categorized into three general conditions. The host is regarded to be resistant if it successfully curbed the attack and prevented the infection. A tolerant host would be able to minimize the disease from spreading while not necessarily eliminating it. In the case where the pathogen successfully disrupts the plant's defense and advances, the host is said to be susceptible (Swarupa, Ravishankar & Rekha, 2014).

The initial recognition of the pathogen-associated molecular patterns (PAMPs)/microbe-associated molecular patterns (MAMPs) involves membrane-embedded pathogen recognition receptors (PRR) that leads to downstream defense response. The extracellular receptors, such as CERK1 and CEBiP which recognize the chitin structure of the fungus (Miya et al., 2007; Shinya et al., 2012), are said to be less specific (Yadeta & Thomma, 2013). Within the host cell, other intracellular receptors (eg: nucleotide-binding leucine-rich repeat (NB-LRR) proteins) take charge in the recognition of the invader's effectors (Huang & Lindhout, 1997). Altogether, the pathogen recognition leads to PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI) by the host defense (Dodds & Rathjen, 2010). Recent advancement in

the genomics, transcriptomics and proteomics tools allow scientists to verify whether or not this general plant immunity concept applies for *Fusarium*-banana interaction as well. It is found that the defense responses of banana against Foc also involve physical and chemical changes whose players interplay with each other in complex activation pathways (Li et al., 2012; Li et al., 2013b; Wang et al., 2012).

2.3.5.1 Production of reactive oxygen species (ROS)

The underlying interaction between reactive oxygen species (ROS) compounds and ROS scavenging enzymes is often associated with early defense response against invading pathogens. Production of ROS like hydroxyl radical (-OH) and hydrogen peroxide (H_2O_2) increases after initial infection leading to activation of other signaling pathways. Excessive ROS products, nevertheless, may lead to cell damage and thus is controlled by ROS scavenging enzymes (Mittler et al., 2004; Swarupa, Ravishankar & Rekha, 2014). This phenomenon clearly explains the differential expression of ROS scavenging enzyme like catalase in Foc-infected banana (Ravishankar et al., 2011; Van den Berg et al., 2007).

2.3.5.2 Physical modification of the host's cell wall

In preventing penetration via cell wall, the host strengthens its physical mechanism, mainly by the production of gels and tyloses as well as disintegration of vessels in the xylem lumena (Daly & Walduck, 2006). The hosts also generate phenolic compounds, which are the monomers of lignin, to promote lignification and suppress the attack at the current site. Using suppression subtractive hybridization (SSH), Van den Berg and co-workers (2007) showed that peroxidase, a cell wall-strengthening enzyme, was up-regulated in the roots of FocTR4-infected tolerant banana cultivar 'GCTCV-218' (AAA). This was also exemplified by Ravishankar and co-workers (2011) in their study with Foc-tolerant *M. acuminata* ssp. *burmannicoides* 'Calcutta-4'. At the same time, the

plants also target the cell-wall degrading enzymes by releasing more inhibitor proteins such as polygalacturonase inhibitor protein (PGIP) (Ravishankar et al., 2011).

2.3.5.3 Production of defense-related genes

As for defense-related genes, disease resistance-genes (R-genes) are of particular interest and have been fairly covered in *Fusarium*-banana interaction studies (Chen et al., 2007; Miller et al., 2008; Peraza-Echeverria et al., 2008; Peraza-Echeverria et al., 2009; Sutanto, Sukma & Hermanto, 2014). The expression of other defense-related genes, mostly of PR-genes, is also notably increased. For example, differential expression of *CERK1*, *CEBiP* and *PR-1* was observed between Foc-infected tolerant *M. acuminata* cv. _Yueyoukang' and susceptible *M. acuminata* cv. _Brazilian' (Bai et al., 2013). Similar up-regulated *PR-1* pattern had also been displayed in tolerant *M. acuminata* _GCTCV-218' (Cavendish) (Van den Berg et al., 2007) and *M. acuminata* cv. _Baxi' (Cavendish) (Li et al., 2013b). In the same study, Li and co-workers (2013a) also observed up-regulation of other PR genes including *PR-5* and unannotated *PR* candidates upon Foc infection. Wang and co-workers (2012) revealed another consistent discovery of PR proteins abundance following Foc race 4 (FocR4) infection in *M. acuminata* cv. _Brazillian' (Cavendish) that include PR-1, PR-5 as well as PR-10. PR-3 (β-1,3-glucanase) and PR-4 (endochitinase) are another common PR proteins associated with *Fusarium* infection in banana (Li et al., 2013b; Van den Berg et al., 2007).

2.3.5.4 Summary of the plant's response mechanism against pathogen attack

In summary, pathogen-associated molecular patterns (PAMPs)/microbe-associated molecular patterns (MAMPs)/Damage-/danger-associated molecular pattern (DAMPs) released by the fungus are recognized by the extracellular, membrane-embedded pathogen recognition receptors (PRR) (Figure 2.5). The recognition activates MAMP-triggered immunity (MTI) which leads to downstream defense activation responses that include physical modification of the host's cell wall, release of defense-related proteins such as PR proteins, production of ROS, various signal transduction pathways and secondary metabolites. In a successful attack, the activation of MTI can be suppressed by the pathogen's toxins/effectors leading to effector-triggered susceptibility (ETS). Inside the cytoplasm, effectors are recognized by one of the intracellular receptors (eg: NB-LRR proteins) that elicits the effector-triggered immunity (ETI), which is an upgraded version of MTI that leads to hypersensitive response (HR) at the infection site.

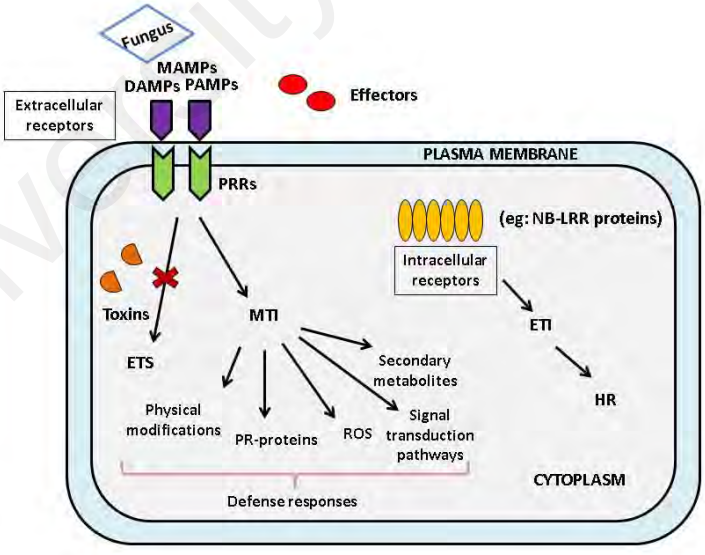


Figure 2.5: Elicitation of plant's defense pathways in response to fungus attack

2.4 Pathogenesis-related (PR) 10 protein

2.4.1 General background

Pathogenesis-related (PR) proteins are proteins that are induced by various biological and non-biological conditions including pathogen invasion, abiotic stresses, hypersensitive responses (HR), systemic acquired resistance (SAR), signaling molecules, chemical compounds or any related situations (Agarwal & Agarwal, 2014; Liu & Ekramoddoullah, 2006; Van Loon & Van Strien, 1999). Early studies in tobacco discovered five classes of PR proteins, PR-1 to PR-5 (Bol et al., 1990) before extended research and re-arrangement of the PRs nomenclature revealed new sub-groupings. To date, 17 subgroups of PR proteins have been identified in monocotyledonous, dicotyledonous and conifers based on similarity in amino acid sequences, serological relationship and enzymatic activity (Christensen et al., 2002; Liu & Ekramoddoullah, 2006; Van Loon et al., 1994) (Table 2.6).

2.4.2 Structure analysis and subclasses of PR-10 proteins

PR-10, one of the members in the PR protein families, was first associated with low molecular weight, acidic proteins found in cultured parsley cells following treatment by fungal elicitor (Somssich et al., 1988). Similarly, Fristensky and co-workers (1988) reported a group of small disease resistance response proteins that were highly expressed in *Pisum sativum* cv. Alaska (pea) upon induction by pathogens (*Fusarium solani* and *Pseudomonas syringae* pv *pisi*) and fungal elicitor (chitosan). A new subclass of PR-10, *Bet v 1*, was later discovered which has become the basis of current PR-10 studies. *Bet v 1* encodes for major birch pollen allergen and shared homology (≈55%) with the previously found pea disease resistance gene despite being derived from two unrelated plant species (Breiteneder et al., 1989; Fristensky et al., 1988). Rapid evolvement of PR-10 studies establish more subclasses of PR-10 which

Table 2.6: Subclasses of pathogenesis-related (PR) proteins except for PR-10 (Agarwal & Agarwal, 2014)

Pathogenesis-related (PR) group	Description	References
PR-1	• Roles are still unknown • Expression can be affected by salicylic acid (SA) and pathogen • Commonly used as marker for systemic acquired resistance (SAR)	Mitsuhara et al., 2008
PR-2	• Consists of β-1,3-glucanase • Catalyzes the hydrolysis of β-1,3-glucans, which is a component of fungal cell wall • Generates elicitors which contribute to host's defense against fungal	Van Loon & Van Strien, 1999
PR-3	• Hydrolyzes the β-1,4 glycosidic bonds that connects the *N*-acetylglucosamine residues of chitin • Possesses antimicrobial activity	Van Loon & Van Strien, 1999
PR-4	• Endochitinases with C-terminal domain called Barwin • Induced upon pathogen attack	Neuhaus et al., 1996
PR-5	• Includes permatins, osmotins, zeamatins, thaumatin-like protein (TLP) • TLP: Causes osmotic breakage of transmembrane pore on the fungal plasma membranes	Sels et al., 2008
PR-6	• Proteinase inhibitors • Disrupts utilization of fungal's lytic enzymes and completion of replication cycle	Sels et al., 2008
PR-7	• Acts as endoproteinase • Characterized in tomato	Vera & Conejero, 1988
PR-8	• Chitinases	Van Loon & Van Strien, 1999
PR-9	• Peroxidases • Key enzymes in cell wall building processes • Catalyzes lignin deposition and strengthen plants' cell wall	Taheri & Tarighi, 2011
PR-11	• Tobacco „class V" chitinase	Melchers et al., 1994
PR-12	• Plant defensins • Precise roles are unclear, but predicted to inhibit the growth of fungi and yeasts	Sels et al., 2008
PR-13	• Thionins • Antimicrobial effects by causing permeabilization of cell membranes	Sels et al., 2008
PR-14	• Lipid transfer proteins • Involves in cutin synthesis, β-oxidation, plant defense signaling and plant defense	Sels et al., 2008
PR-15 & PR-16	• Isolated from barley • Induced by *Erysiphe graminis* • Possesses oxalate oxidase property	Ebrahim, Usha & Singh, 2011
PR-17	• Tobacco PRp27 as type member	Ebrahim, Usha & Singh, 2011

include major latex protein (MLP) (Chen & Dai, 2010; Lytle et al., 2009; Sun et al., 2010), cytokinin-specific binding proteins (CSBPs) (Pasternak et al., 2006; Zubini et al., 2009), (S)-norcoclaurine synthase (NCS) (Lee & Facchini, 2010) and Hyp-1 from *Hypericum perforatum* (Michalska et al., 2010; Śliwiak, Dauter & Jaskolski, 2013) despite having fairly low sequence similarity with the classic PR-10 (Fernandes et al., 2013). Liu and Ekramoddoullah (2006) simplified the PR-10 classification into two groups namely intracellular pathogenesis-related protein (IPR) and (S)-norcoclaurine synthase (NCS).

Size of the coding sequences (cds) of *PR-10* genes varies between 453-489 bp encoding for 151-163 amino acid residues (Agarwal & Agarwal, 2014; Fernandes et al., 2013). They are characterized as small, acidic proteins of about 15-18 kDa with protease-resistant property (Agarwal & Agarwal, 2014; Liu and Ekramoddoullah, 2006; Walter et al., 1990). This is particularly true for IPR proteins. Cds of *PR-10* NCS group members, on the other hand, are generally longer (633-696 bp) with predicted molecular weight of 26 kDa. PR-10 NCS group members shared 28-38% sequence identity with IPR proteins (Agarwal & Agarwal, 2014).

The genomic constitution of most PR-10 proteins consists of two exons interrupted by an intron (Lebel et al., 2010; Liu & Ekramoddoullah, 2006). At least four PR-10 subclasses are reported in apple (*Malus domestica*) which lack of intron and appear to be a unique case of PR-10 (Gao et al., 2005). Unlike other extracellular PR proteins, PR-10 does not have specific signal peptide sequences. Thus, the proteins will not be secreted from cells and are localized in the cytosol (Jain et al., 2012).

Typically, PR-10 proteins share specific regions like Y-shaped hydrophobic ligand binding site (Marković-Housley et al., 2003; Radauer, Lackner & Breiteneder**,** 2008) and glycine-rich loop motif (Liu & Ekramoddoullah, 2006). The glycine-rich loop motif is highly conserved in PR-10 sequences even in the distant PR-10 subfamilies such as

CSBP and MLP (Fernandes et al., 2013). Sequence variations of the motif include GxGGxGxxK (Lebel et al., 2010; Xu et al., 2014), GxGxxG (Jwa et al., 2001), GxGGxG (He et al., 2014; Kim et al., 2014) and GxG (Park et al., 2004). The motif is referred as phosphate-binding (P-loop) due to the strikingly comparable features found in nucleotide-binding proteins. The loop is predicted to be the binding sites for ATP and GTP and presumed to be responsible for the ribonuclease activity of the protein (Liu & Ekramoddoullah, 2006). However, inconsistency has been observed in the ribonuclease properties of PR-10 in which some of the proteins do not have affinity for ATP (Koistinen et al., 2005).

2.4.3 General three-dimensional (3D) structure

Three-dimensional (3-D) structures of PR-10 proteins have been demonstrated mostly from classical and Bet v 1 groups (Biesiadka et al., 2002; Gajhede et al., 1996; Kim et al., 2014; Pasternak et al., 2005). Despite the small size and dense folding format, the proteins are comprised of several components that include α-helices (α1-3), anti-parallel β-sheets (β1-7) and loops. α3 helix and β1 sheet are both attached to the C-terminal and N-terminal of the protein, respectively. Two short α1 and α2 helices are located next to each other and are separated from α3 by anti-parallel, successive β2-β7 sheets. β2-β7 sheets are organized in a successive manner enabling contact between β1 and β7 again and forming five hairpin loops. The major opening to the internal cavity of PR-10, where the ligands attach, are guarded by odd-numbered loops (L3-L9) (Figure 2.6 and Figure 2.7). There are also some other entrances available that allow access to the internal pocket of the protein that is largely hydrophobic (Fernandes et al., 2013).

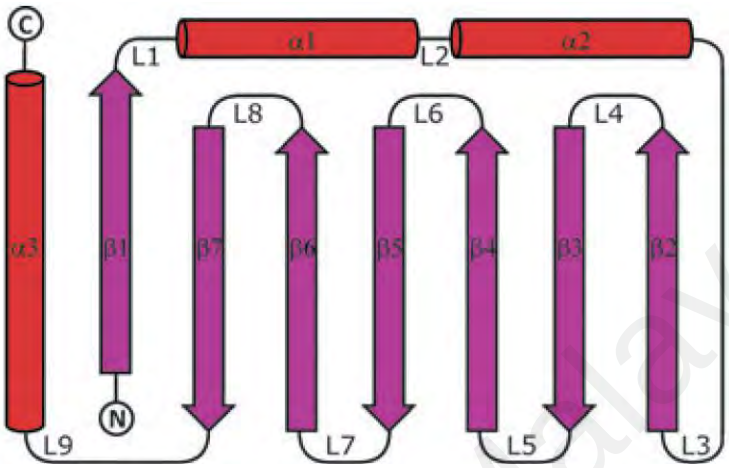


Figure 2.6: α-helicases and β-sheet structures of PR-10 protein. The α-helicases (α1-3) (cylinders) and β-sheet strands (β1-7) (arrows) are linked by structurally organized loops (L1-9) (adapted from Fernandes et al., 2013)

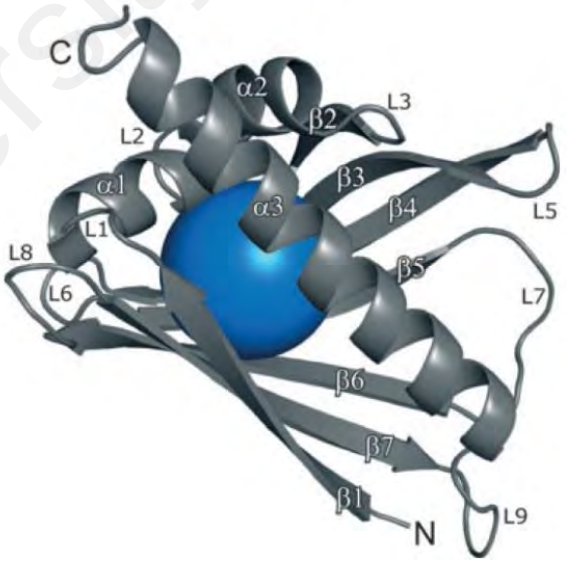


Figure 2.7: 3-D representation of PR-10 protein. The secondary structure elements are labeled and the protein cavity is shown by a sphere (adapted from Fernandes et al., 2013)

2.4.4 Expression pattern of *PR-10*

2.4.4.1 Spatial expression

In general, in the absence of external stimuli, *PR-10* transcript is barely detected or expressed at basal level. In *Oryza sativa* L., Jwa et al. (2001) observed no jasmonate inducible *PR-10* (*JIOsPR10*) transcript in healthy leaves prior to stress induction. Differential gene expression is also observed in different plant parts. For instance, *SsPR10* of *Solanum surattense* is highly expressed in the roots but has a weaker expression in the stems (Liu et al., 2006). By comparing three different parts of soybean plants (*Glycine max*), Xu et al. (2014) reported a more constitutive and stronger expression of *GmPR10* in the leaves compared to the roots and stems.

2.4.4.2 Biotic- and abiotic-induced expression

Differential expression upon biotic and abiotic stresses supports the significant roles played by *PR-10* in the plant system, mainly in the defense mechanism pathway and growth regulation. Typically, biotic factors were shown to increase the expression of *PR-10*. However, abiotic factors and signaling molecules showed mixed results in influencing the expression pattern of *PR-10*. For example, the expression of *PR-10* in plants can be both induced and suppressed in response to salicylic acid (SA) and abscisic acid (ABA) (Table 2.7).

Table 2.7: Expression pattern of *PR-10* from various plant species in response to different biotic and abiotic factors

Gene source/donor	Nomenclature	Up-regulated expression by		Down-regulated expression by		References
		Abiotic factors /Signaling molecules	Biotic factors	Abiotic factors/Signaling molecules	Biotic factors	
Oryza sativa	*JIOsPR10*	Jasmonate, salicylate, H_2O_2, light, protein phosphatase inhibitors	*Magnaporthe grisea*	Dark, ethylene, ABA	-	Jwa et al., 2001
Lithospermum erythrorhizon	*LePR1*	Wounding, salicylic acid (SA), aspirin, benzothiadiazole (BTH), H_2O_2, abscisic acid (ABA), jasmonic acid (JA), linolenic acid	*Erwinia stewarti, Pseudomonas syringae, Aspergillus nidulans, Penicillium funiculosum*	-	-	Hwang et al., 2003
Pinus monticola	*PmPR10*	Wounding, cold hardening, methyl jasmonate (MeJa), okadaic acid	*Cronartium ribicola*	SA, ABA	-	Liu, Ekramoddoullah & Yu, 2003
Panax ginseng	*PgPR10-2*	SA, H_2O_2, ABA, NaCl, JA, chilling	*Colletotrichum gloeosporioides, Phytophthora capsici, Alternaria solani*	-	-	Pulla et al., 2010
Glycine max	*GmPR10*	ABA, gibberellic acid (GA_3), SA, H_2O_2,	*Phytophthora sojae*	-	-	Xu et al., 2014
Panax ginseng	*PgPR10-4*	JA	*Botrytis cinerae, Colletotrichum gloeosporoides, Rhizoctonia solani, Phythium ultimum*	ABA (strongly reduced), light, UV, salinity, chilling, SA & H_2O_2 (showed later increase)	-	Kim et al., 2014
Arachis hypogea L.	*AhSIPR10*	NaCl, $ZnCl_2$, mannitol-induced water deficit stress, ABA, MeJa, SA, H_2O_2	-	-	-	Jain et al., 2012

2.4.5 Putative roles in defense mechanism

Elevated expression of PR-10 upon pathogen attack has been observed in many plant species including rice (McGee et al., 2001), plum (El-kereamy et al., 2009), white birch (Swoboda et al., 1995) and oil palm (Al-Obaidi et al., 2014), suggesting its role as anti-bacterial, anti-viral and anti-fungal protein. Moiseyev and co-workers (1994) discovered high sequence similarity between PR-10 and ribonuclease (RNase) isolated from phosphate-deprived ginseng cells. Due to its homologous structure with ginseng ribonuclease, PR-10 is speculated to also have ribonuclease activity. However, no similar report was published with regards to sequence homology between PR-10 and ribonuclease from other plant species except for the P-loop motif found in both proteins (Saraste et al., 1990; Soh et al., 2012).

Besides ginseng, RNase activity of PR-10 has also been shown in other plants including *Lupines albus* (Bantignies et al., 2000), *Arachis hypogaea* L. (peanut) (Chadha & Das, 2006), *Astragalus mongholicus* (Astragali radix) (Yan et al., 2008) and *Zea mays* (Xie et al., 2010). As a type of nuclease that degrades RNA into smaller components, RNase is able to inhibit translational activity and thus, protecting the plant cells from any harmful action induced by the pathogen. However, RNase property of PR-10 has not been finalized due to inconsistent results. For example, LlPR10.1A isolated from yellow lupine does not exhibit RNase catalytic activity unlike its homologous counterpart, LlPR10.1B (Biesiadka et al., 2002). In another study incorporating hyphal extension inhibition assay of PR-10 protein isolated from hot pepper, CaPR-10, 2-3 mm inhibition zone of growing hyphae was observed after infection with *Phytophthora capsici* (Park et al., 2004).

Manipulating the anti-fungal property of PR-10, over-expression of *PR-10* gene in several plants have been reported. For instance, transgenic tobacco and soybean plants harboring soybean *PR-10* (*GmPR-10*) demonstrated improved resistance against

Phytophthora nicotianae and *Phytophthora sojae* infection, respectively (Xu et al., 2014). Similar results were also reported in *Physcomitrella patens* (moss) over-expressing *PpPR-10* following *Phytium irregulare* infection (Castro et al., 2016). In banana, involvement of *PR-10* as a part of defense strategies against *Fusarium* wilt has never been reported. Thus, this project is probably the first to elaborate the component of *PR-10* gene in this plant as well to demonstrate the response of *PR-10*-over-expressing banana plants against *Fusarium* wilt.

2.5 Disease management

After almost 140 years since the first discovery of *Fusarium* wilt in bananas and plantains, no definite solution has been able to solve the problem. Though fungicide is commonly used to control the disease spread, it poses effect on the environment and does not offer total protection from the disease besides showing selective effectiveness on certain races only (Buddenhagen, 2007). Disease control and improvement strategies also become a hard task mainly due to the ―perennial nature" of the plants, the ―polycylic" disease activity (Ploetz, 2015b), high sterility and lack of highly resistant clones particularly in significant subclones such as Cavendish (Aguilar Morán, 2011).

In terms of management, every personnel associated with the farming areas must be adequately equipped with information about the disease since the pathogen can easily be transferred through soils or plant materials attach to equipment, vehicles, clothes or footwear. Good farming practices, that include consistent crop monitoring, biosecurity protocols of entering, working and leaving the plantation, stringent quarantine and movement procedures are required to ensure a disease-free farm.

While complete eradication of *Fusarium* wilt is still questionable and out-of-reach (Gondolini, 2014), a number of disease control strategies have been implemented aiming mainly on improving the plants‘ tolerance or suppressing the infection. At

plantation level, some farmers opt for crop rotation (Li et al., 2011), rice hull burning (Molina et al., 2010) as well as biological soil disinfestation (BSD) (Huang et al., 2015). Biocontrol agents, compound-supplemented soil, somaclonal variants and genetic manipulation via induced mutagenesis and transformation are also among the alternatives that have been suggested to circumvent the threat (Table 2.8).

Table 2.8: Different strategies for controlling the spread of *Fusarium* wilt in banana

Strategy	Examples	Advantages	Disadvantages	References
Biocontrol agents	• *Trichoderma* spp. • *Actinomycetes* • *Pseudomonas* spp. • Non-pathogenic *F. oxysporum* • *Streptomyces griseus* (St 4)	• Environmental friendly	• Inoculum dependent • Mixed results • Mostly ended at laboratory or greenhouse stage	Belgrove, Steinberg & Viljoen, 2011; Cao et al., 2004; Mohd Fishal, Meon & Wong, 2010; Nel et al., 2006; Thangavelu & Jayanthi, 2009; Zacky & Ting, 2013
Exogenous application of certain compounds	• Silicon • Salicylic acid	• Environmental friendly	• Time and energy laborious • Inoculum dependent	Fortunato, Rodrigues & Baroni et al., 2012; Wang et al., 2015
Somaclonal variation	• GCTCV-215-1 (Tai Chiao No. 1) • Formosana • ‗Mutiara' (a *M. acuminata* cv. ‗Rastali' variant)	• Feasible and simple procedures • Fruits produced invited positive market response due to comparable agricultural traits (in certain aspects)	• Large number of samples are required for screening • Laborious • Time-consuming • Uncontrollable frequency of successful clones • Occurrence of epigenetic and off-types variants • Negative traits were sometimes observed (eg: longer maturation period, lower fruit production as well as reduced fruit quality	Hwang & Ko, 1986; Hwang & Ko, 2004; Hwang & Tang, 1999; Tang, Hwang & Jones, 1994
Chemical mutagenesis	• Ethyl methanesulphonate (EMS) • Sodium azide (NaN_3) • Diethyl sulphate (DES)	• Enable genetic alteration	• Require large number of samples for screening • Require optimization of mutagens	Bhagwat & Duncan, 1998a
Physical mutagenesis	• Gamma rays			Bhagwat & Duncan, 1998b

Of these, transgenic approach has become popular and is regarded as one of the most effective strategies (Buddenhagen, 2007; Gondolini, 2014) (Table 2.9). To date, two most widely used transformation methods for plants are *Agrobacterium*-mediated transformation and particle bombardment (Becker et al., 2000; Ganapathi et al., 2001; Maziah, Sariah & Sreeramanan, 2007; Pei et al., 2005). In earlier attempts, sources of explants were usually tissues that resulted in mixture of transformed and non-transformed cells (chimerism). With the introduction of embryogenic cell suspension (ECS), reports showed that chimeric transformants were minimized as the transgene was presumably incorporated into a single cell (Ghag, Shekhawat & Ganapathi, 2012; Mohandas et al., 2013). ECS, derived from meristems (Dhed'a et al., 1991) and male inflorescence (Côte et al., 1996), was regenerable and could be maintained over a long period. Nevertheless, the major bottlenecks for ECS are its long establishment process, low plant regeneration efficiency and declining embryogenic potential over time (Jain, 2008).

The success of gene integration is commonly confirmed by polymerase chain reaction (PCR) or Southern blot. Expression profile can then be determined by either qualitative analysis like reverse transcriptase PCR and northern blot or by quantitative measure such as real-time PCR. Protein expression is analyzed by Western blot, enzyme-linked immunosorbent assay (ELISA) or two-dimensional gel electrophoresis (2D electrophoresis). More importantly, in any transgenic plant research involving pathogen infestation, disease response of the new clones must be evaluated. To date, no standardized Foc bioassay challenge protocols are available. Each experiment showed variation mostly in terms of inoculum concentration, treatment hour, age of plants and disease scoring index. Establishment of a reference assay will be a great contribution towards the advancement of transgenic banana research against *Fusarium* wilt.

Table 2.9: Development of transgenic banana clones against *Fusarium* wilt

Cultivar	FOC isolate	Donor	Transgene	Approach	References
Taijiao (AAA)	Race 4	Human	Lysozyme	• Particle bombardment and *Agrobacterium*-mediated transformation • Explant: Corm slice	Pei et al., 2005
Rastali (AAB)	N/A	*Oryza sativa* (rice) and *Glycine max* (soybean)	chitinase and β-1,3-glucanase	• Co-bombardment • Explant: Tiny single meristem bud	Sreeramanan et al., 2006
Rastali (AAB)	Race 1	*Glycine max* (soybean)	β-1,3-glucanase	• *Agrobacterium*-mediated transformation • Explant: Single bud	Maziah, Sariah & Sreeramanan, 2007
Pei Chiao (AAA) and Gros Michel (AAA)	Race 4	*Arabidopsis thaliana*	Arabidopsis root-type ferredoxin gene (Atfd3)	• *Agrobacterium*-mediated transformation • Explant: Multiple bud clumps (MBC)	Yip et al., 2011
		N/A	Ferredoxin-like protein (pflp)		
Lady Finger (AAB)	Race 1 (VCG 0124/5)	Chicken, *Caenorhabditis elegans* and human	Anti-apoptosis genes (*Bcl-xL*, *Ced-9* and *Bcl-2* 3‘ UTR)	• Co-cultivation of *Agrobacterium*-embryogenic cell suspension (Apoptosis-related gene)	Paul et al., 2011
Rasthali (AAB)	Race 1	*Petunia hybrida*	Defensin	• Co-cultivation of *Agrobacterium*-embryogenic cell suspension (*Petunia* defensin)	Ghag, Shekhawat & Ganapathi, 2012
Nangka (AAB)	Race 4	*Oryza sativa* (rice)	Thaumatin-like protein	• Particle bombardment on cauliflower-like bodies' • Explant: meristemic male inflorescence	Mahdavi, Sariah & Maziah, 2012
Furenzhi (AA)	Race 4	*Trichoderma harzianum*	endochitinase gene chit42	• Co-cultivation of *Agrobacterium*-embryogenic	Hu et al., 2013

				cell suspension	
Rasthali (AAB)	Race 1	*F. oxysporum* f. sp. *cubense*	intron hairpin RNA-mediated expression of two important Foc genes: 1. ftf1: *Fusarium* transcription factor 1 2. vel (velvet): involved in the regulation of sexual and asexual development, secondary metabolism and virulence in *Fusarium*	• Co-cultivation of *Agrobacterium*-embryogenic cell suspension	Ghag, Shekhawat & Ganapathi, 2014
Rasthali (AAB)	Race 1	Rasthali	Cell-death related genes *(MusaDAD1*, *MusaBAG1* and *MusaBI1)*	• Co-cultivation of *Agrobacterium*-embryogenic cell suspension	Ghag, Shekhawat & Ganapathi, 2014a
Rasthali (AAB)	Race 1	*Stellaria media* (common chickweed)	Seed defensin (Sm-AMP-D1)	• Co-cultivation of *Agrobacterium*-embryogenic cell suspension	Ghag, Shekhawat & Ganapathi, 2014b

Table 2.9 continued

CHAPTER 3: MATERIALS & METHODS

3.1 Materials

When this study started, the banana genome project was still on-going. Thus, pathogenesis-related 10 gene (*PR-10*) of *M. acuminata* cv. _Mutiara' (*MamPR-10*) was selected as the reference sequences for this experiment. The *MamPR-10* sequences were obtained from a microarray study to compare gene expression of two banana cultivars, susceptible *M. acuminata* cv. _Rastali' and tolerant *M. acuminata* cv. _Mutiara', upon infection with *Fusarium oxysporum* f. sp. *cubense* (Foc) (Lim, 2006). In that study, high quality RNA was extracted from roots of both *M. acuminata* cv. _Rastali' and *M. acuminata* cv. _Mutiara'. Complementary DNA (cDNA) libraries of both cultivars were constructed using ZAP-cDNA® Gigapack® III Gold cDNA library synthesis kit (Stratagene, U.S.A.). cDNA inserts in the range of 500 bp and 3 kb in-length were ligated into pBluescript SK (-) and maintained in UniZAP vector plasmid (lambda ZAP II vector predigested with EcoR1 and Xho1), packaged using Gigapack III Gold packaging extract and plated on NZY plates. The core plaques containing the cDNA insert were used as templates in the PCR amplification using T3 and T7 primers. Clones with cDNA inserts which showed multiple amplified bands and were not of the required size (shorter than 500 bp or longer than 3 kb) were discarded. Out of 5,000 plaques cored and amplified, a total of 4,000 clones (80%) from each *M. acuminata* cv. _Mutiara' and *M. acuminata* cv. _Rastali' cDNA libraries fulfill the required criteria (single band in the range of 500 bp and 3 kb). These clones were then directly amplified from the plaques, purified and prepared for spotting onto slides for subsequent microarray expression analysis. Hybridization experiment of non-infected *M. acuminata* cv. _Rastali' target and *Fusarium*-infected *M. acuminata* cv. _Mutiara' was performed. Data was generated from ScanArray Express software (PerkinElmer, U.S.A.) and analyzed using GeneSpring 6.1 analysis software (Silicon Genetics, U.S.A.). From the

analysis, *PR-10* was selected by comparing the differential gene expression of *M. acuminata* cv. ‗Mutiara' infected for 24 hours against non-*Fusarium* infected *M. acuminata* cv. ‗Rastali'. In total, a number of 50 genes which demonstrated more than 2-fold up-regulated and more than 2-fold down-regulated in expression level were identified. *MamPR-10* sequence was then used to design the primers for rapid amplification of cDNA ends (RACE) experiment of *M. acuminata* ssp. malaccensis Based on the assembled RACE sequences, the full-length sequence of *PR-10* cDNA was amplified from young leaves of *M. acuminata* ssp. malaccensis grown in PALAPES, University of Malaya (voucher number: KLU48226). This gene is designated as *MaPR-10*.

pCAMBIA1304 (www.cambia.org) was used in this study as plant expression vector whilst TOPO® vector was used for direct cloning of gene of interest (GOI). *M. acuminata* cv. ‗Berangan' cells suspension was provided by Plant Biolotechnology Research Laboratory (PBRL), University of Malaya and was maintained at 25±2°C on a rotary shaker at 90 rpm prior to transformation experiment (Chin et al., 2014).

Tissue culture-derived *M. acuminata* cv. ‗Berangan' plants used for establishment of baseline disease response were obtained from CEBAR, University of Malaya, Malaysia. The plantlets were cultured in MS medium for one month before they were transferred to M5 (Appendix B9xi) for root development. The plantlets were acclimatized in sterile mixed soil and sand (3:1) for about two months under greenhouse conditions (27±2°C) with 16 hours photoperiod. Healthy plants with the following requirements were selected for bioassay experiment: 1) minimum of 3-5 green leaves, 2) vigorous white roots of at least 5 cm in length, and 3) sturdy stem with about 0.5 to 1.0 cm in diameter.

Foc race 4 (FocR4) isolate, namely C1 HIR, was provided by Prof. Dr. Baharuddin Salleh of Plant Pathology and Mycology Laboratory, School of Biological Science, Universiti Sains Malaysia (USM), Malaysia, and was maintained as pure culture at the

Phytomycology Laboratory, University of Malaya. The fungus was isolated from the inner stem of an infected *M. acuminata* cv. Cavendish in Kuala Terengganu, Terengganu, Malaysia. The overview of this study is summarized in Figure 3.1.

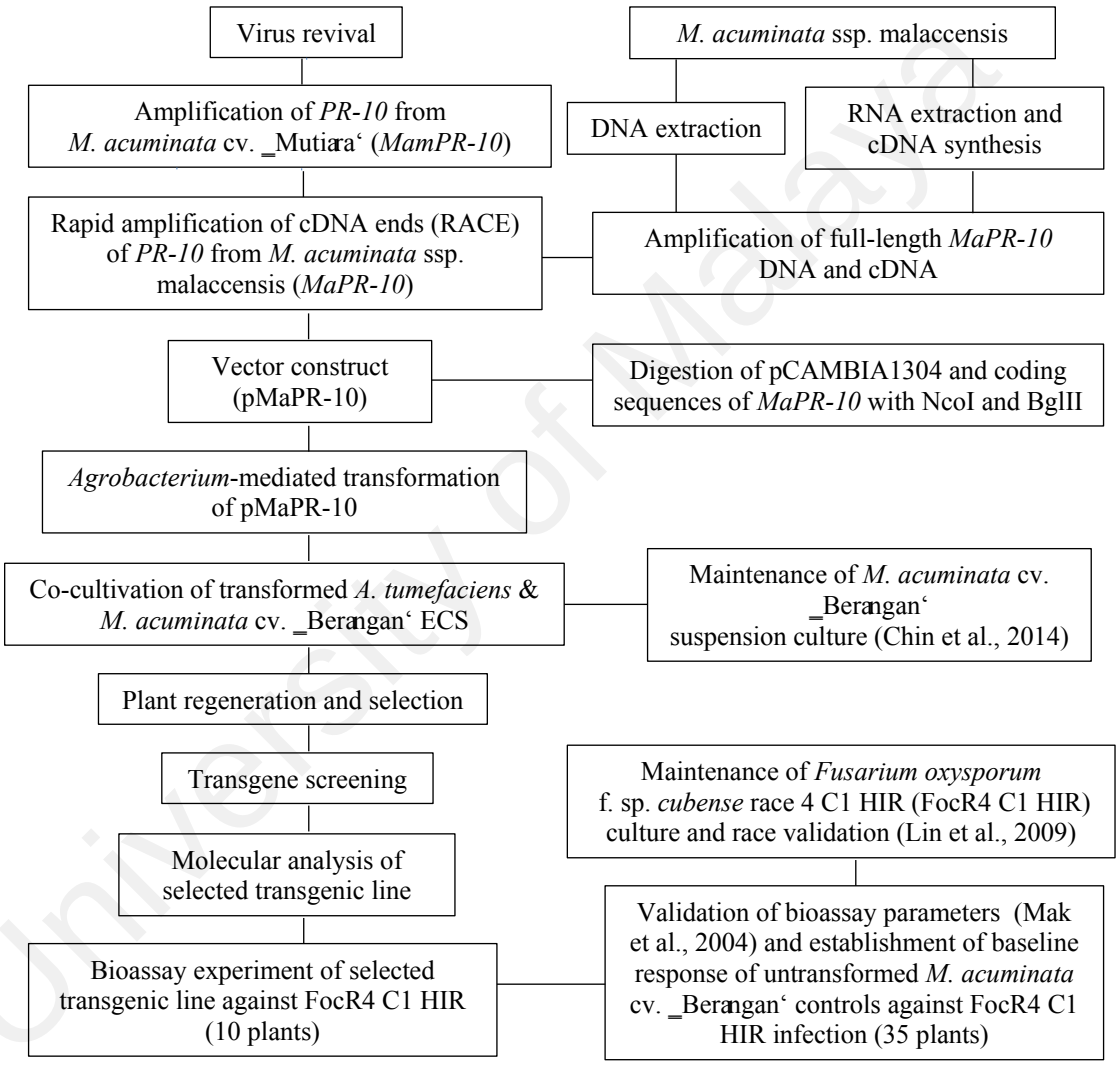


Figure 3.1: Project overview

3.2 General molecular techniques

3.2.1 DNA extraction using CTAB method

DNA extraction was performed according to modified CTAB method (Al-Obaidi et al., 2010). About 0.3-0.5 g of young banana leaf was weighed out and ground in a chilled mortar. The powder was collected in 1 ml CTAB buffer (Appendix B1ii) pre-heated at 65°C for 10 min. The tube was then vortexed and heated at 65°C for 10 min. Equal volume of chloroform:isoamyl alcohol (C:I) (24:1) (Thermo Fisher Scientific, U.S.A.) was added before the mixture was vortexed for 3 min. The mixture was then centrifuged for 15 min at 11,200 *g*. The aqueous phase (top layer) was transferred into newly labeled, sterile 1.5 ml microcentrifuge tube. Washing step was repeated three times. In the final washing step, the amount of aqueous phase transferred was recorded. A total of 0.1 V of 3 M sodium acetate (BDH, U.K.) (Appendix B3viii) was added and the mixture was topped up to 1.5 ml using ice-cold absolute ethanol (Sigma-Aldrich, U.S.A.). After overnight incubation at 4°C, the tube was centrifuged at 4°C, 11,200 *g* for 30 min. Supernatant was discarded and the pellet was rinsed in 1 ml of ice-cold 70% (v/v) ethanol (Sigma-Aldrich, U.S.A.). The mixture was centrifuged at 4°C, 11,200 *g* for 7 min. Supernatant was discarded and the pellet was air-dried. The pellet was dissolved in 30 µl of sterile distilled water (sdH_2O) and the extracted DNA was kept at -20°C prior to RNase treatment. RNase treatment was performed by adding 1 µl of RNase A (20 mg/ml) (Thermo Scientific, U.S.A.) into the extracted DNA. The mixture was mixed well and incubated at 37°C for 30 min to 1 hour. RNase-treated DNA sample was stored at -20°C until further use.

3.2.2 RNA extraction using CTAB method

Total RNA was extracted from young banana leaves using modified protocol described previously (Al-Obaidi et al., 2010). Briefly, RNA was precipitated out after 2- to 3-night incubation at -80°C. Following centrifugation, the pellet was dissolved in 20 µl of DEPC-treated water and the extracted RNA was kept at -80°C prior to DNase treatment.

3.2.3 DNase treatment

DNase treatment was performed according to modified manufacturer's manual (Thermo Scientific, U.S.A). A maximum of 2 µg of extracted RNA sample, 5 µl of DNase I, 2 µl of 10 X DNase I buffer and nuclease-free water (up to 20 µl) were added in a sterile 1.5 ml microcentrifuge tube and placed on ice. All components were mixed briefly and incubated at 37°C for 45 min. A total of 0.1 V EDTA (50 mM) was added and mixed well to stop the reaction. The reaction mixture was then heated at 65°C for 10 min for inactivation. The treatment was repeated until all DNA traces were eliminated. DNase-treated RNA sample was stored at -80°C until further use.

3.2.4 Evaluation on the integrity of DNA and RNA extract

A mixture of 1 V of extracted RNA, 1 V of 2 X loading dye (Thermo Scientific, U.S.A.) and 1 V of DEPC-treated water was prepared and incubated at 65°C for 10 min. For evaluation of DNA quality, about 1 µl of 6 X loading dye (Thermo Scientific, U.S.A.) was added into 3 µl of DNA extract. The mixtures were loaded onto 1% (w/v) agarose gel and electrophoresed at 125 volts for 25 min. The gel was briefly stained with 0.5 µg/ml ethidium bromide and de-stained in tap water for about 3-5 min or up to

30 min for DNA and RNA, respectively. The gel was viewed under UV light to evaluate the success of the extraction. Purity ($A_{260/280}$) and concentration of extracted samples were also determined using NanoDrop 2000 (Thermo Scientific, U.S.A.).

3.2.5 cDNA preparation

First strand cDNA synthesis was performed using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, U.S.A.) in a sterile 0.2 ml tube. The mixture was incubated for 5 min at 25°C followed by 60 min at 42°C. The reaction was stopped by heating at 70°C for 5 min (Appendix C1). The reverse transcription reaction mixture was directly used for downstream analysis or kept at -20°C for less than one week.

3.2.6 Amplification of target gene using GoTaq® Flexi buffer (Promega, U.S.A.)

PCR reaction mixtures were prepared as described in Appendix B6i. Thermocycling conditions were as follows: initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 1 min, primer annealing for 20 sec, and extension at 72°C for 1 min. Then, a final extension was performed at 72°C for 7 min, followed by cooling at 25°C for 5 min (Applied Biosystems® Veriti® 96-Well Thermal Cycler platform, U.S.A.). Purified plasmid harboring the target gene was used as template for positive control if available. Meanwhile, sdH_2O was used as the template for negative control PCR mixture.

3.2.7 Amplification of target gene using HotStarTaq DNA Polymerase (Qiagen, Germany)

PCR reaction mixtures were prepared as described in Appendix B6ii. Thermocycling conditions were as follows: initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, primer annealing for 20 sec, and extension at 72°C for 1 min. Then, a final extension was performed at 72°C for 7 min followed by cooling at 25°C for 5 min (Applied Biosystems® Veriti® 96-Well Thermal Cycler platform, U.S.A.). Purified plasmid harboring the target gene was used as template for positive control if available. Meanwhile, sdH_2O was used as the template for negative control PCR mixture.

3.2.8 Amplification of target gene using Phusion® High Fidelity Taq polymerase (Thermo Scientific, U.S.A.)

PCR reaction mixtures were prepared as described in Appendix B6iii. Thermocycling conditions were as follows: initial denaturation at 98°C for 10 sec, followed by 35 cycles of denaturation at 98°C for 1 sec, primer annealing for 5 sec, and extension at 72°C for 12 sec. Then, a final extension was performed at 72°C for 1 min followed by cooling at 25°C for 5 min (Applied Biosystems® Veriti® 96-Well Thermal Cycler platform, U.S.A.). Purified plasmid harboring the target gene was used as template for positive control if available. Meanwhile, sdH_2O and DNA of untransformed plantlet were used as the template for negative control PCR and transformation, respectively.

3.2.9 Analysis of PCR products

A mixture of 4 µl of PCR product and 1 µl of 6 X loading dye (Thermo Scientific, U.S.A.) was loaded onto 1% (w/v) agarose stained by ethidium bromide (0.5 µg/ml) alongside 4 µl of GeneRuler 1 kb or 100 bp DNA ladder (Thermo Fisher Scientific, U.S.A.). The gel was electrophoresed at 125 volts for 25 min and viewed under UV light to confirm the presence of bands. PCR products were purified using QIAquick Gel Extraction Kit (Qiagen, Germany) and stored at -20°C prior to further analysis.

3.2.10 Cloning of target gene

Cloning of the target gene was performed using TOPO® TA Cloning® Kit according to the manufacturer‘s protocols (Invitrogen, U.S.A.). Briefly, a cloning reaction mixture was prepared in a sterile 1.5 ml microcentrifuge tube and placed on ice (Appendix C2). The reaction mixture was mixed by gentle tapping and then left at room temperature for 5-15 min. The tube was either placed on ice prior to *Escherichia coli* (*E. coli*) transformation or stored at -20°C overnight.

3.2.11 Introduction of purified target gene into *E. coli* TOP 10 competent cells

About 2 µl of TOPO® cloning reaction mixture (section 3.2.10) was added into a vial of One Shot® chemically competent *E. coli* (Invitrogen, U.S.A.). The mixture was incubated on ice for 20 min followed by heat-shock at 42°C for 30 sec. The tube was immediately placed on ice for 2 min. About 250 µl of SOC medium (room temperature) (Invitrogen, U.S.A.) was added into the tube. The tube was placed on a shaker at 220 rpm, 37°C for about an hour. About 50 µl of transformation product added with 20 µl of

fresh SOC medium (Invitrogen, U.S.A.) was spread evenly on LB agar (Conda, Spain) supplemented with 50 µg/ml kanamycin. The plates were incubated overnight at 37°C.

3.2.12 PCR colony of transformed *E. coli* TOP cells harboring target gene

Colonies that grow on the selective agar plate following overnight incubation at 37°C were gently scraped out by sterile toothpicks before the latter were touched onto grid LB agar plates (50 µg/ml kanamycin) with designated numbers. The same toothpicks were dipped into corresponding PCR tubes containing 30 µl of sdH_2O. The tubes were heated at 98°C for 10 min. The boiled cultures were used as templates for PCR screening whilst the grid plates were incubated at 37°C for subsequent plasmid preparation. PCR colony was performed using GoTaq® DNA polymerase (Promega, U.S.A.) (section 3.2.6). The PCR products were analyzed according to the protocol described earlier (section 3.2.9).

3.2.13 Plasmid extraction

Plasmid extraction was performed according to the methods described by Sambrook and Russell (2001). Selected positive transformants harboring target gene were cultured in 10 ml LB broth (Conda, Spain) supplemented with 50 µg/ml kanamycin. The cultures were incubated on a rotary shaker at 37°C, 220 rpm overnight. Overnight cultures were transferred into sterile 15 ml Falcon tubes and centrifuged at 4,032 *g* for 15 min. The supernatant was discarded and 200 µl of solution I (Appendix B3i) was added. The tube was then vortexed to dissolve the pellet. The dissolved pellet was transferred into new sterile 1.5 ml microcentrifuge tube. A total of 200 µl of solution II (Appendix B3ii) was added and the tube was inverted a few times. The tube was left at room temperature for

4 min before adding in 200 µl of solution III (Appendix B3iii). The mixture was inverted a few times followed by incubation at -20°C for at least 30 min. The tube was then centrifuged at 18,928 *g* for 10 min. The supernatant was transferred into a new sterile 1.5 ml microcentrifuge tube. About 1.5 µl of RNase A (20 mg/ml) (Invitrogen, U.S.A.) was added and the mixture was incubated at 37°C for 1-3 hours. The tube was briefly spun down before adding in 600 µl of phenol (Sigma-Aldrich, U.S.A.) followed by centrifugation at 18,928 *g* for 3 min. The aqueous layer was transferred into a new sterile 1.5 ml microcentrifuge tube. A total of 600 µl of chloroform (Fisher Thermo Scientific, U.S.A.) was added into the tube. The mixture was vortexed followed by centrifugation at 18,928 *g* for 3 min. The volume of aqueous layer transferred into a new sterile 1.5 ml microcentrifuge tube was recorded. About 0.1 V of 3 M sodium acetate (Sigma-Aldrich, U.S.A.) and 2.5 V of absolute ethanol (Sigma-Aldrich, U.S.A.) were added and the solutions were mixed well. The tube was incubated at -20°C for 30 min followed by centrifugation at 18,928 *g* for 15 min. The supernatant was discarded before adding in 1 ml of chilled 70% (v/v) ethanol (Sigma-Aldrich, U.S.A.). The tube was inverted a few times and centrifuged at 18,928 *g* for 5 min. The supernatant was discarded and the pellet was dried using vacuum centrifuge. Appropriate volume of sdH_2O (normally 30-50 µl) was added to dissolve the pellet. The purified plasmid was stored at -20 °C until further use.

3.2.14 Sequencing analysis

All purified PCR products/target genes/plasmids were sequenced (AITbiotech, Singapore) and analyzed using BLAST software (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequence alignment was performed using

specialized BLAST software (bl2seq) and/or ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/) if reference sequences were available.

3.3 Analysis of *MamPR-10* cDNA sequence (*M. acuminata* cv. 'Mutiara')

3.3.1 Phage viability

'Economical phage rescue method' was performed to test the viability of *MamPR-10* cDNA (*M. acuminata* cv. 'Mutiara') phage stock. In this method, the phage stock was scraped out from corresponding ELISA plate stored at -80°C and inoculated into 1.5 ml microcentrifuge tube containing 100 µl of SM buffer (Appendix B2iv). The mixture was stored at 4°C. A single colony of *E. coli* XL1-Blue MRF' grown on Luria-Bertani (LB) agar (Conda, Spain) supplemented with tetracyline (12.5 µg/ml) was inoculated into 10 ml of LB broth (Conda, Spain) supplemented with 0.5 M $MgSO_4$ and 10% (w/v) maltose. The culture was incubated on a rotary shaker at 37°C, 220 rpm overnight. A total of 200 µl of the overnight culture was inoculated into fresh 10 ml LB broth (Conda, Spain) containing the same supplements. The culture was incubated on a rotary shaker at 37°C, 220 rpm until OD_{600} reading was approximately 0.5. Tubes with different dilution folds (DF), ranging from 10^0 to 10^{-5}, were prepared by diluting the phage stock in SM buffer. A total of 200 µl of fresh XL1-Blue MRF' culture (OD_{600}~0.5) was added into melted yeast-triptone (YT) agar (~20 ml) (Appendix B2iii). The mixture was swirled gently and quickly poured into the prepared LB plate pre-divided into four different sections with respective dilution factors. A total of 5 µl of the undiluted phage stock (10^0) and other dilution mixtures were spread onto corresponding section. The plates were wrapped with parafilm and incubated overnight at 37°C. Plaque formation was observed the following morning (~16 hours).

3.3.2 Plaque forming unit (pfu) calculation

Plaque forming unit (pfu) of the original phage stock was estimated by repeating the phage rescue experiment (section 3.3.1) with minor modification (Maniatis et al., 1982). A total of 10 µl of selected dilution mixtures were directly added into 200 µl of fresh XL1-Blue MRF‘ culture (OD_{600}~0.5). The mixtures were incubated in hybridization oven at 37°C for either 30 min or 1 hour before they were added into the melted YT agar and poured onto the prepared LB plates. The plates were incubated overnight at 37°C. Plaque formation was recorded the following morning (~16 hours) and pfu of the phage stock was calculated (Formula 1).

Formula 1: Pfu calculation

$$\text{pfu/ml} = \frac{\text{No. of plaques formed}}{\text{Dilution factor x Volume of phage stock}}$$

3.3.3 Amplification of *MamPR-10* (*M. acuminata* cv. ‘Mutiara’)

PR-10 cDNA sequences of *M. acuminata* cv. ‗Mutiara‘ (*MamPR-10*) were amplified from pBluescript SK (-) vector using T7/T3 and pBSF/pBSR primer pairs (Appendix D). T7/T3 were the universal primer sequences of pBluescript SK (-) vector while pBSF/pBSR primers were designed based on the vector sequences located upstream and downstream of Xho1 and EcoR1 sites, respectively (Figure 3.2). A total of 10 µl of the undiluted *PR-10* phage stock was added into 40 µl of sdH_2O in a PCR tube. The mixture was briefly spun down and incubated at 98°C for 10 min. The boiled culture was used as template in the PCR reaction mixtures using GoTaq® DNA polymerase (Promega, U.S.A.) (section 3.2.6) (Table 3.1). All purified PCR products were cloned, sequenced and analyzed (section 3.2.9-3.2.14).

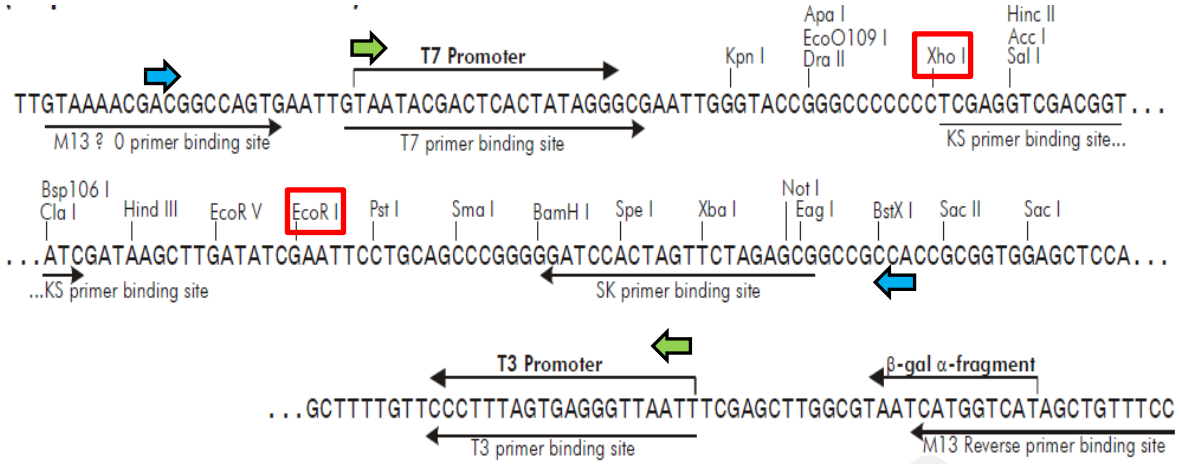


Figure 3.2: Multiple cloning site region of pBluescript SK (-). Primer positions for T7/T3 and pBSF/pBSR pairs are indicated by green and blue arrows, respectively. *MamPR-10* insertion sites at Xho1 and EcoR1 are shown in red box

Table 3.1: Optimal PCR conditions for amplification of *MamPR-10* (*M. acuminata* cv. _Mutiara') using T3/T7 and pBSF/pBSR primer pairs

Primer pair	Optimal Tm (°C)	Concentration of $MgCl_2$ (mM)
T3/T7	45	1.5
pBSF/pBSR	65	1.5

3.4 Rapid amplification of cDNA ends (RACE) of *PR-10* (*M. acuminata* ssp. malaccensis) (*MaPR-10*)

3.4.1 Preparation of 5' RT product for 5' RACE

3.4.1.1 CIP treatment (Dephosphorylating RNA)

RNA of young *M. acuminata* ssp. malaccensis leaves was extracted (section 3.2.2) and treated using DNase I (section 3.2.3). CIP treatment was performed on the DNase-treated RNA sample according to the manufacturer's protocol (GeneRacer™ kit, Invitrogen, U.S.A.). A mixture of 1 µg of high quality DNase-treated RNA, 1 µl of 10 X CIP buffer, 1 µl of RNaseOut (400 U/µl), 1 µl of CIP (10 U/µl) and DEPC-treated water

(up to 10 µl) was prepared in a RNase-free microcentrifuge tube and placed on ice. The reaction mixture was pipetted, vortexed and spun down to mix well. It was incubated at 50°C for 1 hour and placed on ice prior to RNA precipitation.

RNA was precipitated out by adding 900 µl of DEPC-treated water and 100 µl of phenol-chloroform (P:C) into the tube. The reaction mixture was vortexed vigorously for 30 sec followed by centrifugation at 18,928 *g* for 5 min. The aqueous phase was transferred into a new microcentrifuge tube (~100 µl). Next, 2 µl of 10 mg/ml mussel glycogen, 10 µl of 3 M sodium acetate (pH 5.2) and 220 µl of 95% (v/v) ethanol (Sigma-Aldrich, U.S.A.) were added into the tube. The reaction mixture was vortexed and stored at -20°C overnight.

RNA pellet was obtained by centrifugation at 4°C, 18,928 *g* for 20 min. The supernatant was discarded before 500 µl of 70% (v/v) ethanol (Sigma-Aldrich, U.S.A.) was added. The tube was inverted several times and briefly vortexed. Centrifugation was repeated at 4°C, 18,928 *g* for 2 min. Supernatant was carefully removed without disturbing the pellet. The tube was air-dried for 1-2 min before the pellet was dissolved in 7 µl of DEPC-treated water. The sample was placed on ice or stored at -20°C prior to TAP treatment.

3.4.1.2 TAP treatment (Removing the mRNA cap structure)

TAP treatment was performed on the CIP-treated RNA according to the manufacturer‘s protocol (GeneRacer™ kit, Invitrogen, U.S.A.). A total of 7 µl of dephosphorylated RNA (section 3.4.1.1), 1 µl of 10 X TAP buffer, 1 µl of RNaseOut (400 U/µl) and 1 µl of TAP (0.5 U/µl) were prepared in a sterile 1.5 ml RNase-free microcentrifuge tube and placed on ice. The mixture was pipetted, vortexed and spun down to mix well. The reaction mixture was then incubated at 37°C for 1 hour. RNA was precipitated (section 3.4.1.1) and directly used in RNA ligation step (section 3.4.1.3).

3.4.1.3 RNA ligation (Ligating the RNA oligo to the decapped mRNA)

A total of 7 µl of dephosphorylated, decapped RNA (section 3.4.1.2) was added into pre-aliquoted, lyophilized GeneRacer RNA oligo (0.25 µg). The reaction mixture was pipetted, spun down and incubated at 65°C for 5 min to relax the RNA secondary structure. The tube was placed on ice (~2 min) and briefly centrifuged before the following reagents were added: 1 µl of 10 X ligase buffer, 1 µl of ATP (10 mM), 1 µl of RNaseOut (400 U/µl) and 1 µl of T4 RNA ligase (5 U/µl). The reaction mixture was incubated at 37°C for 1 hour and placed on ice prior to RNA precipitation. RNA was precipitated (section 3.4.1.1) and the pellet was re-suspended in 10 µl of DEPC-treated water.

3.4.1.4 Reverse transcription of 5' ligated-RNA

Reverse transcription (RT) of 5' ligated-RNA was performed according to the manufacturer's protocol (GeneRacer™ kit, Invitrogen, U.S.A.). A total of 1 µl of random primer, 1 µl of dNTP mix and 1 µl of sdH_2O was added into 10 µl of ligated RNA (section 3.4.1.3). The reaction mixture was incubated at 65°C for 5 min to remove any RNA secondary structure. It was then chilled on ice for 1 min and briefly centrifuged before the following reagents were added: 4 µl of 5 X first strand buffer, 1 µl of 0.1 M DTT, 1 µl of RNaseOut (40 U/µl) and 1 µl of SuperScript III reverse transcriptase (200 U/µl). The reaction mixture was mixed well by pipetting and incubated under the following conditions: primer binding at 25°C for 5 min; followed by elongation at 50°C for 30-60 min and lastly heat-inactivation at 70°C for 15 min. The reaction mixture was chilled on ice for about 2 min and briefly spun down. A total of 1 µl of RNase H (2 U) was added to the mixture followed by incubation at 37°C for 20 min. The reaction mixture was stored at -20°C.

3.4.2 Preparation of 3' RT product for 3' RACE

Reverse transcription of DNase-treated RNA for 3' RACE was performed according to the manufacturer's manual (GeneRacer™ kit, Invitrogen, U.S.A.). A total of 1 µl of GeneRacer™ OligodT primer (50 µM) (Invitrogen, U.S.A.), 1 µl of dNTP mix (10 mM), 1 µl of sdH_2O and 10 µl of high-quality DNase treated RNA was mixed in a sterile PCR tube. The mixture was incubated at 65°C for 5 min to remove any RNA secondary structure and then chilled on ice for 1 min. The mixture was briefly spun down before the following reagents were added: 4 µl of 5 X First Strand Buffer, 1 µl of 0.1 M DTT, 1 µl of RNaseOut™ (40 U/µl) and 1 µl of SuperScript™ III RT (200 U/µl). The mixture was mixed well and incubated at 50°C for 60 min, followed by heat-inactivation at 70°C for 15 min. Next, the reaction mixture was chilled on ice for 2 min and briefly spun down before 1 µl of RNaseH (2 U/µl) was added. The mixture was incubated at 37°C for 20 min and stored at -20°C until further use.

3.4.3 Primer design for RACE of *MaPR-10*

Forward (PR10_3'O and PR10_3'I) and reverse (PR10_5'O) gene-specific primers were designed for both 3' and 5' RACE of *PR-10* (*M. acuminata* ssp. malaccensis) (*MaPR-10*), respectively, from the *MamPR-10* cDNA (*M. acuminata* cv. _Mutiara') sequences obtained (PCR Primer Stats software, http://www.bioinformatics.org/sms2/pcr_primer_stats.html) (Figure 3.3). Meanwhile, the other primers (GeneRacer™ 3', GeneRacer™ 3' Nested and GeneRacer™ 5') were supplied with the GeneRacer™ kit (Invitrogen, U.S.A.) (Appendix D).

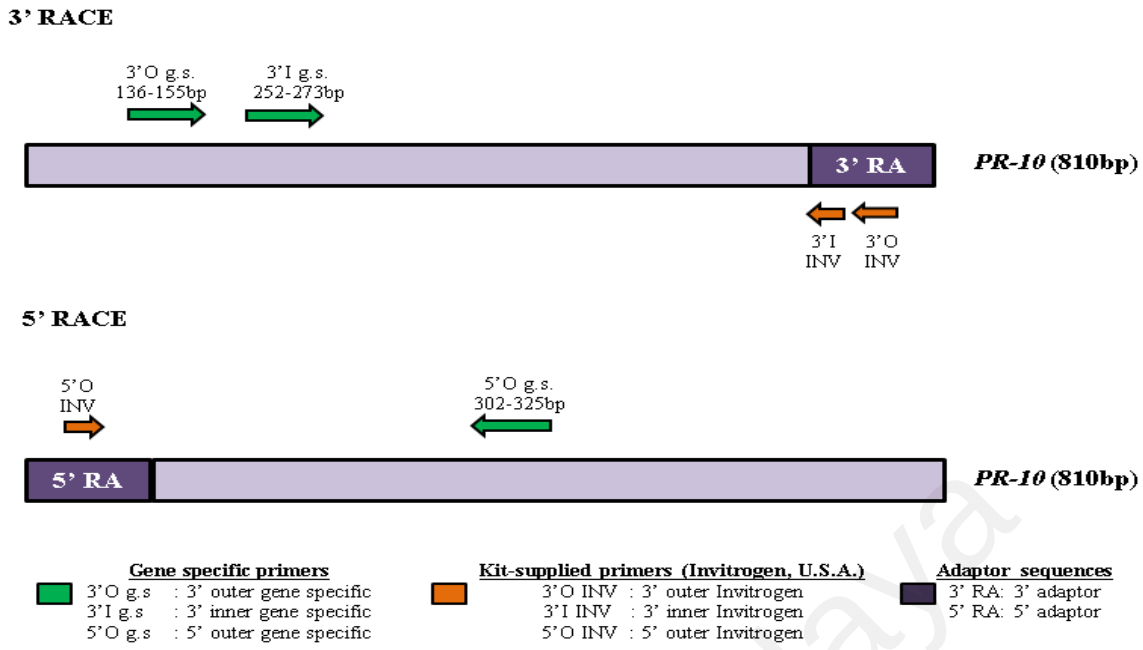


Figure 3.3: Nucleotide position of gene-specific primers designed for 3' and 5' RACE of *MaPR-10* from cDNA sequences of *M. acuminata* cv. 'Mutiara' (*MamPR-10*)

3.4.4 5' RACE

PCR reaction mixtures for 5' RACE were prepared using HotStarTaq buffer (Qiagen, Germany) as described previously (section 3.2.7) which included the use of 1.5 mM of $MgCl_2$, 0.8 µM of PR10_5'O and GeneRacer™ 5' primers (Invitrogen, U.S.A.) as well as 2% (v/v) of 5' RT products (section 3.4.1.4). The annealing temperature was set in the range of 64°C to 70°C. All purified PCR products were cloned, sequenced and analyzed (section 3.2.9-3.2.14).

3.4.5 3' RACE

In the amplification of 3' end of *MaPR-10*, the first round of RACE reaction mixtures (3' outer RACE) were prepared using HotStarTaq buffer (Qiagen, Germany) as described previously (section 3.2.7) which included the use of 0.8 µM of PR10_3'O

and GeneRacer^{TM} 3‘ primer (Invitrogen, U.S.A.) as well as 2% (v/v) of 3‘ RT (section 3.4.2). The annealing temperature was set in the range of 47°C to 53°C.

The PCR product of 3‘ outer RACE was not sequenced and directly used as template in the second round of RACE reaction (3‘ inner) to improve the specificity of 3‘ end amplification. PCR reaction mixtures were prepared using HotStarTaq buffer (Qiagen, Germany) as described previously (section 3.2.7) which included the use of 1 mM of $MgCl_2$, 0.8 µM of PR10_3‘I and GeneRacer^{TM} 3‘ nested primers (Invitrogen, U.S.A.) as well as 2% (v/v) of 3‘ outer product as template. The annealing temperature was set in the range of 53°C to 59°C. All purified PCR products were cloned, sequenced and analyzed (section 3.2.9-3.2.14).

3.5 Analysis of putative full-length *MaPR-10* DNA and cDNA

3.5.1 Sample preparation

Crude DNA and RNA were extracted from young *M. acuminata* ssp. malaccensis leaves (section 3.2.1 and section 3.2.2, respectively) and treated accordingly (section 3.2.3). First strand cDNA synthesis was performed using treated RNA extract (section 3.2.5).

3.5.2 Amplification of putative full-length *MaPR-10* DNA and cDNA

Primer pairs were designed based on the assembled 5‘ and 3‘ RACE sequences to amplify the full-length *MaPR-10* sequences (Appendix D). PCR reaction mixtures containing 1.5 mM of $MgCl_2$, 0.8 µM of PR10 full F/PR10 full R primers and corresponding DNA/cDNA templates were prepared and run as described previously (section 3.2.6). The annealing temperature was set at 62°C. All purified PCR products were cloned. Two clones were sent for sequencing and analyzed (section 3.2.9-3.2.14).

3.6 Analysis of *MaPR-10* coding sequences (*MaPR-10* cds)

3.6.1 Amplification of *MaPR-10* cds

Coding sequences (cds) of *MaPR-10* (*MaPR-10* cds) were predicted using protein translation tool (EMBOSS Transeq) as well as alignment of *PR-10* cds from other plant species. A primer pair to read *MaPR-10* cds (PR10 cds F/PR10 cds R) (Appendix D) was designed as such to contain NcoI and BglII restriction sites in the forward and reverse primers, respectively (Appendix D). Amplification of *MaPR-10* cds was performed on *M. acuminata* ssp. malaccensis cDNA using GoTaq® DNA polymerase (Promega, U.S.A.) with $MgCl_2$ concentration of 1.0 mM and Tm at 58.5°C (section 3.2.6). All purified PCR products were cloned, sequenced and analyzed (section 3.2.9-3.2.14). Selected positive colonies were also maintained in 40% (v/v) glycerol stocks (BDH, U.K.) and stored at -80°C.

3.6.2 pMaPR-10 vector construct

Amplified *MaPR-10* cds fragment was purified using QIAquick Gel Extraction Kit (Qiagen, Germany) while pCAMBIA1304 plasmid was extracted from available glycerol stock (section 3.2.13). Purified *MaPR-10* and pCAMBIA1304 were digested using NcoI and BglII restriction enzymes (New England Biolabs, U.S.A.) (Appendix C3). Digestion reaction mixtures were incubated at 37°C for 4 hours. Both digested *MaPR-10* and pCAMBIA1304 were ligated using T4 DNA ligase (Fermentas, U.S.A.) at 4°C overnight (Appendix C4). The mixture was terminated by heating at 65°C for 10 min. The reaction mixture can be directly used for *E. coli* transformation or kept at -20°C. The resulting construct was designated as pMaPR-10.

In addition, the integrity of pMaPR-10 construct was confirmed by the presence of hygromycin phosphotransferase II (*hptII*) gene as well as the flanking regions between

cauliflower mosaic virus (CaMV35S) promoter-*MaPR-10* cds and *MaPR-10* cds-mgfp5 using PCR (Promega, U.S.A.) (section 3.2.6) (Table 3.2 and Figure 3.4) (Appendix D).

Table 3.2: Optimal PCR conditions to amplify different regions of pMaPR-10 construct

Amplified region and primer pair	Optimal Tm (°C)	Concentration of $MgCl_2$ (mM)
hptII (hpt F/hpt R)	52	2.0
CaMV35S-*MaPR-10* cds (1304F/PR10 cds R)	57	1.0
MaPR-10 cds -mgfp5 (PR10 S F/1304R)	52	1.0

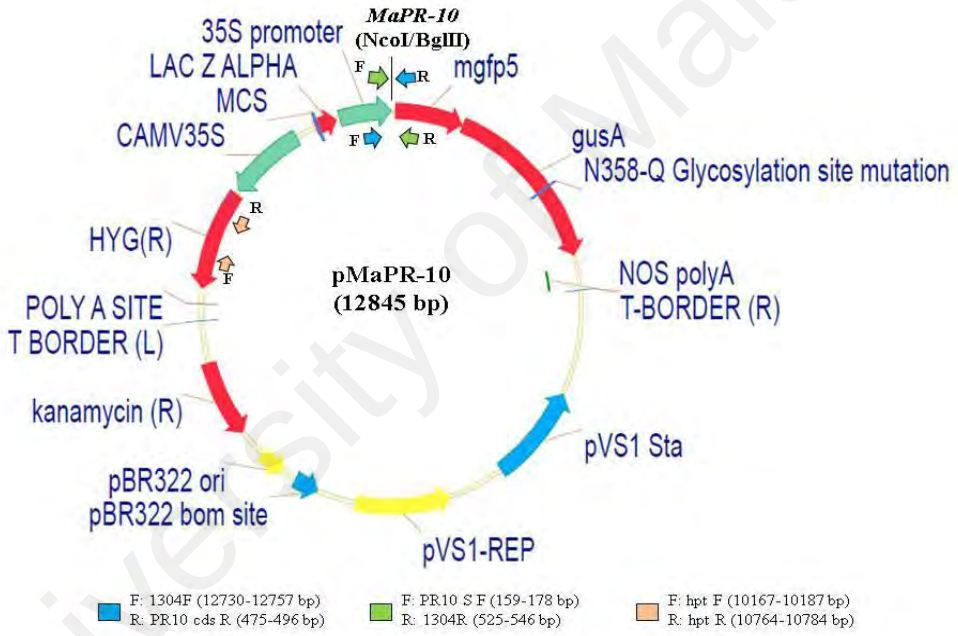


Figure 3.4: Primer positions to check the integrity of pMaPR-10 construct (modified from www.cambia.org)

3.6.3 Analysis of MaPR-10 protein

The molecular weight and isoelectric point (IE) of MaPR-10 protein were predicted using ExPASy (Compute pI/Mw) tool (Gasteiger et al., 2005). Putative domains were predicted using blastp software (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and ScanProsite (De Castro et al., 2006). The evolutionary analyses of MaPR-10 and 16 other PR-10s

from selected plant species were conducted in MEGA5 (Tamura et al., 2011) and the evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1987). The evolutionary distances were computed using the Poisson correction method (Zuckerkandl & Pauling, 1965) and in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated. The presence of signal peptide and transmembrane domain was predicted by SignalP 4.0 (Petersen et al., 2011) and TMbase (Hofmann & Stoffel, 1993), respectively. Putative phosphorylation sites were analyzed using NetPhos 2.0 (Blom, Gammeltoft & Brunak, 1999).

3.6.4 Introduction of pMaPR-10 construct into *E. coli* TOP 10 cells

pMaPR-10 construct was introduced into One Shot® chemically competent *E. coli* TOP 10 cells (Invitrogen, U.S.A.) (section 3.2.11). Positive colonies were verified by PCR using PR10 cds F/PR10 cds R primer pair (Appendix D) (section 3.2.12). Plasmids of selected clones were purified (section 3.2.13), sent for sequencing and analyzed (section 3.2.14). Selected positive colonies were also maintained in 40% (v/v) glycerol stocks (BDH, U.K.) and stored at -80°C.

3.7 *Agrobacterium*-mediated transformation of pMaPR-10 construct

3.7.1 Preparation of competent *Agrobacterium tumefaciens* (*A. tumefaciens*) strain LBA4404 cells

A. tumefaciens strain LBA4404 was streaked on LB agar (Conda, Spain) supplemented with 50 µg/ml rifampicin. The plate was incubated in the dark at room temperature for two days. A single colony was inoculated into 5 ml LB broth (Conda,

Spain) supplemented with 50 µg/ml rifampicin and incubated at 28°C, 200 rpm overnight on a rotary shaker. About 2 ml of the overnight culture was transferred into 50 ml of LB broth (Conda, Spain) without antibiotic supplement. The culture was placed on a rotary shaker at 28°C, 200 rpm until OD_{600} was approximately 0.5 followed by centrifugation at 4°C, 2,800 *g* for 10 min**.** Supernatant was discarded and the pellet was re-suspended in 20 ml of ice-cold 1 mM HEPES (pH 7.0) (Sigma-Aldrich, U.S.A.) (Appendix B4i). Centrifugation step was repeated using the same setting. Re-suspension and centrifugation steps were repeated. The pellet was finally re-suspended in 500 µl of ice-cold 10% (v/v) LB-glycerol (Appendix B2vii). Freshly prepared competent cells were stored in 100 µl aliquots in sterile, ice-cold 1.5 ml tubes. All tubes were dipped briefly in liquid nitrogen prior to storage at -80°C.

3.7.2 *Agrobacterium*-mediated transformation of pMaPR-10 construct

A maximum of 10 µl (<1 µg) of purified pMaPR-10 plasmids (section 3.6.4) were added into competent *A. tumefaciens* LBA4404 cells and mixed well. The reaction mixture was placed on ice for 30 min and then dipped in liquid nitrogen for 1 min followed by incubation at 37°C for 4 min. The mixture was finally chilled on ice for 1 min. A total of 900 µl of LB broth (Conda, Spain) was added into the reaction mixture followed by incubation at 28°C for two hours. About 100-200 µl of the incubated reaction mixture was spread on LB agar (Conda, Spain) supplemented with 50 µg/ml rifampicin and 100 µg/ml kanamycin. Positive colonies were verified by PCR using PR10 cds F/ PR10 cds R primer pairs (section 3.2.12). Selected positive colonies were maintained in 40% (v/v) glycerol stocks (BDH, U.K.) and stored at -80°C. pCAMBIA1304 was used as a positive control.

3.8 Co-cultivation of *M. acuminata* cv. 'Berangan' cells suspension

Positive *A. tumefaciens* colonies were cultured in 3 ml LB broth (Conda, Spain) (supplemented with 100 µg/ml kanamycin and 50 µg/ml rifampicin) and placed on a rotary shaker at 28°C, 220 rpm overnight. The overnight cultures were maintained in 40% (v/v) glycerol stocks and stored at -80°C. The remaining cultures were inoculated into 30 ml LB broth (Conda, Spain) without antibiotic supplement. The cultures were placed on a rotary shaker at 28°C, 220 rpm until the OD_{600} was approximately 0.5 followed by centrifugation at 4°C, 2,800 *g* for 10 min. The pellet was re-suspended in 30 ml of M2 medium supplemented with 100 µM acetosyringone (Appendix B9vi). Depending on the size of *M. acuminata* cv. _Berangan' cells, the suspension was either initially sieved through 425 µM mesh or settled directly to 1:2 (cell volume:liquid medium) in a sterile 50 ml Falcon tube. About 500 µl of the settled *M. acuminata* cv. _Berangan' cells were added into 10 ml of fresh M2 medium containing 1 ml of transformed *A. tumefaciens* culture. The infected culture was incubated in the dark on a rotary shaker at 25±2°C, 100 rpm for 30 min. After incubation, the liquid medium was completely removed and replaced with 10 ml of fresh M2 medium containing the same supplement. The culture was co-cultivated on a rotary shaker in the dark at 80 rpm. After 4 days, the liquid medium was completely removed and replaced with 10 ml of fresh M2 supplemented with 50 µg/ml cefotaxime. The culture was maintained on a rotary shaker with the same incubation conditions. After 48 hours, liquid medium was completely removed and replaced with 20 ml of M3 medium supplemented with 0.2 mg/L hygromycin and 50 µg/ml cefotaxime (Appendix B9vii). The culture was maintained on a rotary shaker with the same incubation conditions. Untransformed *A. tumefaciens* and *A. tumefaciens* culture harboring pCAMBIA1304 vector were used as negative and positive controls, respectively.

3.9 Maintenance and regeneration of transformed cells

In M3 medium, the cells were sub-cultured every 10-14 days for about 7-9 weeks until somatic embryos developed. From the third sub-culture cycle, the infected cells were cultured separately in 0.2 mg/L-, 2 mg/L- and 5 mg/L-hygromycin-supplemented M3 medium. The immature somatic embryos were transferred to M4 (8 mg/L BAP or M8B) medium supplemented with 0.2 mg/L hygromycin and 50 µg/ml cefotaxime (Appendix B9viii). Potential somatic embryos were maintained in the dark at 25±2°C for 4-8 weeks with 2-week-subculturing interval. The immature somatic embryos with protruding shoots were transferred to M4B (M4 medium supplemented with 4 mg/L BAP, 0.2 mg/L hygromycin and 50 µg/ml cefotaxime) (Appendix B9ix). They were maintained at 25±2°C in light conditions (light intensity: 1450 lux) with 2-week-subculturing interval until shoots regenerated. The regenerated shoots were transferred to M2B (M4 medium supplemented with 2 mg/L BAP) and maintained under the same conditions for four weeks (Appendix B9x). Single shoots were transferred to M5 medium for root elongation and maintained under the same conditions until transgene verification.

3.10 Analysis of putative transgenic *M. acuminata* cv. 'Berangan' harboring *MaPR-10*

3.10.1 Primer design

To verify the integration of T-DNA region, the plantlets were screened for the integration of *hptII* and *MaPR-10* transgene using hpt F/hpt R and PR10 S F/1304R primer pairs, respectively. PR10 S F/1304R pair was designed as such to amplify both pCAMBIA1304 and *MaPR-10* regions. For estimation of transgene copy number using quantitative real-time PCR (qPCR), similar primer design was adopted as such the

forward and reverse primer would anneal to the *MaPR-10* and mgfp5 regions of pCAMBIA1304, respectively (Appendix D).

3.10.2 Screening of putative transgenic plantlets

DNA extraction was performed on the leaves of putative transgenic plantlets from all regenerated lines using DNeasy Plant Mini Kit (Qiagen, Germany). The quality of the extracted DNA was evaluated using gel electrophoresis and NanoDrop 2000 (Thermo Scientific, U.S.A.) (section 3.2.4). Primary and secondary PCR were performed using Thermo Scientific Phusion® High Fidelity PCR Master Mix (Thermo Scientific, U.S.A.) (section 3.2.8) (Appendix B6iii) (Table 3.3). All purified PCR products were cloned, sequenced and analyzed (section 3.2.9-3.2.14).

Table 3.3: Optimal PCR conditions to amplify antibiotic resistance gene (*hptII*) and gene-specific region (*MaPR-10*-mgfp5) in putative transgenic Line-19 plantlets harboring *MaPR-10*

Amplified region and primer pair	**Primary PCR**		**Secondary PCR**	
	Optimal Tm (°C)	**Concentration of $MgCl_2$ (mM)**	**Optimal Tm (°C)**	**Concentration of $MgCl_2$ (mM)**
hptII (hpt F/hpt R)	52	1.0	52	2.0
MaPR-10 cds-mgfp5 (PR10 S F/1304R)	69	1.0	52	1.0

3.10.3 Transgene copy number analysis

Using pMaPR-10 construct (12,845 bp) with the initial concentration of 100 ng/µl, five serial dilutions were prepared starting from $1/10^3$ to $1/10^7$ (SD1-SD5). qPCR reaction mixtures containing 1 X Power SYBR Green (Applied Biosystems, U.S.A.), 0.2 µM of forward primer, 0.2 µM of reverse primer, 1 µl of diluted plasmid template

and sterile ultra-pure water were prepared in a total of 15 µl aliquot. For unknown genomic DNA samples extracted from the leaves of putative transgenic plantlets, qPCR reaction mixtures were prepared using 30 ng template. All standards and unknown samples were run in triplicates under the following parameters (QuantStudio^{TM} 12K Flex Real-time PCR platform, Life Technologies, U.S.A.):

Hold stage:	95°C, 10 min (1 cycle)
PCR setting:	95°C, 15 sec followed by 60°C, 1 min (40 cycles)
Melt curve stage:	95°C, 15 sec; 60°C, 1 min; 95°C, 15 sec (1 cycle)

Utilizing information on the concentration of the plasmids as well as their nucleotide sizes (in bp), a linear relationship between C_T of serial dilution standards and log10 of plasmid copy numbers was established. From the linear equation obtained and mean C_T values of unknown DNA samples, copy numbers of *MaPR-10* transgene in 30 ng of DNA was calculated. By assuming the size of A genome to be 523 Mbp (D'Hont et al., 2012; Droc et al., 2013), total number of genomes of *M. acuminata* cv. _Berangan' in 30 ng genomic DNA was estimated. The transgene copy number of *MaPR-10* was finally projected by correlation with the number of *M. acuminata* cv. _Berangan' genomes present in 30 ng DNA (Sreedharan, Shekhawat & Ganapathi, 2012). Dissociation curve was generated to verify the presence of single peak target.

3.10.4 Expression analysis of *MaPR-10* in Line-19 plants

Young leaves of 1-month-old Line-19 plants and untransformed controls grown under the same conditions (26±2°C, 16-8 hour photoperiod) were harvested simultaneously. RNA extraction was performed (section 3.2.2) followed by DNase I treatment (section 3.2.3). The integrity of DNase treated RNA was assessed (section 3.2.4) and first strand cDNA sample was prepared (section 3.2.5). qPCR reaction

mixtures of three randomly selected Line-19 plants (PR16, PR27 & PR35) and untransformed control including non-template control (NTC) and non-RT samples (NRT) were prepared in triplicates and placed on ice (section 3.10.3) (Appendix B7i). Ribosomal protein S 2 (*RPS2)* (Chen et al., 2011) was used as reference gene in which the *MaPR-10* expression was normalized to untransformed controls (Appendix D). All data were analyzed using ExpressionSuite Software v1.0.4 (Applied Biosystem, U.S.A.). Dissociation curve was generated to verify the presence of single peak target.

3.11 Establishment of baseline disease response for *M. acuminata* cv. 'Berangan' plants against *Fusarium oxysporum* f. sp. *cubense* (Foc) C1 HIR infection

Prior to Foc challenge of transgenic *M. acuminata* cv. ‗Berangan' plants, the infection parameters of Foc C1 HIR bioassay adopted from Mak et al. (2004) were first validated and the baseline disease response were determined using wild-type ‗Berangan' plants. Foc C1 HIR isolate was used as the source of infection throughout the bioassay experiment.

3.11.1 Single spore isolation

To obtain pure culture of C1 HIR isolate, a loopful of water agar containing C1 HIR was scraped out from the original stock tube using aseptic techniques and cultured in the middle of potato dextrose agar (PDA) plate (Difco^{TM}, BD, France). The plate, labeled as PDA1, was incubated in the dark at room temperature (26±2°C) for about 3-5 days until healthy mycelia growth was observed. One loopful of the mycelia was scraped out and re-suspended in 1.5 ml microcentrifuge tube containing 1.5 ml of sdH_2O. The tube was gently flicked to ensure complete resuspension of mycelia. One loopful of the mycelia suspension was then streaked evenly on a fresh PDA plate named PDA2. PDA2 was

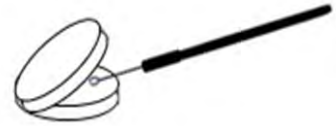
incubated in the dark at room temperature for about two days until distinct mycelia spots were observed. Single mycelia spot was selected and carefully scraped out using sterile loop or blade before it was cultured onto another fresh PDA plate named PDA3. PDA3 was incubated in the dark at room temperature for 3-5 days. All PDAs used throughout the process were supplemented with 0.5 g/L streptomycin (Figure 3.5).

3.11.2 Spore germination and pure culture storage

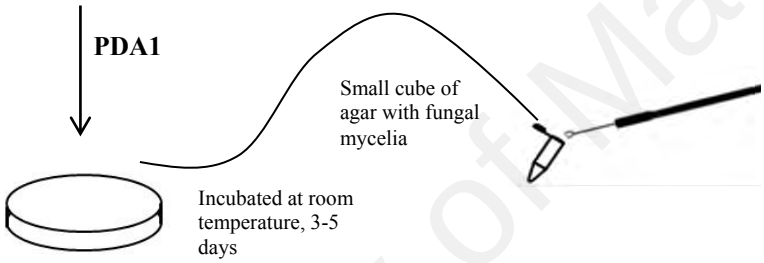
To confirm the viability of the pure culture and simultaneously prepare the long-term storage stock, one loopful of mycelia from PDA3 was cultured in the middle of a fresh PDA plate. The plate was incubated in the dark at room temperature for 3-5 days until vigorous mycelia growth was observed. The mycelia can be directly used for subsequent analysis or stored at room temperature for a maximum of one week.

For long-term storage, about 10 ml of sdH_2O was added onto the mycelia grown on PDA. Using sterile L-stick, the mycelia suspension was mixed evenly. A sterile filter paper (~2 cm x ~2 cm) was dipped into the suspension and placed into a sterile universal bottle. The bottle cap was losen and the sterile filter paper was air-dried in the laminar flow hood for a few days. The dried filter paper containing the pure culture can be stored in the dark at room temperature for a maxium of six months.

Aseptic technique was applied throughout the isolation process. All steps were carried out inside laminar flow hood. Inoculation loop was sterilized by flaming over a spirit methyl lamp until the wire became red-hot. A small chunk of water agar containing C1 HIR culture was scraped out from the original C1 HIR stock tube.



C1 HIR chunk was inoculated onto potato dextrose agar (PDA) and incubated at room temperature for 3-5 days. Every PDA plate was labeled with initial, date and name of the culture. The first PDA plate was labeled as PDA1. All PDAs used throughout the isolation process contain the same nutrient composition.



Healthy mycelia culture appeared on the agar surface after 3-5 days of incubation. The mycelia was scraped out and diluted into 1.5 ml microcentrifuge tube containing 1.5 ml sterile distilled water.

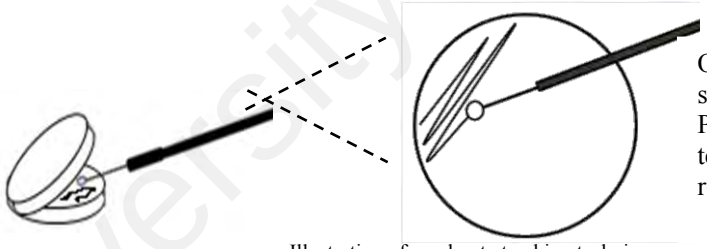
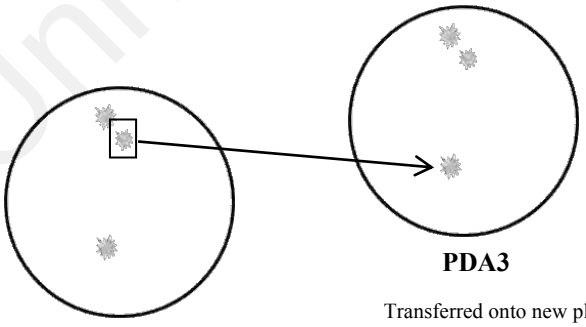


Illustration of quadrant-streaking technique

One loopful of the diluted sample was streaked onto fresh PDA (assigned as PDA2) using quadrant-streaking technique. PDA2 plate was incubated at room temperature for two days.



A distinct single colony was picked and transferred onto a new PDA (assigned as PDA3). PDA3 was incubated at room temperature for another 3-5 days to obtain pure C1 HIR culture.

Figure 3.5: Schematic diagram of single spore isolation method (modified from Pérez-Vicente et al., 2014)

3.11.3 DNA extraction of C1 HIR isolate

DNA extraction methods of C1 HIR isolate were modified from Lin and co-workers (2009). One loopful of C1 HIR mycelia grown on PDA was gently placed on the surface of fresh potato dextrose broth (PDB) (Difco^{TM}, BD, France). The culture was incubated in the dark at room temperature (26±2°C). After 5-7 days of incubation, the mycelia was collected, blotted-dry on sterile C-fold towel and then wrapped in aluminium foil. The mycelia was frozen-dry in liquid nitrogen before it was ground into fine powder. The ground mycelia (~0.2-0.4 g) was transferred into a pre-weighed sterile 1.5 ml microcentrifuge tube. The tube was weighed again to estimate the weight of the mycelia. About 1 ml of fungal DNA extraction buffer (Appendix B1iii) was added into the tube. The mixture was vortexed and incubated at 65^{o}C for 15 min followed by centrifugation at 4^{o}C, 21,952 *g* for 5 min. The supernatant was transferred into a new sterile 1.5 ml microcentrifuge tube before 20 µl of RNase A (20 mg/ml) (Thermo Scientific, U.S.A.) was added. The mixture was mixed well and incubated at 37^{o}C for three hours before it was transferred into a new sterile 2 ml microcentifuge tube. An equal volume of phenol:chloroform:isoamyl alcohol (PCIA) (Thermo Fisher Scientific, U.S.A.) was added and the mixture was inverted a few times to mix well (Note: The mixture cannot be mixed by vortexing). The mixture was incubated at 65^{o}C for 5 min, cooled down to room temperature and then centrifuged at 4^{o}C, 21,952 *g* for 5 min. The aqueous layer was carefully transferred to a new sterile 2 ml microcentrifuge tube without disrupting the organic phase before an equal volume of ice-cold absolute ethanol (Sigma-Aldrich, U.S.A.) was added. The mixture was incubated at -20^{o}C. On the next day, the mixture was centrifuged at 4^{o}C, 21,952 *g* for 10 min and the pellet was washed in 1 ml of ice-cold 75% (v/v) ethanol (Sigma-Aldrich, U.S.A.). The mixture was centrifuged at 4^{o}C, 21,952 *g* for 10 min. The washing step was repeated. The pellet was

dried using vacuum centrifuge and re-suspended in about 30 µl sdH_2O prior to storage at -20^oC.

3.11.4 Verification of the race group of C1 HIR

Verification of the race group of C1 HIR isolate was performed using Foc4F and Foc4R primer pair (Lin et al., 2009) (Appendix D). The target fragment was amplified using GoTaq® DNA polymerase (Promega, U.S.A.) (Appendix B6iv) (section 3.2.6). The annealing temperature was set at 60°C. All purified PCR products were cloned, sequenced and analyzed (section 3.2.9-3.2.14).

3.11.5 Preparation of C1 HIR spore suspension

Pure C1 HIR isolate was cultured on PDA in the dark at room temperature for about 3-4 days until mycelia formation was observed. One loopful of mycelia was inoculated into 1 L of fresh PDB (Difco^{TM}, BD, France). The culture was incubated at room temperature (26±2°C) and swirled twice a day for one week. The viability test was conducted on the 5th day post-inoculation by spreading about 100 µl of diluted spore suspension onto a PDA plate. The plate was incubated at room temperature in the dark for 36-48 hours for spore germination. The concentration of the spores was measured on the 7th day post-inoculation using haemocytometer and calculated using Formula 2. The final concentration was adjusted to 10^6 spores/ml using sdH_2O.

Formula 2: Calculation of C1 HIR spore concentration

$$\frac{\text{Total of cells in all squares counted}}{\text{No. of squares counted}} \times 2 \text{ (dilution factor)} \times 1000$$

3.11.6 Bioassay challenge of wild-type 'Berangan' plants against C1 HIR isolate

After two months of acclimatization under greenhouse conditions (29±1°C, 16-8 hour photoperiod), 35 wild-type _Berangan' plants were carefully uprooted and their roots were rinsed with tap water to remove the soil traces (Figure 3.6). The plants' roots were fully soaked in the prepared spore suspension (section 3.11.5) for two hours before the plants were replanted into the same potting mixture. Negative control plants were treated with sdH_2O.

Bioassay experiment was performed using _double tray' techniques in which about 4-6 potted plants were placed in the upper, smaller tray (43×29×9 cm) that fit snuggly into the larger, bottom tray (46×31×20 cm). The plants were watered with tap water every other day and with Hoagland's solution (Sigma-Aldrich, U.S.A.) once a week. Additionally, half tea-spoon of nitrogen:phosphorus:potassium (NPK) (15:15:15) fertilizers was added into each pot once every fortnight.

The contaminated water contained in the bottom tray was treated using sodium hypochlorite and left overnight before it was discarded the next day. All materials used in the experiment including plants and soils were autoclaved at 121°C, 15 psi for 45 min prior to disposal. Waste materials were disposed in double-layered biohazard plastic bags. C1 HIR bioassay challenge procedures were summarized in Figure 3.7.

Figure 3.6: Two month-old healthy *M. acuminata* cv. _Berangan' plant for bioassay study. Bar represents 3 cm

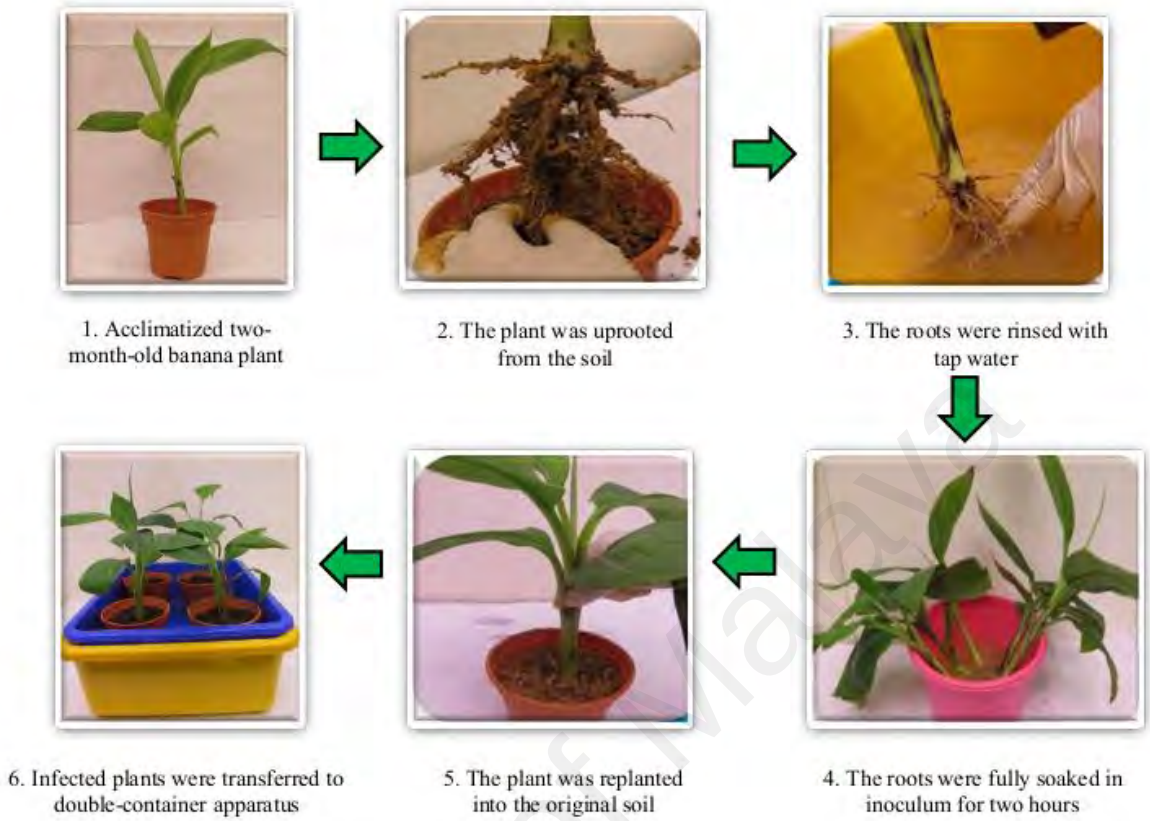


Figure 3.7: Flow chart illustrating the *Fusarium* wilt bioassay procedures

3.11.7 Field emission scanning electron microscope (FESEM) observation

C1 HIR-infected rhizome samples were prepared following the methods of Rahman and co-workers (2010) for FESEM observation on day 0, 2 and 4 post-inoculation. The specimens were dehydrated with normal dry air and adhered onto aluminum specimen mounts with carbon tapes. The specimens were coated with platinum for 30 sec and photographed using FEG Quanta 450, EDX-OXFORD electron microscope.

3.11.8 Leaf symptoms and rhizome discoloration index (LSI and RDI) of infected plants

Appearance of yellowing and wilting leaf symptoms on the infected plants was observed at least once a week. At the end of the 5th week-post-inoculation, the number of leaves showing yellowing and wilting symptoms per plant were recorded. Infected plants were carefully uprooted and their rhizomes were vertically cut into half to observe the brownish/darkish discoloration. The severity of the symptoms on leaves and rhizomes of wild-type *M. acuminata* cv. _Berangan‘ plants against C1 HIR was determined based on leaf symptoms index (LSI) and rhizome discoloration index (RDI), respectively (Table 3.4). The scores were then used to calculate the disease severity index (DSI) for each LSI and RDI, separately (Formula 3).

Table 3.4: Leaf symptoms index (LSI) and rhizome discoloration index (RDI) used in the evaluation of plants' response against C1 HIR (Brake et al., 1995; Mak et al., 2004)

Leaf symptoms index (LSI)	1) No streaking or yellowing of leaves. 2) Slight streaking and/or yellowing of lower leaves. Plant appears healthy. 3) Streaking and/or yellowing of most of the lower leaves. 4) Extensive streaking and/or yellowing on most or all of the leaves. 5) Dead plant
Rhizome discoloration index (RDI)	1) No discoloration of tissue of stele region of rhizome or surrounding tissue. 2) No discoloration of stele region of rhizome; discoloration at junction of root and rhizome. 3) Trace to 5% of stele region discolored. 4) 6-20% of stele region discolored. 5) 21-50% of stele region discolored. 6) More than 50% of stele region discolored. 7) Discoloration of the entire rhizome stele. 8) Dead plant

Formula 3: Formula for disease severity index (DSI). DSI is calculated based on the LSI and RDI scores to determine the disease response of the infected plants from leaves and rhizomes, respectively

$$\text{DSI} = \frac{\sum (\text{Number on scale x Number of seedlings in that scale})}{\sum (\text{Number of treated seedlings})}$$

The final DSI values for LSI and RDI were translated into one of the four designations (Table 3.5). In the case when one of the indexes was translated as susceptible, the test plants were regarded as susceptible against that particular isolate. Similarly, if one of the scores turned out to be tolerant while the other was resistant, the cultivar was considered tolerant. Only when both disease translations were resistant, then the cultivar was considered resistant.

Table 3.5: Translation of DSI scales (Mak et al., 2004)

DSI Scales for LSI	DSI Scales for RDI	Translation
1.0	1.0	Resistant
Between 1.1 and 2	Between 1.1 and 3	Tolerant
Between 2.1 and 3	Between 3.1 and 5	Susceptible
Between 3.1 and 5	Between 5.1 and 8	Highly susceptible

3.12 Analysis of the transgenic *MaPR-10* plants' response against C1 HIR isolate

Prior to bioassay challenge, the identities of all putative transgenic *M. acuminata* cv. _Berangan' plants were confirmed by PCR (section 3.10.2). Acclimatization and bioassay challenge of all plants were performed as described previously (section 3.11.4-3.11.8). Four biological groups were prepared according to treated transgenic Line-19

M. acuminata cv. ‗Berangan', untreated transgenic Line-19 *M. acuminata* cv. ‗Berangan', treated untransformed *M. acuminata* cv. ‗Berangan' and untreated untransformed *M. acuminata* cv. ‗Berangan' each containing at least 10 plants.

In compliance with Biosafety Act 2007 and Biosafety Regulations 2010, the execution of this project which involved contained use of living modified organisms (LMOs) has been notified to the Director General of the Department of Biosafety, Malaysia using Form E (NBB/N/CU/13/FORM E) (Appendix E). In this form, details on the project such as information of all the researchers involved, description of the activities and the LMOs as well as the premises at which the activities took place were provided. Risk assessment and risk management concerning human health and safety, decontamination of equipment used and disposal of LMOs/waste were also discussed. Besides, we have also included the emergency response plans to be undertaken during the unintended release of the LMOs and occurrence of undesirable effects. The emergency response plans are essential to protect the human and animal health as well as the environment, ensure appropriate arrangement to remove the LMOs in the affected areas and dispose the plants, animals or any other organisms exposed and prepare proper isolation procedures (including quarantine and evacuation). The notification was submitted to Institutional Biosafety Committee of University of Malaya (IBC UM) which is registered with the National Biosafety Board (NBB). The preliminary screening was carried out by IBC UM before the project approval was obtained with the IBC reference number of IBCUM14-007.

CHAPTER 4: RESULTS

4.1 Analysis of *MamPR-10* (*M. acuminata* cv. 'Mutiara')

4.1.1 Phage viability and pfu calculation

After overnight incubation at 37°C, plaque formation of *MamPR-10* phage clone (*M. acuminata* cv. _Mutiara') was observed on YT agar plate mixed with *E. coli* XL1-Blue MRF' which indicated successful bacterial lysis and thus proving the viability of the phage (Figure 4.1). More plaques were produced on plates in which the cells underwent 1–hour-incubation compared to a 30-minute-incubation. Countable number of plaques were produced from mixture with dilution factor of 10^{-5} which underwent 30-minute-incubation at 37°C. Pfu of *MamPR-10* phage stock was calculated to be 5 x 10^{7} pfu/ml.

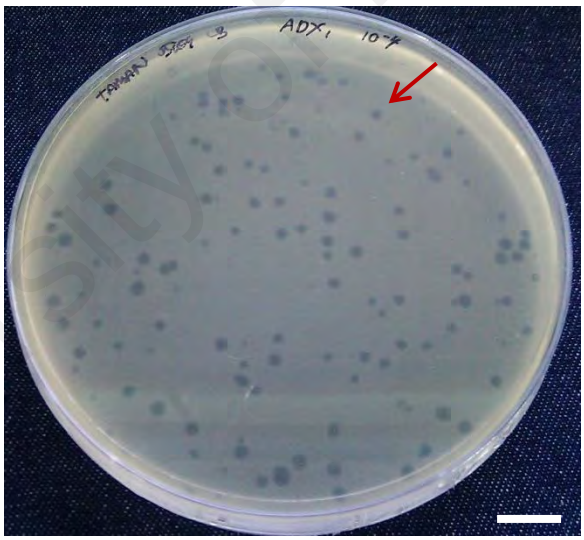


Figure 4.1: Typical viral plaque formation (arrow) observed on YT agar plate. Bar represents 1 cm

4.1.2 Amplification of *MamPR-10* (*M. acuminata* cv. 'Mutiara')

Amplification of *PR-10* cDNA from *M. acuminata* cv. _Mutiara' (*MamPR-10*) library using T7/T3 primer pair produced a double-band-product about 800 bp and 1.5 kb (Figure 4.2). When amplification was performed using pBSF/pBSR primers, one specific band of 810 bp was produced (Figure 4.3, Appendix F1 and Appendix J1). BLASTX results of the *MamPR-10* cDNA sequence most similarly matched *PR-10* of *Pinus monticola* (Accession number: AAL50005.1) with 58% coverage and 38% identity at the amino acid level (Figure 4.4). These results verified the identity of the sequences previously described by Lim (2006). The 1.5-kb-band, on the other hand, did not match any *PR-10* sequences and was considered as an unspecific product.

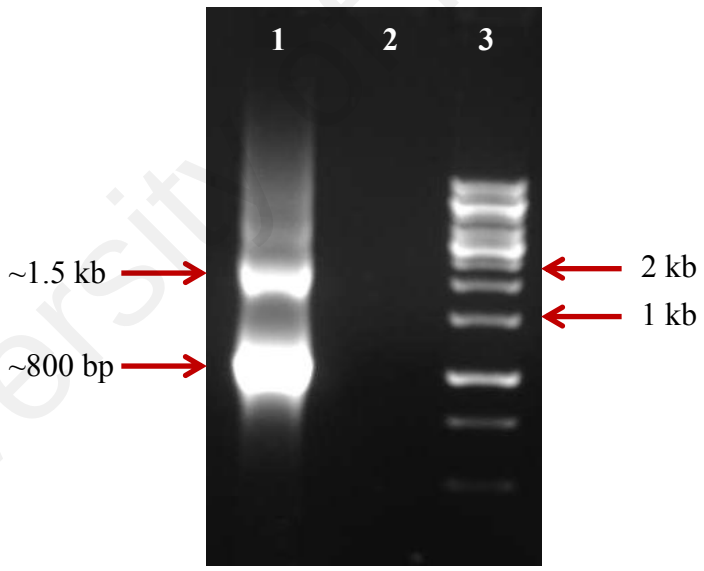


Figure 4.2: Amplification of *MamPR-10* cDNA (*M. acuminata* cv. _Mutiara') using T3/T7 primer pair. Amplified products are around 800 bp and 1.5 kb

Lane 1: *MamPR-10* cDNA (*M. acuminata* cv. _Mutiara')
Lane 2: Negative control PCR
Lane 3: 1 kb DNA marker

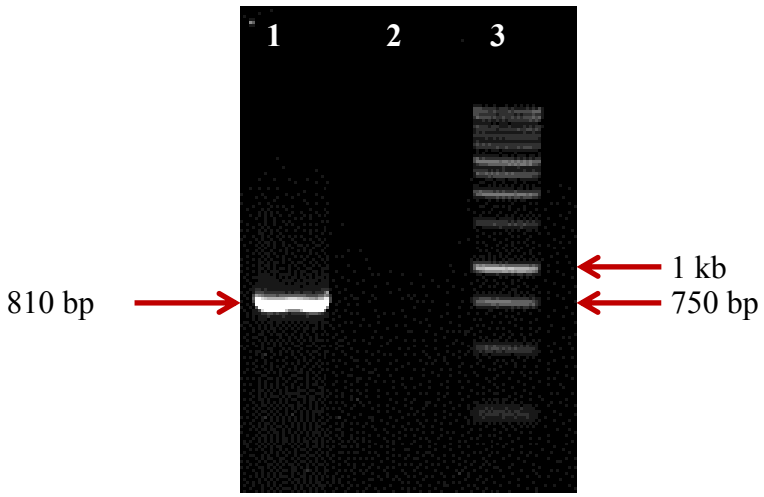


Figure 4.3: Amplification of *MamPR-10* cDNA (*M. acuminata* cv. _Mutiara') using pBSF/pBSR primer pair. Amplified product is 810 bp in-length

Lane 1: *MamPR-10* cDNA (*M. acuminata* cv. _Mutiara')
Lane 2: Negative control PCR
Lane 3: 1 kb DNA marker

Description	Max score	Total score	Query cover	E value	Ident	Accessior
PR10 protein [Pinus monticola]	117	117	58%	3e-28	36%	AAL50002.1
PR10 protein [Pinus monticola]	117	117	58%	3e-28	36%	AAL50001.1
intracellular pathogenesis-related protein PinmIII [Pinus monticola]	116	116	58%	4e-28	36%	AAC33531.1
PR10 protein [Pinus monticola]	119	119	58%	4e-29	36%	AAL50006.1
PR10 protein [Pinus monticola]	119	119	58%	5e-29	37%	AAL49998.1
PR10 protein [Pinus monticola]	119	119	58%	5e-29	38%	AAL50005.1

Figure 4.4: BLASTX results of *MamPR-10* cDNA (*M. acuminata* cv. _Mutiara') (810 bp)

4.2 Rapid amplification of cDNA ends (RACE) of *MaPR-10*

4.2.1 RNA extraction

High quality RNA extracted from *M. acuminata* ssp. malaccensis was demonstrated by the appearance of distinct 25S and 18S bands in the desired purity range ($A_{260/280}$ of 1.8-2.0). All DNA traces had been completely eliminated following DNase treatment (Figure 4.5). *PR-10* derived from *M. acuminata* ssp. malaccensis was later designated as *MaPR-10*.

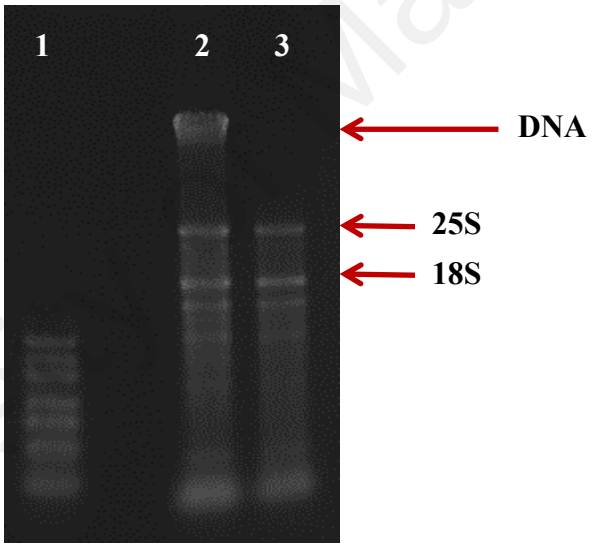


Figure 4.5: RNA extracts from young leaves of *M. acuminata* ssp. malaccensis (wild-type banana)

Lane 1: Low Range RNA marker
Lane 2: Crude RNA of *M. acuminata* ssp. malaccensis
Lane 3: DNase-treated RNA of *M. acuminata* ssp. malaccensis

4.2.2 3' and 5' RACE

3' outer RACE of *MaPR-10* produced double bands around 400 bp and 800 bp (Figure 4.6) while 3' inner RACE produced one specific band of 532 bp (Figure 4.7, Appendix F2 and Appendix J2). Top BLAST match for 3' RACE of amplified *MaPR-10* sequences was *PR-10* of *Zea mays (Z. mays)* (Accession number: NP_001147373.1) with 50% coverage and 39% identity at amino acid level. 3' RACE of *MaPR-10* sequences also showed 98% identity to the *PR-10* cDNA sequences of *M. acuminata* cv. 'Mutiara' (*MamPR-10*) with 63% coverage (Figure 4.8 and Appendix F3).

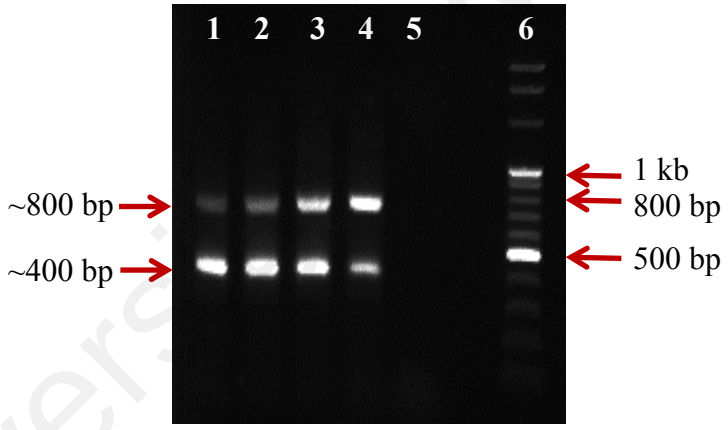


Figure 4.6: 3' RACE (outer) of *MaPR-10.* Amplification produced double bands around 400 and 800 bp

Lane 1-4:	Temperature gradient of 3' RACE (outer) of *MaPR-10* (47, 49, 51 and 53°C, respectively)
Lane 5:	Negative control PCR
Lane 6:	100 bp DNA marker

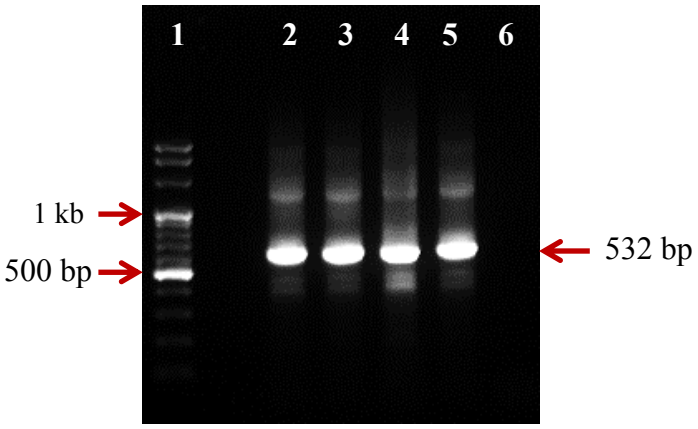


Figure 4.7: 3‘ RACE (inner) of *MaPR-10*. Amplification produced a prominent 532 bp-band

Lane 1:	100 bp DNA marker
Lane 2-5:	Temperature gradient of 3‘ RACE (inner) of *MaPR-10* (53, 55, 57 and 59°C, respectively)
Lane 6:	Negative control PCR

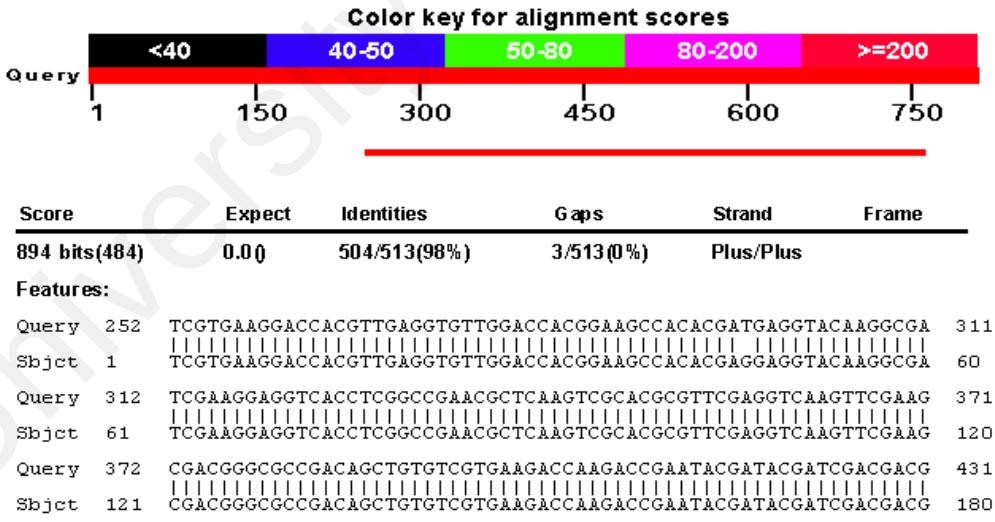


Figure 4.8: Sequence alignment of 3‘ inner RACE of *MaPR-10* and *MamPR-10*

5‘ RACE of *MaPR-10* produced one specific band of 335 bp (Figure 4.9, Appendix F4 and Appendix J3). BLAST results of 5‘ RACE most significantly matched *PR-10* of *P. monticola* with 43% identical and 80% coverage at amino acid level (Accession number: AAL50005.1) and was in accordance with the previous report (Lim, 2006). Sequence alignment between 5‘ RACE product and *PR-10* cDNA of *M. acuminata* cv. _Mutiara‘ (*MamPR-10*) showed 99% identity with 39% coverage (Figure 4.10 and Appendix F5). About 74 bp overlapping nucleotides were discovered from the sequence alignment of 3‘ and 5‘ RACE results (Appendix F6). Completion of 3‘ and 5‘ RACE was verified by the detection of polyA tail and 5‘ RACE adapter sequences, respectively. Summary of 3‘ and 5‘ RACE of *MaPR-10* was shown in Table 4.1.

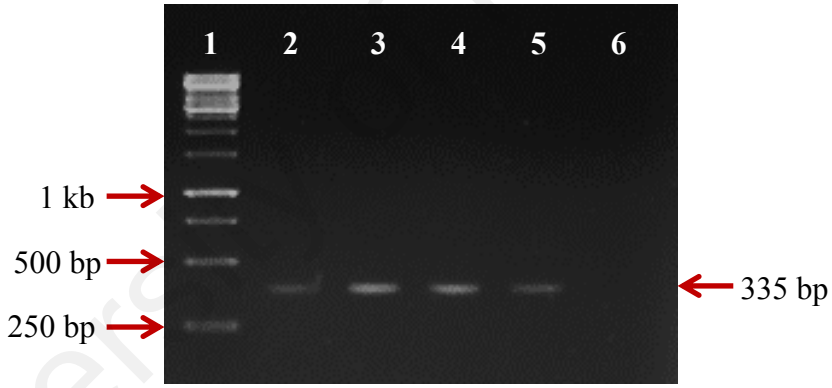
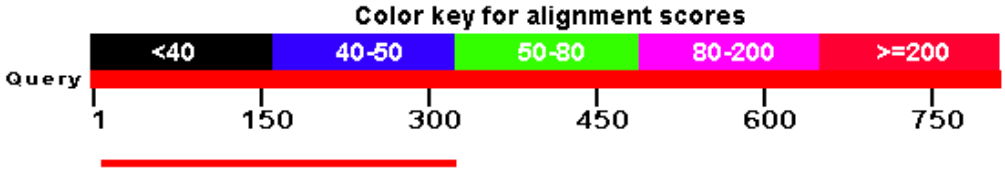


Figure 4.9: 5‘ RACE of *MaPR-10*. Amplification produced a 335 bp-band

Lane 1:	1 kb DNA marker
Lane 2-5:	Temperature gradient of 5‘ RACE of *MaPR-10* (64, 66, 68 and 70°C, respectively)
Lane 6:	Negative control PCR



Sequence ID: lcl|Query_149725 Length: 335 Number of Matches: 1
Range 1: 20 to 335

Score	Expect	Identities	Gaps	Strand	Frame
573 bits(310)	6e-168()	314/316(99%)	0/316(0%)	Plus/Plus	

Features:

```
Query  10   GAGCTTTCCTTCCCTTCATTAAGGTACTACGACTCACCACAACATGGTCACCGGCTGCTG  69
           ||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||
Sbjct  20   GAGCTTTCCTTCCCTTCATTAAGGTACTACGACTCACCACAACATGGTCGCCGGCTGCTG  79

Query  70   CACCAACGAGGTGACGGTCAACGTCAGCGTCCACAGGATGTGGAAGGCGGCCGCCTGCGA  129
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  80   CACCAACGAGGTGACGGTCAACGTCAGCGTCCACAGGATGTGGAAGGCGGCCGCCTGCGA  139

Query  130  GGATCACATCCTGCTGCCAAAGATCATTCCTGAGTACTTCGCTGGCGCAGAGCTCGTCGG  189
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  140  GGATCACATCCTGCTGCCAAAGATCATTCCTGAGTACTTCGCTGGCGCAGAGCTCGTCGG  199
```

Figure 4.10: Sequence alignment of 5‘ RACE of *MaPR-10* and *MamPR-10*

Table 4.1: Summary of 3‘ and 5‘ RACE of *MaPR-10*

3’ RACE	**5’ RACE**
• 532 bp • 98% identical and 63% coverage with *MamPR-10* (*M. acuminata* cv. _Mutiara‘) • 39% identical and 50% coverage with PR-10 of *Z. mays*	• 335 bp • 99% identical and 39% coverage with *MamPR-10* (*M. acuminata* cv. _Mutiara‘) • 43% identical and 80% coverage with PR-10 of *P. monticola*

4.3 Analysis of putative full-length *MaPR-10* DNA and cDNA

Full-length *MaPR-10* DNA and cDNA encoded for 949 bp (Figure 4.11 and Appendix F7) and 773 bp nucleotides (Figure 4.12, Appendix F8 and and Appendix J4), respectively. Full-length *MaPR-10* DNA and cDNA covered about 92% of *MamPR-10* (*M. acuminata* cv. _Mutiara') sequences with high nucleotide similarity (98% and 99% for *MaPR-10* DNA and cDNA, respectively) (Figure 4.13 and Figure 4.14). By comparing the size of full-length *MaPR-10* DNA and cDNA, the gene was revealed to have a 176-bp-intron. The coding sequences (cds) of *MaPR*-10 predicted using protein translation tools (EMBOSS Transeq) as well as the alignment of *PR*-10 cds from other plant species (Appendix F10) were revealed to be 483 bp (including stop codon TGA). Using all the information obtained, the putative full-length *MaPR-10* cDNA gene is comprised of two exons with 62-bp 5'UTR and 228-bp 3'UTR, respectively (Figure 4.15 and Table 4.2). The presence of expected band (949 bp and 773 bp for DNA and cDNA fragments, respectively) in the putatively transformed colonies verified the success of *E. coli* transformation (Figure 4.16 and Figure 4.17).

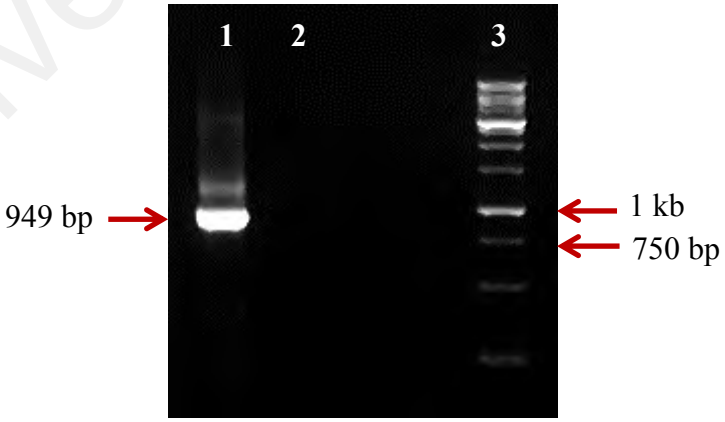


Figure 4.11: Amplification of putative full-length *MaPR-10* DNA. Amplified product is 949 bp in-length

Lane 1: Putative full-length *MaPR-10* DNA
Lane 2: Negative control PCR
Lane 3: 1 kb DNA marker

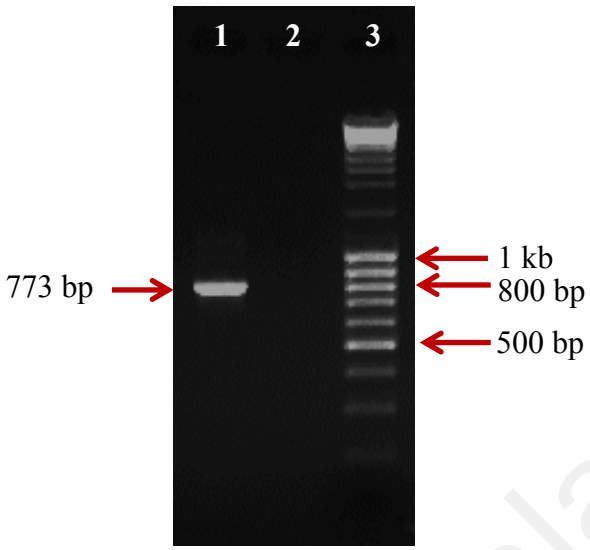
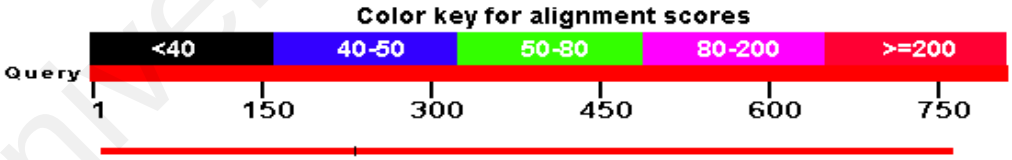


Figure 4.12: Amplification of putative full-length *MaPR-10* cDNA. Amplified product is 773 bp in-length

Lane 1: Putative full-length *MaPR-10* cDNA
Lane 2: Negative control PCR
Lane 3: 100 bp plus DNA marker



Sequence ID: lcl|Query_83907 Length: 949 Number of Matches: 2
Range 1: 413 to 949

Score	Expect	Identities	Gaps	Strand	Frame
929 bits(503)	0.0()	527/538(98%)	3/538(0%)	Plus/Plus	

Features:

```
Query  227  ACTCCAGCCGCAGAGCCACTGACCTTCGTGAAGGACCACGTTGAGGTGTTGGACCACGGA  286
            ||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  413  ACTGCAGCCGCAGAGCCACTGACCTTCGTGAAGGACCACGTTGAGGTGTTGGACCACGGA  472

Query  287  AGCCACACGATGAGGTACAAGGCGATCGAAGGAGGTCACCTCGGCCGAACGCTCAAGTCG  346
            |||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  473  AGCCACACGAGGAGGTACAAGGCGATCGAAGGAGGTCACCTCGGCCGAACGCTCAAGTCG  532

Query  347  CACGCGTTCGAGGTCAAGTTCGAAGCGACGGGCGCCGACAGCTGTGTCGTGAAGACCAAG  406
            |||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
Sbjct  533  CACGCGTTCGAGGTCAAGTTCGAAGCGGCGGGCGCCGACAGCTGTGTCGTGAAGACCAAG  592
```

Figure 4.13: Sequence alignment of full-length *MaPR-10* DNA (*M. acuminata* ssp. malaccensis) and *MamPR-10* cDNA (*M. acuminata* cv. _Mutiara‘)

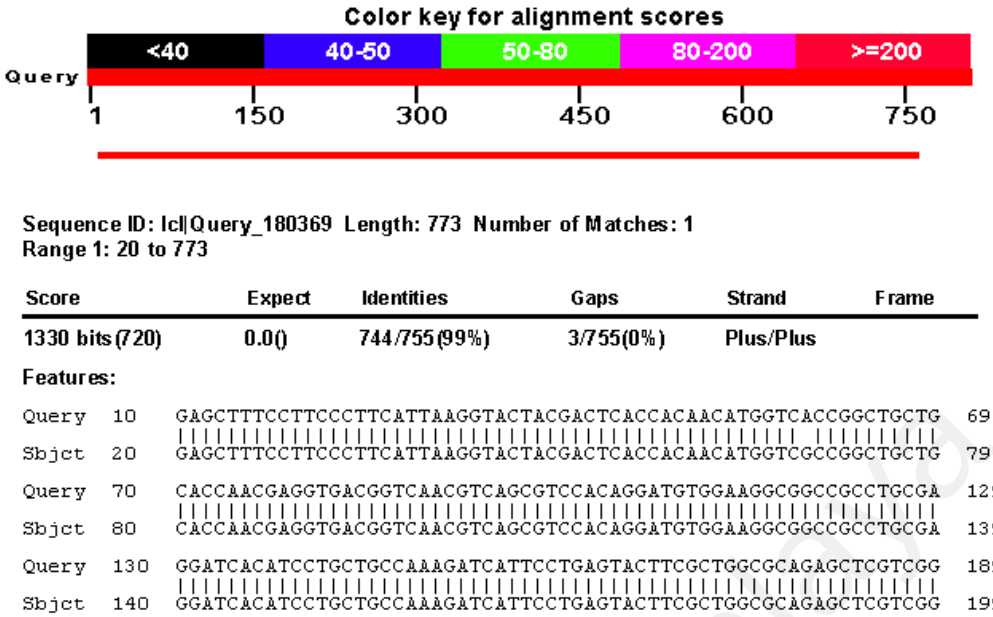


Figure 4.14: Sequence alignment of full-length *MaPR-10* cDNA (*M. acuminata* ssp. malaccensis) and *MamPR-10* cDNA (*M. acuminata* cv. _Mutiara‘)

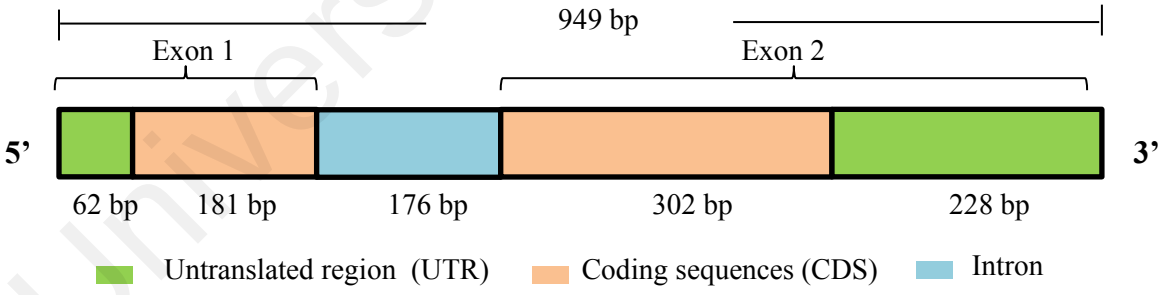


Figure 4.15: Components of full-length *MaPR-10* DNA sequence

Table 4.2: Summary of *MaPR-10* DNA and cDNA sequences

MaPR-10 DNA	*MaPR-10* cDNA
949 bp	773 bp
Intron: 176 bp 5‘ UTR: 62 bp 3‘ UTR: 228 bp	

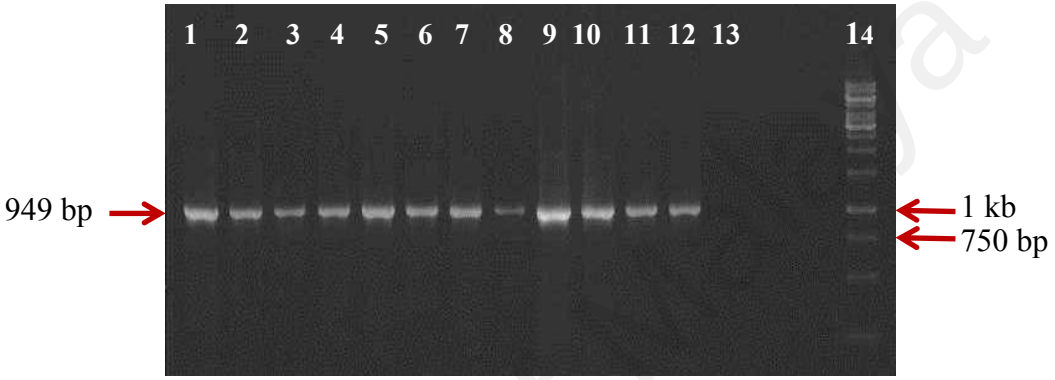


Figure 4.16: PCR colony of *E. coli* TOP10 clones harboring full length *MaPR-10* DNA

Lane 1-12: *E. coli* TOP10 clones harboring full length *MaPR-10* DNA
Lane 13: Negative control PCR
Lane 14: 1 kb DNA marker

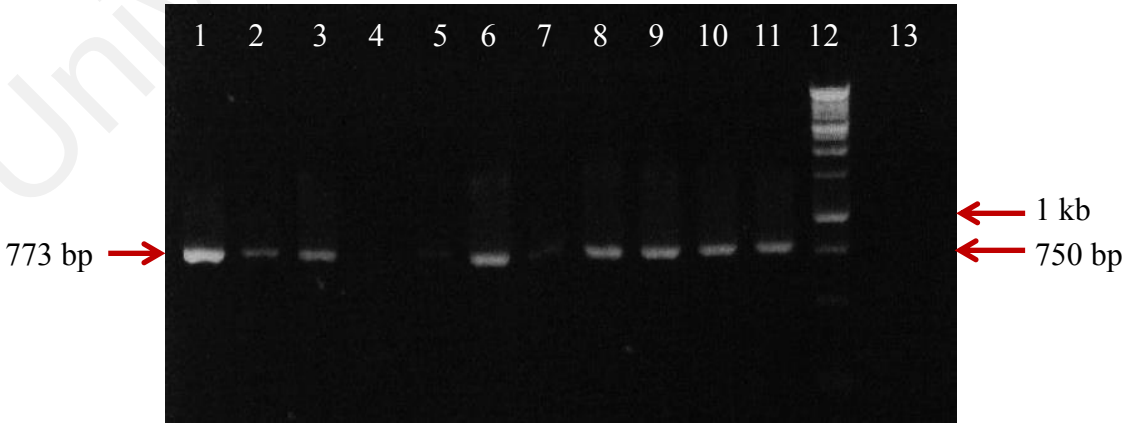


Figure 4.17: PCR colony of *E. coli* TOP10 clones harboring full length *MaPR-10* cDNA

Lane 1-11: *E. coli* TOP10 clones harboring full length *MaPR-10 cDNA*
Lane 12: 1 kb DNA marker
Lane 13: Negative control PCR

4.4 Analysis of *MaPR-10* coding sequences (*MaPR-10* cds)

Coding sequences of *MaPR-10* gene (483 bp in length including stop codon TGA) were successfully amplified using PR10 cds F/PR10 cds R flanked with NcoI and BglII restriction sites, respectively (Figure 4.18, Figure 4.19 and Appendix J5). The presence of the expected *MaPR-10* fragment in the putative transformed colonies verified the success of *E. coli* transformation (Figure 4.20). Comparison between these sequences with the banana genome database (D'Hont et al., 2012; Droc et al., 2013) showed 99% identity, spanning from 34783670-34784336 of chromosome 8 (Figure 4.21 and Figure 4.22).

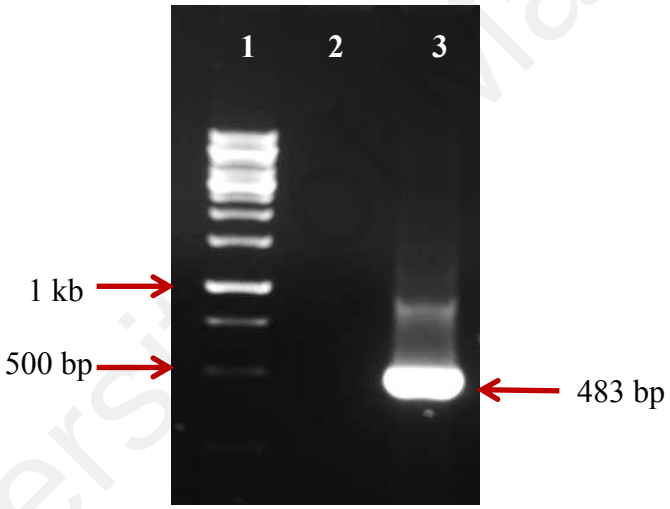


Figure 4.18: Amplification of *MaPR-10* cds. Amplified product is 483 bp in-length

Lane 1: 1 kb DNA marker
Lane 2: Negative control PCR
Lane 3: *MaPR-10* cds

```
ATGGTCGCCGGCTGCTGCACCAACGAGGTGACGGTCAACGTCAGCGTCCACAGG
ATGTGGAAGGCGGCCGCCTGCGAGGATCACATCCTGCTGCCAAAGATCATTCCT
GAGTACTTCGCTGGCGCAGAGCTCGTCGGCGATGGCGAAGCTGGCAGCACAAAG
ACCTTCCACTTCACTCCAGCCGCAGAGCCACTGACCTTCGTGAAGGACCACGTT
GAGGTGTTGGACCACGGAAGCCACACGAGGAGGTACAAGGCGATCGAAGGAGGT
CACCTCGGCCGAACGCTCAAGTCGCACGCGTTCGAGGTCAAGTTCGAAGCGACG
GGCGCCGACAGCTGTGTCGTGAAGACCAAGACCGAATACGATACGATCGACGAC
GCGCCGCTGCCGGAGGACGAGGTGCAGAAGATGACGGACGTGCCAGTACGGATG
ATGAAGTCGGTGGAAGCCTATCTGATAGCCAACCCCGGTGTTTGTGCCTGA
```

Figure 4.19: Sequences of *MaPR-10* cds (483 bp). Start (ATG) and stop codon (TGA) are highlighted in green and red, respectively

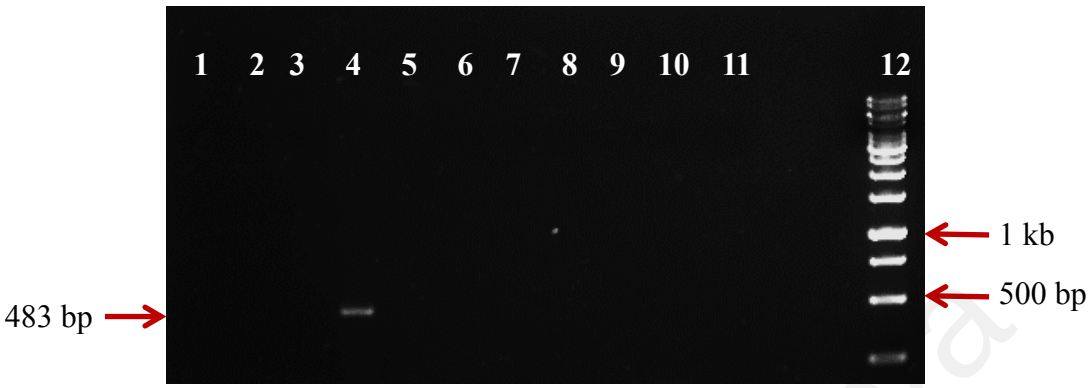


Figure 4.20: PCR colony of *E. coli* TOP10 clones harboring *MaPR-10* cds

Lane 1-10: *E. coli* TOP10 clones harboring *MaPR-10* cds
Lane 11: Negative control PCR
Lane 12: 1 kb DNA marker

```
Score =  351 bits (177), Expect = 4e-95
Identities = 180/181 (99%)
Strand = Plus / Plus

Query: 1        atggtcgccggctgctgcaccaacgaggtgacggtcaacgtcagcgtccacaggatgtgg 60
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 34783670 atggtcgccggctgctgcaccaacgaggtgacggtcaacgtcagcgtccacaggatgtgg 34783729

Query: 61       aaggcggccgcctgcgaggatcacatcctgctgccaaagatcattcctgagtacttcgct 120
                 |||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct: 34783730 aaggcggccgcctgcgaggatcacatcctgctgccaaagatcattcctcagtacttcgct 34783789

Query: 121      ggcgcagagctcgtcggcgatggcgaagctggcagcacaaagaccttccacttcactcca 180
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 34783790 ggcgcagagctcgtcggcgatggcgaagctggcagcacaaagaccttccacttcactcca 34783849
```

Figure 4.21: blastn results of *MaPR-10* cds (The Banana Genome Hub) (http://banana-genome.cirad.fr/)

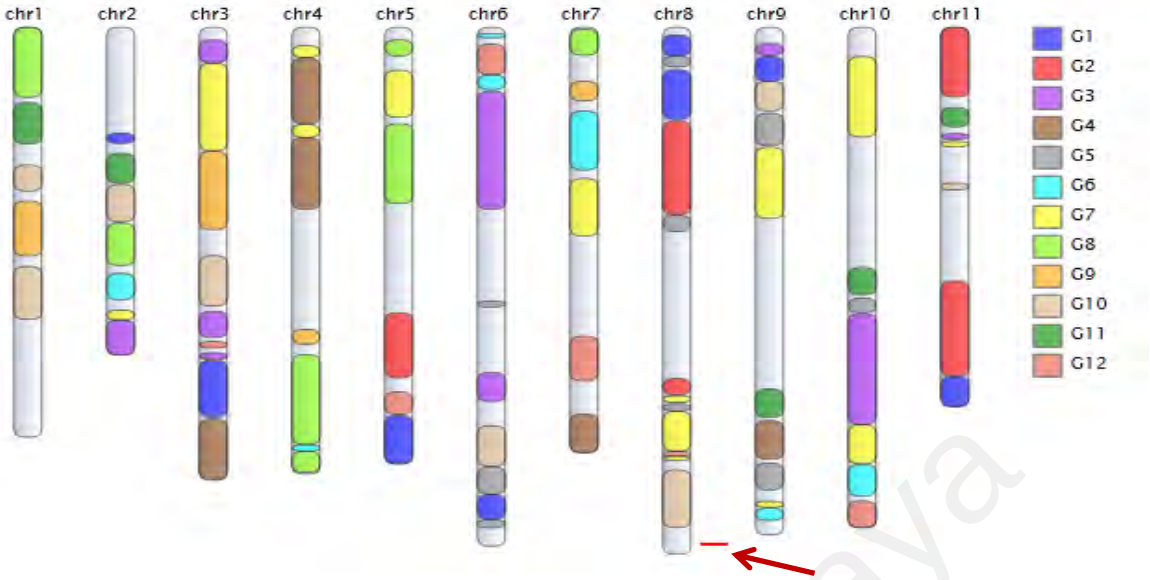


Figure 4.22: Location of *MaPR-10* on banana genome as mapped by The Banana Genome Hub (http://banana-genome.cirad.fr/). Twelve color codes shown on the right panel correspond to the twelve *Musa* α/β ancestral blocks (D' Hont et al., 2012; Droc et al., 2013)

MaPR-10 encodes 160 amino acids (Figure 4.23) with a predicted molecular mass of 17.46 kDa and isoelectric point of 5.42. MaPR-10 protein belongs to SRPBCC superfamily with Bet_v1-like domain (blastp, NCBI). Consistent with PR-10 proteins from other plants, hydrophobic ligand binding sites were also found virtually throughout the sequences (Figure 4.24). Alongside Bet_v1-like domain, four additional motifs were also predicted including four N-myristoylation sites (4 - 9 amino acid (a.a.), 51 - 56 a.a., 89 - 94 a.a. and 109 - 114 a.a.), one N-glycosylation site (13 - 16 a.a.), three protein kinase C phosphorylation sites (52 - 54 a.a., 81 - 83 a.a. and 95 - 97 a.a.) and four casein kinase II phosphorylation sites (108 - 111 a.a., 117 - 120 a.a., 119 - 122 a.a. and 123 - 126 a.a.). One serine (S52), two threonine (T119 and T123) and two tyrosine (Y121 and Y151) were identified as putative phosphorylation sites.

```
MVAGCCTNEVTVNVSVHRMWKAAACEDHILLPKIIPEYFAGAELVGDGEAGSTK
TFHFTPAAEPLTFVKDHVEVLDHGSHTRRYKAIEGGHLGRTLKSHAFEVKFEAT
GADSCVVKTKTEYDTIDDAPLPEDEVQKMTDVPVRMMKSVEAYLIANPGVCA*
```

Figure 4.23: Predicted sequence of MaPR-10 protein (160 a.a.)

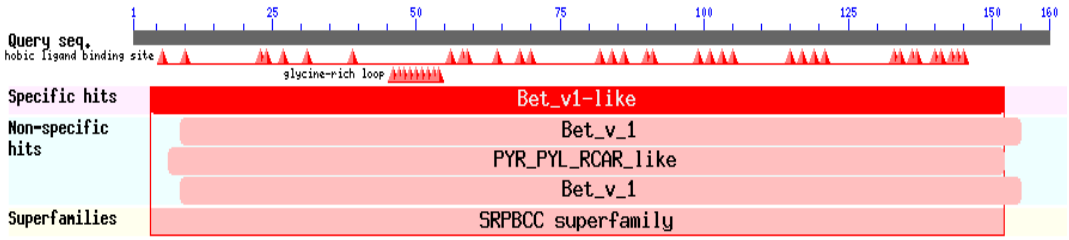


Figure 4.24: Domain search results by blastP (NCBI) software. Conserved domains of MaPR-10 included Bet_v1-like and START-like domains

Phyologenetic analysis of various plant-derived PR-10s revealed that MaPR-10 is more closely related to those from monocots such as *Oryza sativa* (OsPR-10), *Zea mays* (ZmPR-10a) and *Hordeum vulgare* (HvPR-10) (Figure 4.25). Alignment between MaPR-10 and PR-10 of selected plant species, mainly of monocotyledonous taxa, depicted 12 highly conserved amino acid residues, comprising mostly of glycine. Putative P-loop motif, GxGxxG and three amino acid sites (E102 (glutamate or Glu), E149 and Y151 (tyrosine or Tyr)) potentially linked to the RNase activity of MaPR-10 protein were also discovered (Figure 4.26). Presence of signal peptide and transmembrane domain was not detected in MaPR-10.

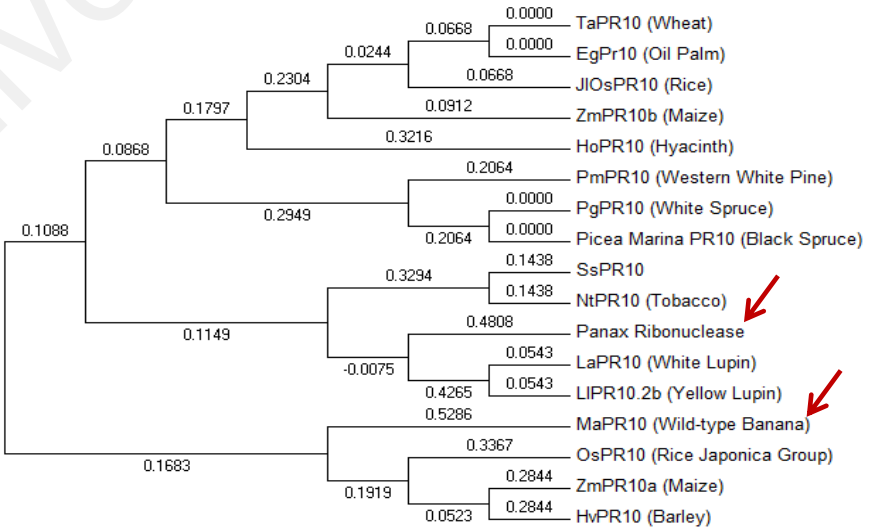


Figure 4.25: Phylogenetic analysis of PR-10 protein from 16 plant species including MaPR-10 and ribonuclease from *Panax ginseng* (red arrows)

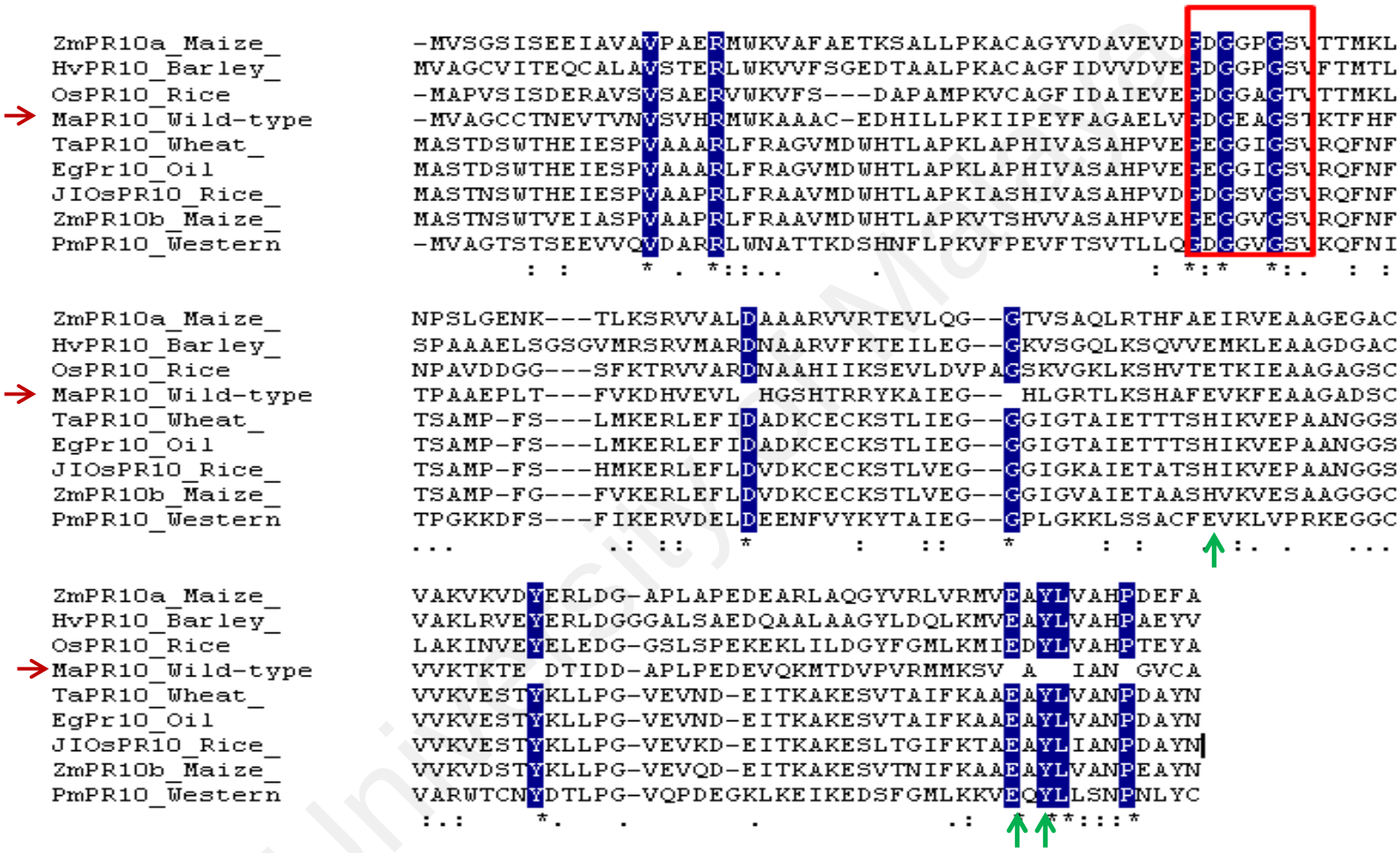


Figure 4.26: Alignment of PR-10 proteins. The plant species involved are *Zea mays* (ZmPR10a: AFW56469.1 and ZmPR10b: DAA44938.1), *Triticum aestivum* (TaPR10: ACG68733.1), *Elaeis guineensis* (EgPR10: AEB96227.1), *Oryza sativa* (OsPR10: ACA50491.1 and JIOsPR10: AAL74406.1), *Hordeum vulgare* (HVPR10: AAP04429.1), *Pinus monticola* (PmPR10: AAL50005.1) and *Musa acuminata* ssp. malaccensis (MaPR10). Strictly conserved amino acid residues are highlighted in blue while conserved motif GxGGxG/GxGxxG are shown in red box. Three putative sites potentially associated with RNase activity of MaPR-10 were shown by green arrow.

4.5 pMaPR-10 vector construct

Successful digestion of both purified *MaPR-10* cds fragment and pCAMBIA1304 plasmid by NcoI and BglII restriction enzymes was demonstrated by the appearance of distinct single band (~483 bp and ~12 kb for *MaPR-10* and pCAMBIA1304, respectively) on the 1% agarose gel when viewed under UV light (Figure 4.27). Expression of pMaPR-10 construct was driven by constitutive CaMV35S dual-promoters. The construct contained kanamycin and hygromycin antibiotic markers for selection in bacterial and plant host cells, respectively (Figure 4.28). The integrity of the construct was confirmed by the presence of 618 bp (Figure 4.29, Appendix F11 and Appendix J6), 618 bp (Figure 4.30, Appendix F12 and Appendix J7) and 388 bp (Figure 4.31, Appendix F13 and Appendix J8) bands, which corresponded to the *hptII* gene, partial CaMV35S promoter-*MaPR-10* and *MaPR-10*-mgfp5 regions at the upstream and downstream of the inserted *MaPR-10*, respectively.

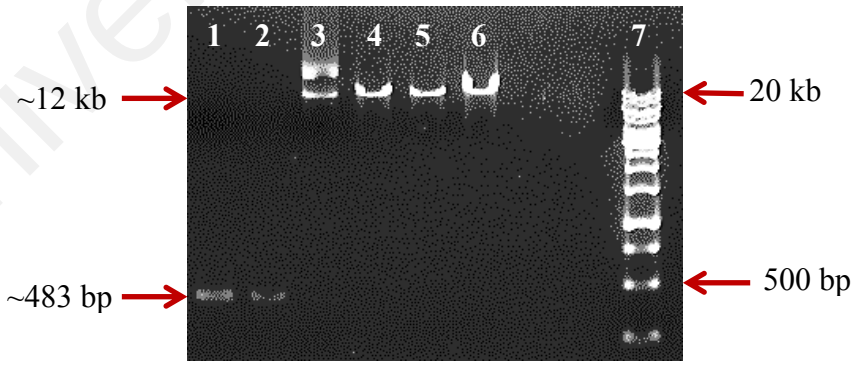


Figure 4.27: Digestion of *MaPR-10* and pCAMBIA1304 plasmids using NcoI and BglII restriction enzymes

Lane 1:	Undigested *MaPR-10* cds
Lane 2:	Digested *MaPR-10* cds
Lane 3:	Undigested pCAMBIA1304
Lane4-6:	Digested pCAMBIA1304
Lane 7:	1 kb DNA marker

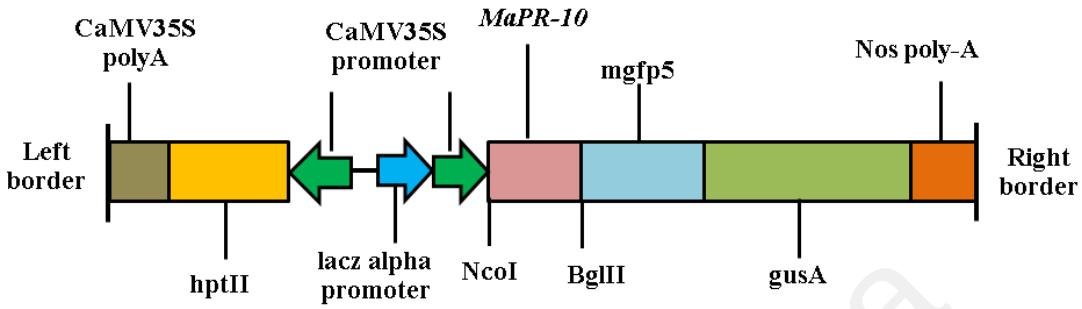


Figure 4.28: Vector construct of pCAMBIA1304 harboring *MaPR-10* cds (pMaPR-10)

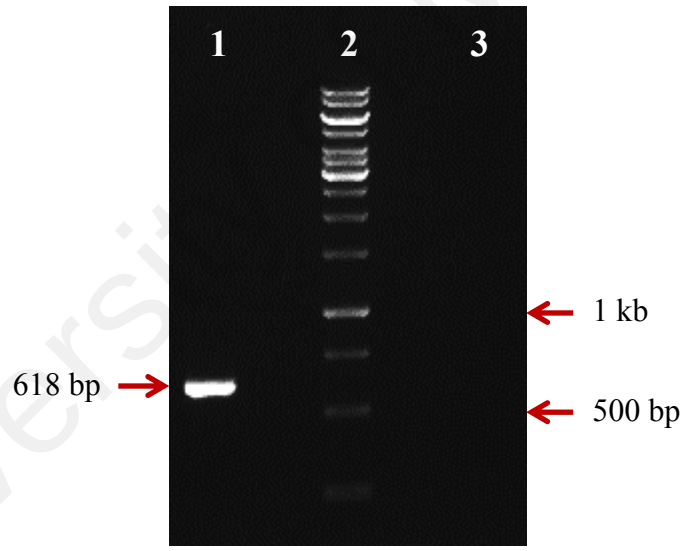


Figure 4.29: Verification of the integrity of pMaPR-10 construct using *hptII* gene (618 bp)

Lane 1: *hptII* gene
Lane 2: 1 kb DNA marker
Lane 3: Negative control PCR

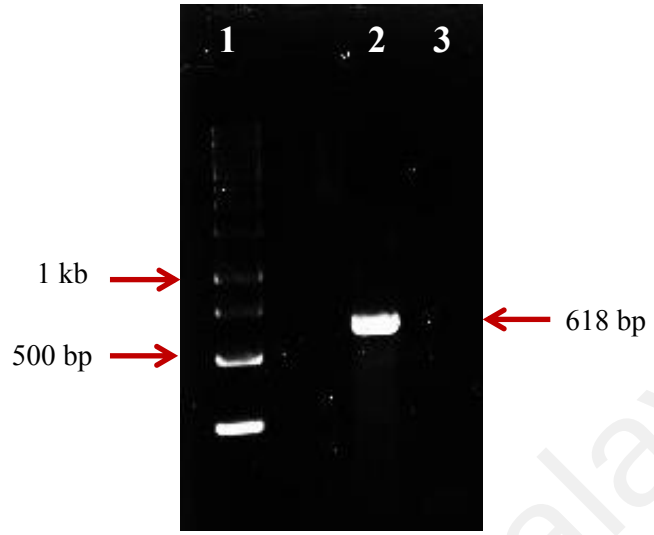


Figure 4.30: Verification of the integrity of pMaPR-10 construct by amplification of the flanking CaMV35S promoter and *MaPR-10* cds region (618 bp)

Lane 1:	1 kb DNA marker
Lane 2:	Flanking CaMV35S promoter and *MaPR-10* cds region
Lane 3:	Negative control PCR

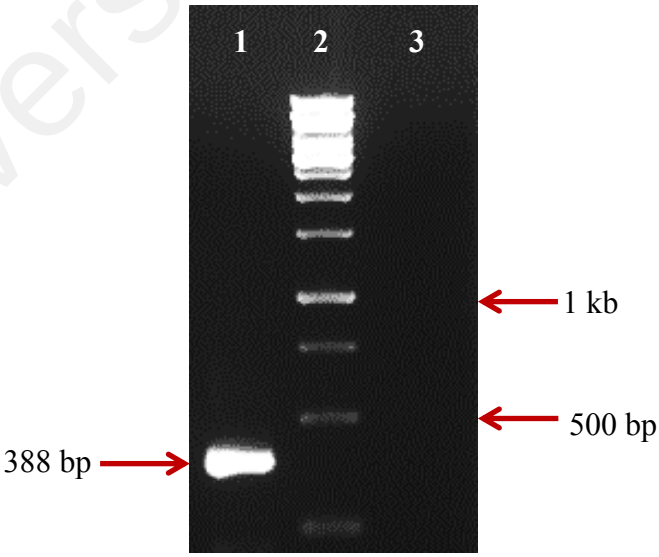


Figure 4.31: Verification of the integrity of pMaPR-10 construct by amplification of the flanking *MaPR-10* cds and mgfp5 region (388 bp)

Lane 1:	Flanking *MaPR-10* cds and mgfp5 region
Lane 2:	1 kb DNA marker
Lane 3:	Negative control PCR

4.6 *Agrobacterium*-mediated transformation of pMaPR-10 construct

Colonies that grew on the selective LB agar plates supplemented with 50 µg/ml rifampicin and 100 µg/ml kanamycin following an overnight incubation at 37°C signified the success of *A. tumefaciens* strain LBA4404 transformation. Expected *MaPR-10* fragment (483 bp) as amplified by PR10 cds F/PR10 cds R primers further verified the presence of pMaPR-10 construct (Figure 4.32). LB broth (50 µg/ml rifampicin and 100 µg/ml kanamycin) inoculated with positively transformed LBA4404 cultures appeared turbid following overnight incubation at 28°C, 220 rpm, thus proving the viability of the cells.

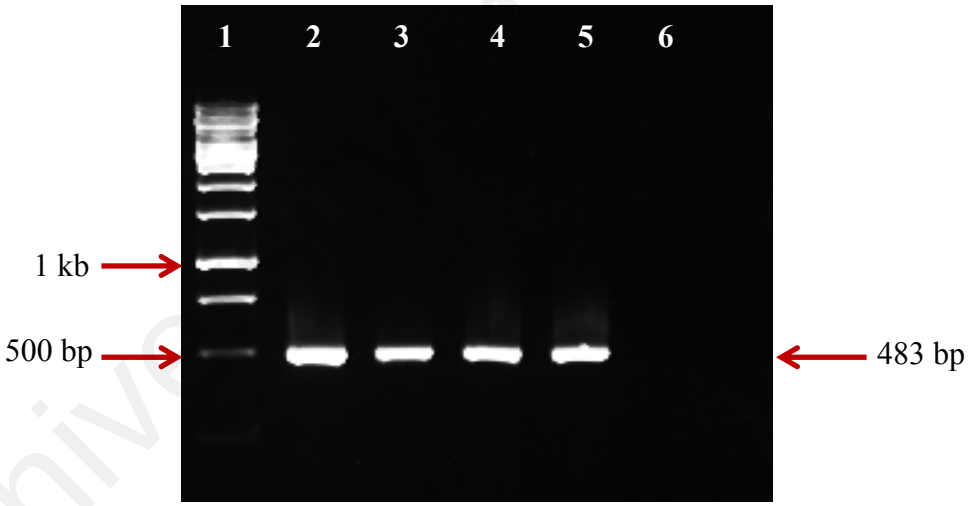


Figure 4.32: PCR colony of selected *A. tumefaciens* strain LBA4404 clones harboring pMaPR-10 construct

Lane 1: 1 kb DNA marker
Lane 2-5: *A. tumefaciens* strain LBA4404 clones harboring pMaPR-10 construct
Lane 6: Negative control PCR

4.7 Maintenance and regeneration of transformed cells

The use of 50 µg/ml cefotaxime in M2 medium managed to suppress the *A. tumefaciens* activity as no contamination was observed during the co-cultivation process. When maintained in M3 medium supplemented with 50 µg/ml of cefotaxime and different hygromycin concentrations, positive control cells harboring empty pCAMBIA1304 vector started showing protruding shoots after about four weeks and were generally more responsive in 2 mg/L hygromycin-supplemented medium. On the other hand, somatic embryos of *MaPR-10* transformed cells were first detected after about five weeks in 5 mg/L hygromycin-supplemented M3 medium. After about three months, somatic embryos induction of *MaPR-10*-transformed cells was most obvious in 0.2 mg/L- followed by 5 mg/L- and 2 mg/L- hygromycin-supplemented M3 medium. Positively transformed cells remained viable and continuously divided forming big cell clumps with protruding shoots whereas untransformed cells became brownish cell debris in the medium.

After about 6-8 weeks of culture on M8B, potential embryogenic calli were able to withstand the antibiotic selection and remained healthy. Globular-stage embryo started to appear on the surface of these calli (Figure 4.33A) while most of the non-surviving cells turned brownish or black and eventually died (Figure 4.33B).

Out of 3418 pMaPR-10 –derived calli cultured on M8B, about 98.4% were non-embryogenic; mostly of watery, compact and yellow nodular types while only about 53 of them or 1.6% were potential embryogenic calli. Some of the potential embryogenic calli showed friable and transluscent proembryo structures (not shown). All potential embryogenic calli with protruding shoots were transferred into M4B medium (Figure 4.33C) for further selection.

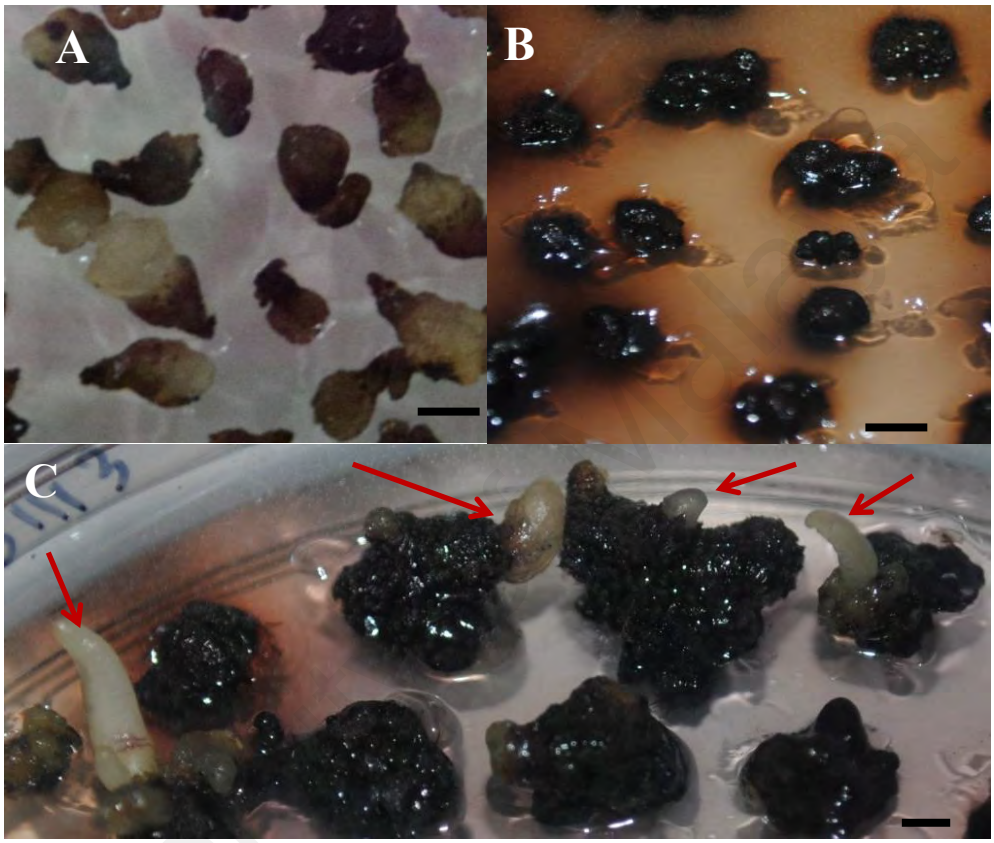


Figure 4.33: Selection of transformed cells on M4 supplemented with 8 mg/L BAP, 0.2 mg/L hygromycin and 50 µg/ml cefotaxime (M8B). A) One month-old potential embryogenic cells culture, B) Somatic embryos turned black and were unable to resist hygromycin selection, C) Protruding shoots observed on potential somatic embryos (arrowed) after 6-8 weeks of culture. Bar represents 0.25 cm

In M4B, potential embryogenic calli started producing multiple shoots as early as one week after culture (Figure 4.34A). Due to continuous antibiotic selection, out of 53 potential transgenic *MaPR-10* lines established, some failed to regenerate new shoots while some turned black finally leading to tissue death (Figure 4.34B). Only 17 lines survived the antibiotic selection at M4B stage, which were Line-3, 8, 13, 17, 18, 19, 20, 22, 23, 24, 29, 39, 42, 45, 50, 51 and 53. All lines depicted different growth and propagation rates. Line-3, 8, 13 and 17 were among the earliest to develop shoots but demonstrated slow shoot propagation. Line-19 plantlets, on the other hand, demonstrated both fast shoot propagation and vigorous growth. Potential shoots emerged from the embryogenic calli continued to grow for about three weeks until they were sub-cultured into new M4B to avoid medium depletion as well for propagation (Figure 4.34C).

After 2-3 weeks of culture in M4B, the shoots grew to about 3 cm in height (Figure 4.34D). Individual shoot was further regenerated in M2B (without antibiotic supplement) and continued to grow until about 5-6 cm in height (approximately 2-3 weeks after culture) (Figure 4.34E) before it was transferred into M5 (rooting medium). After about two weeks of culture in M5, vigorous healthy roots started to develop (Figure 4.34F). The plantlets were maintained in M5 with 1-month-sub-culturing interval prior to transgene screening and acclimatization.

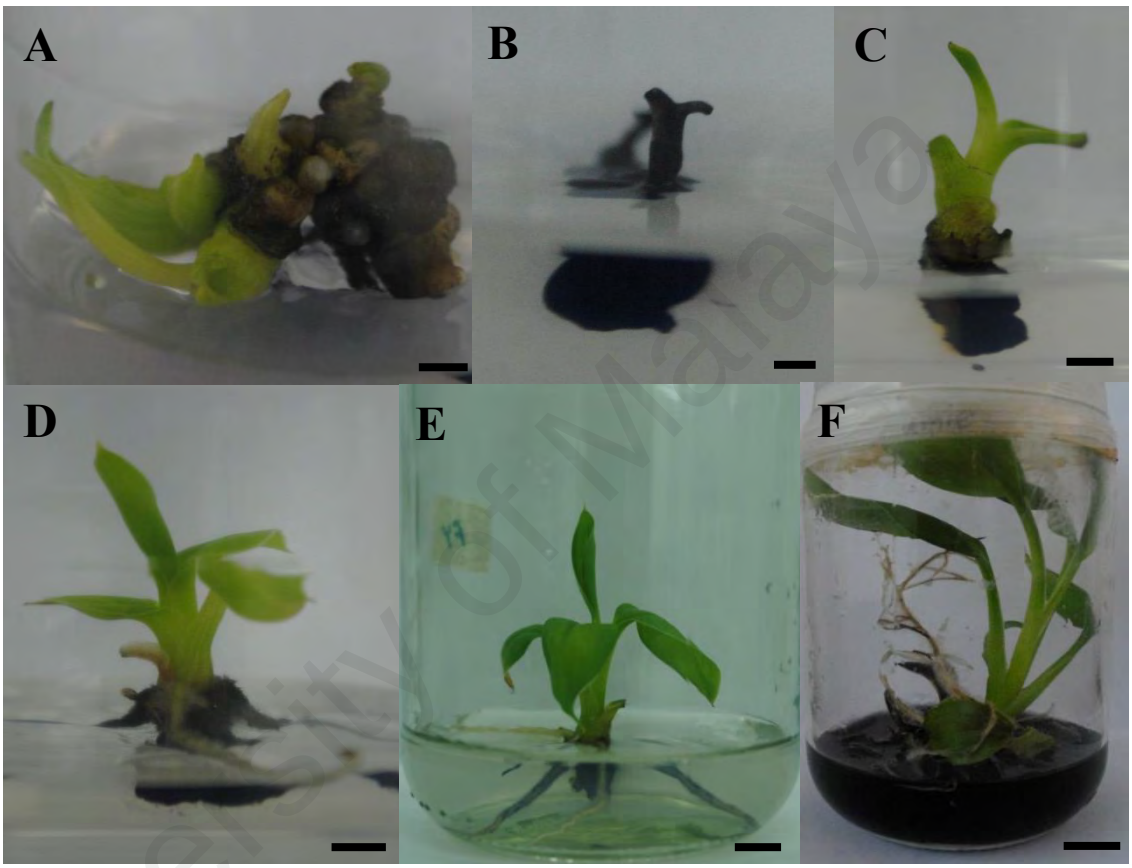


Figure 4.34: Regeneration of *MaPR-10*- harboring plantlet on M4B (supplemented with 4 mg/L BAP, 0.2 mg/L hygromycin and 50 µg/ml cefotaxime), M2B (2 mg/L BAP) and M5 medium. A) Mixture of clumps and shoots formed on M4B, B) Potential embryogenic callus that was unable to resist hygromycin selection turned dark on M4B, C) Shoot of *M. acuminata* cv. 'Berangan' harboring *MaPR-10* cds started to form in M4B, D) Shoot of putative transgenic *M. acuminata* cv. 'Berangan' continued to grow in M4B, E) Shoot of putative transgenic 'Berangan' was cultured in M2B for further shoot regeneration, F) Regenerated shoot was further cultured on M5 for root elongation. Bar A, B= 0.25 cm, C= 0.5 cm, D= 0.3 cm, E= 0.75 cm, F= 1.5 cm

4.8 Analysis of putative transgenic *M. acuminata* cv. 'Berangan' plants harboring *MaPR-10* cds

4.8.1 Screening of putative transgenic plantlets

Out of 17 putative transgenic *MaPR-10* cds lines established on M4B, 11 lines showed 618 bp-*hptII* (Appendix G) and 388 bp-gene specific *MaPR-10* bands (Figure 4.35) when analyzed by PCR. Successful *MaPR-10* integration was observed in Line-18, 19, 20, 22, 23, 29, 39, 42, 45, 51 & 53 whilst Line-3, 8, 13, 17, 24 and 50 were probably escapes. In comparison with other lines, plantlets of Line-19 demonstrated the most rapid shoot propagation and fast growth. In addition, randomly selected Line-19 plantlets also showed positive transgene integration (Figure 4.36)

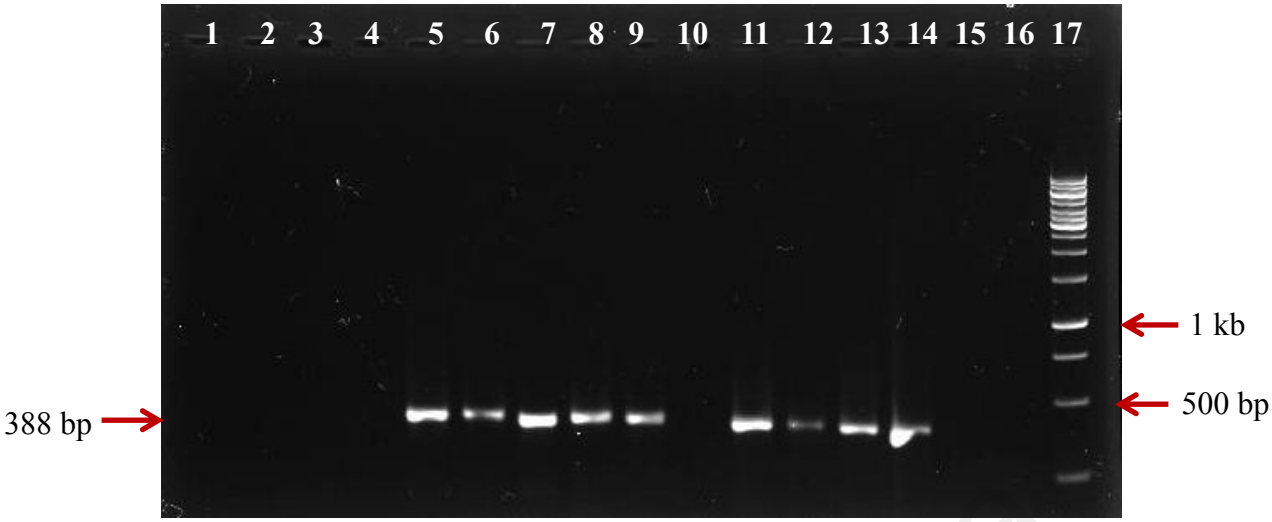


Figure 4.35: Transgene screening of selected *MaPR-10* lines using PR10 S F/1304R primer pair

Lane 1-4, 10:	Negative transformed lines (Line-3, 8, 13, 17 & 24, respectively)
Lane 5-9, 11-13:	Positive transformed lines (Line-18, 19, 20, 22, 23, 29, 39 & 42, respectively)
Lane 14:	Positive control (Purified plasmid 3.8)
Lane 15:	Untransformed control (*M. acuminata* cv. _Berangan‘)
Lane 16:	Negative control PCR
Lane 17:	1 kb DNA marker

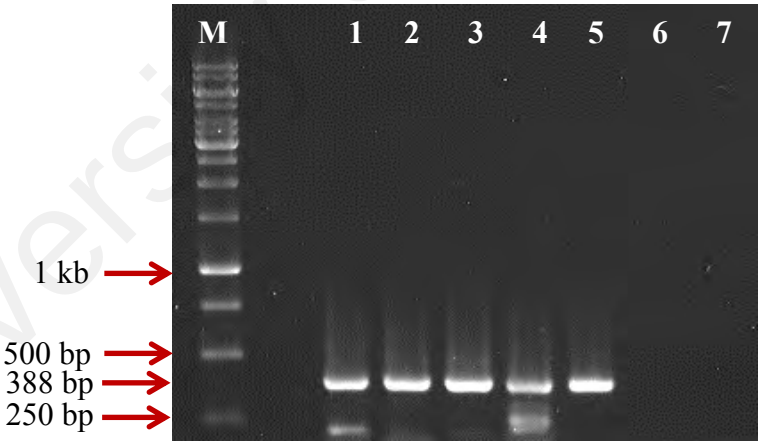


Figure 4.36: Transgene screening of randomly selected Line-19 plantlets using PR10 S F/1304R primer pair

Lane 1-4:	Randomly selected Line-19 plantlets
Lane 5:	Positive control (Purified plasmid 3.8)
Lane 6:	Untransformed control (*M. acuminata* cv. _Berangan‘)
Lane 7:	Negative control PCR
Lane M:	1 kb DNA marker

4.8.2 Transgene copy number analysis

Transgene copy number of *MaPR-10* in Line-19 *M. acuminata* cv. _Berangan' was estimated using qPCR analysis. Single peak generated by dissociation curve denoted the absence of primer dimer as well as the specificity of qpcr_PR10-1304_F2/qpcr_PR10-1304_R2 primers used (Figure 4.37). C_T values of *MaPR-10* for all tested Line-19 samples fell within the C_T range generated by the serial dilution plasmids (Figure 4.38). Based on the linear equation obtained from the standard curve (Figure 4.39), the predicted transgene *MaPR-10* copy number per genome in Line-19 *M. acuminata* cv. _Berangan' was one (Table 4.3).

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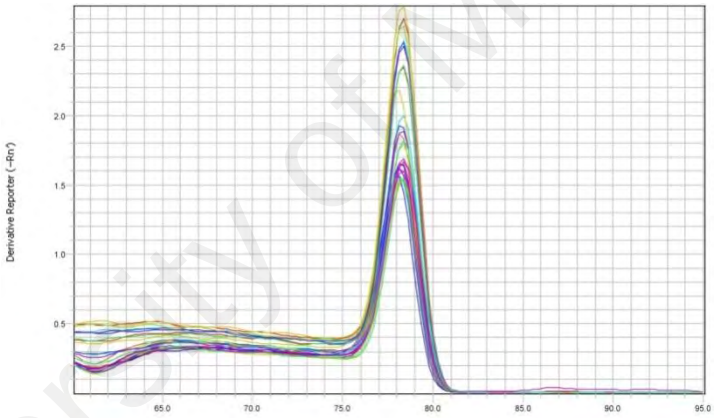

Figure 4.37: Dissociation curve of *MaPR-10*-pCAMBIA1304 region from 5 serial dilution standards (SD1-SD5) and genomic DNA (gDNA) from randomly selected Line-19 plantlets

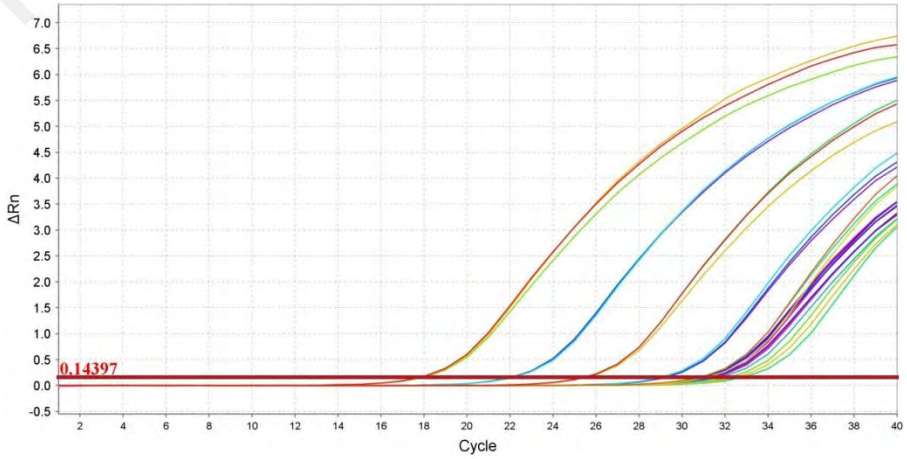

Figure 4.38: Amplification plot of *MaPR-10*-pCAMBIA1304 region from 5 serial dilution standards (SD1-SD5) and genomic DNA (gDNA) from randomly selected Line-19 plantlets

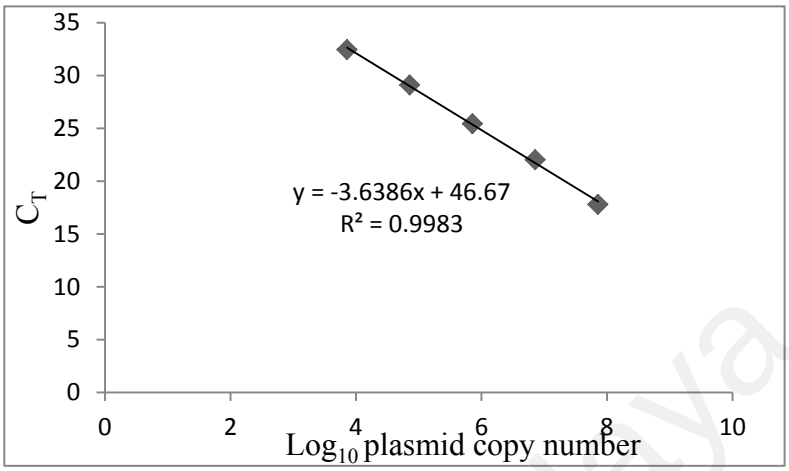

Figure 4.39: Linear equation between C_T of serial dilution standards and log10 of plasmid copy numbers harboring *MaPR-10*

1. Estimated length of one genome of transgenic *M. acuminata* cv. „Berangan" (AAA): 523 Mbp x 3 = 1,569 Mbp
2. Total weight (g) of one genome of transgenic *M. acuminata* cv. „Berangan" (AAA):
   - Assume 1 bp= 660 Da and 1 g of DNA is approximately 6.023×10^{23} Da
   - $\frac{1{,}569 \times 10^6 \text{ bp} \times 660 \text{ Da}}{6.023 \times 10^{23} \text{ Da}} \times 1 \text{ g} = 1.72 \times 10^{-12}$ g
3. No. of copies of *MaPR-10* transgene in gDNA mix (30 ng) calculated from standard curve = ≈15,077
4. No. of genomes of *M. acuminata* cv. „Berangan" in gDNA mix (30 ng)=
$$\frac{30 \times 10^{-9} \text{ g}}{1.72 \times 10^{-12} \text{ g}} = \sim 17{,}441$$
5. No. of copies of *MaPR-10* transgene/genome of *M. acuminata* cv. „Berangan"
$$= \frac{15{,}077}{17{,}441} = 0.86 \sim 1$$

Figure 4.40: Example of calculation for transgene copy number of unknown transgenic sample A

Table 4.3: Summary of *MaPR-10* transgene copy number calculation in unknown Line-19 samples

Unknown samples (Line-19)	C_T mean	No. of copies of *MaPR-10* transgene in gDNA mix (30 ng) from standard curve (X)	No. of genomes of *M. acuminata* cv. _Berangan' in gDNA mix (30 ng) (Y)	Ratio of X/Y	Predicted copy number of *MaPR-10* transgene/genome *M. acuminata* cv. _Berangan'
A	31.47	15,077	17,441	0.86	1
B	30.90	21,523		1.23	1
C	30.87	22,009		1.26	1
D	31.11	18,798		1.08	1

4.8.3 Expression analysis of *MaPR-10*

Expression of *MaPR-10* in Line-19 plants was analyzed using PR10 qpcr F2/PR10 qpcr R2 and RPS2F/RPS2R primer pairs which amplified the target (*MaPR-10*) and reference gene (*RPS2*), respectively. Single peak generated by dissociation curve denoted the absence of primer dimer as well as the primers' specificity (Figure 4.41 and Figure 4.42). Amplification plots for both *RPS2* and *MaPR-10* genes were shown in Figure 4.43 and Figure 4.44, respectively. Relative to the untransformed control, each Line-19 plants tested, PR16, PR27 & PR35 showed variation in the expression of *MaPR-10* (Table 4.4). Using $2^{-\Delta\Delta CT}$ formula, the fold-change of *MaPR-10* in PR16, 27 and 35 was 62.59, 8.74 and 13.66 respectively when normalized to the untransformed control (Figure 4.45).

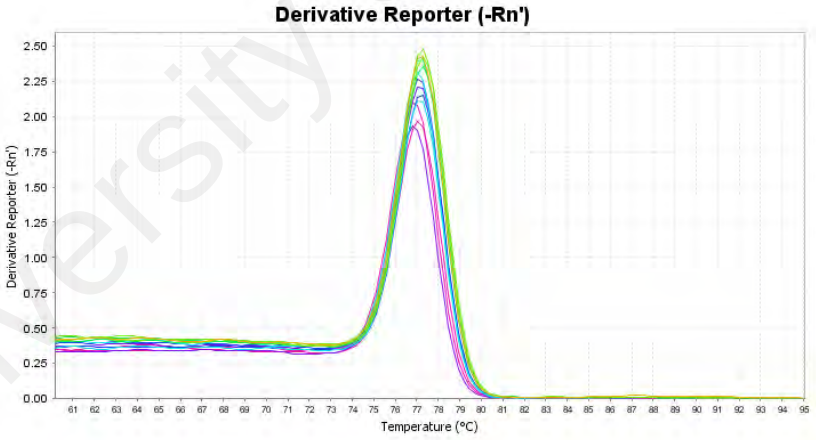

Figure 4.41: Dissociation curve of RPS2F/RPS2R (0.2 µM) primer pair at 60°C for over-expression study of *MaPR-10* in selected Line-19 plants

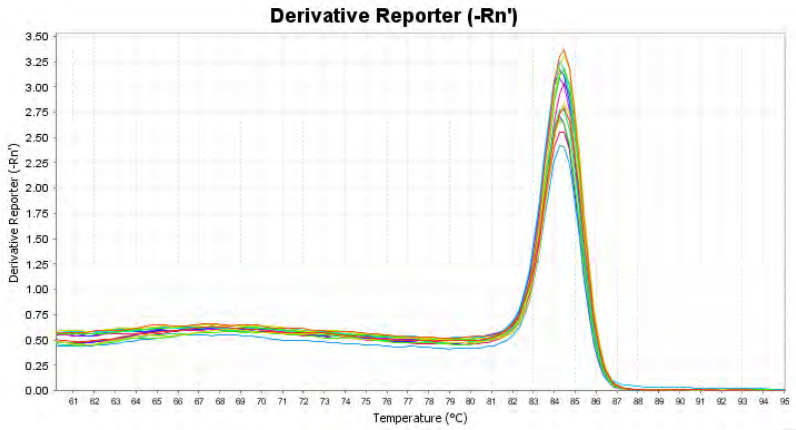

Figure 4.42: Dissociation curve of PR10 qpcr F2/ PR10 qpcr R2 (0.5 µM) primer pair at 60°C for over-expression study of *MaPR-10* in selected Line-19 plants

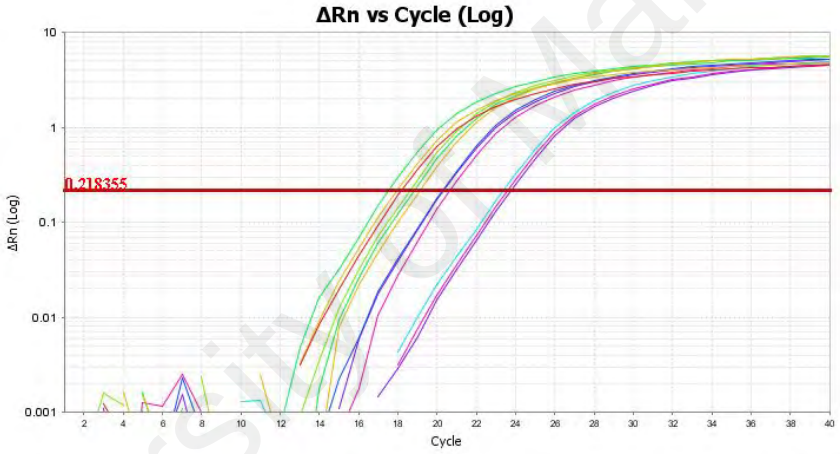

Figure 4.43: Amplification plot of *RPS2* gene (RPS2F/RPS2R) for untransformed control and selected Line-19 cDNA samples

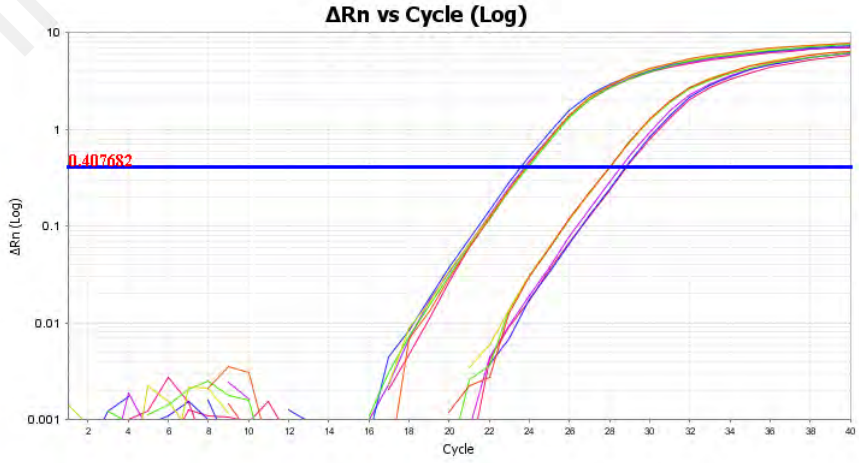

Figure 4.44: Amplification plot of *MaPR-10* transgene (PR10 qpcr F2/ PR10 qpcr R2) for untransformed control and selected Line-19 cDNA samples

Table 4.4: Results summary for over-expression analysis of *MaPR-10* in selected Line-19 plants (PR16, PR27 & PR35) relative to untransformed control (UTC)

Sample	*RPS2* (C_T Mean)	*MaPR-10* (C_T Mean)	ΔC_T Mean (C_T *MaPR-10*-C_T *RPS2*)	ΔC_T SE	$\Delta\Delta C_T$ (ΔC_T Line-19-ΔC_T UTC)	RQ
UTC	18.852	28.004	9.152	0.154	0.000	1.000
PR16	20.441	23.625	3.184	0.175	-5.968	62.590
PR27	17.884	23.909	6.025	0.182	-3.127	8.739
PR35	23.395	28.775	5.380	0.149	-3.772	13.661

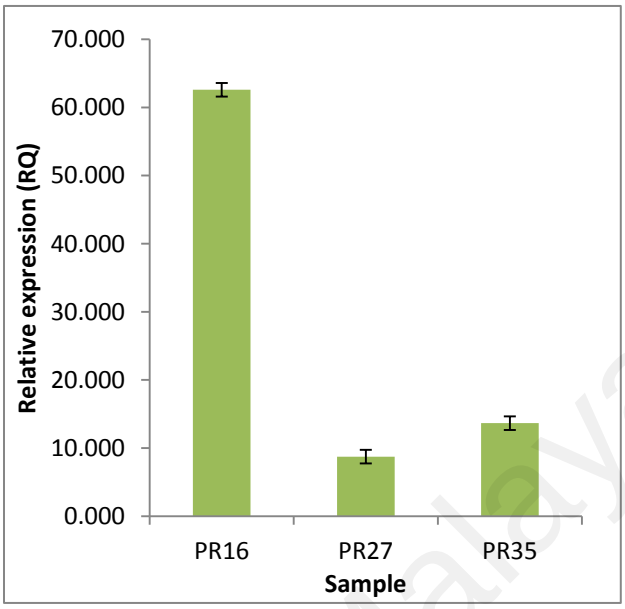

Figure 4.45: qPCR analysis for over-expression of *MaPR-10* in the leaves of selected Line-19 plants (PR16, PR27 and PR35) harvested at the same time. All target gene expression values have been normalized against *Musa RPS2* expression levels. The transgene expression has been calibrated to the untransformed control (UTC), which is assumed to be 1. The data represented are means of three technical replicates

4.9 Establishment of baseline disease response for *M. acuminata* cv. 'Berangan' plants against *Fusarium oxysporum* f. sp. *cubense* (Foc) C1 HIR infection

4.9.1 Verification and spore germination of C1 HIR isolate

FocR4 C1HIR that was cultured on PDA showed extensive white purplish mycelium after 4-5 days of incubation in the dark at room temperature (26±2°C) (Figure 4.46). PDB inoculated with mycelium became turbid and showed significant mycelium growth after 5-7 days of incubation. Germinated spores spotted on PDA confirmed the viability of pure C1 HIR culture that was then used in the bioassay experiments. High quality DNA in the desired purity range ($A_{260/280}$~1.8 to 2.0) was successfully obtained prior to race verification (Figure 4.47). C1 HIR was classified as a race 4 isolate based on the presence of a 242 bp-band upon amplification using the specific Foc4F and Foc4R primer pair (Figure 4.48).

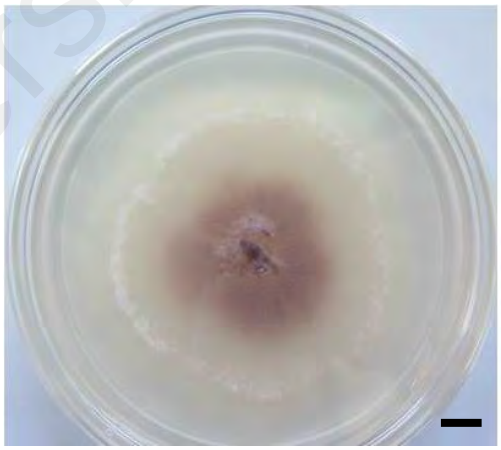

Figure 4.46: CI HIR cultre on PDA. White purplish of FocR4 C1 HIR mycelium grew on PDA after 4-5 days incubation in the dark at room temperature (26±2°C)

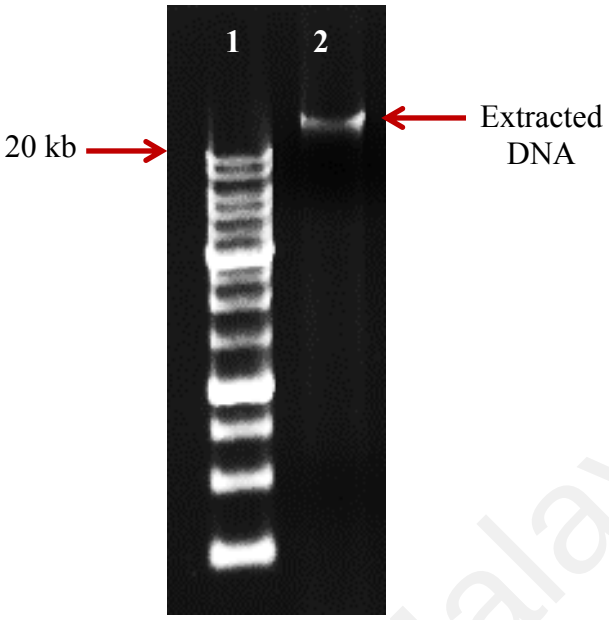

Figure 4.47: DNA extraction of Foc

Lane 1: 1 kb DNA marker
Lane 2: Extracted Foc DNA

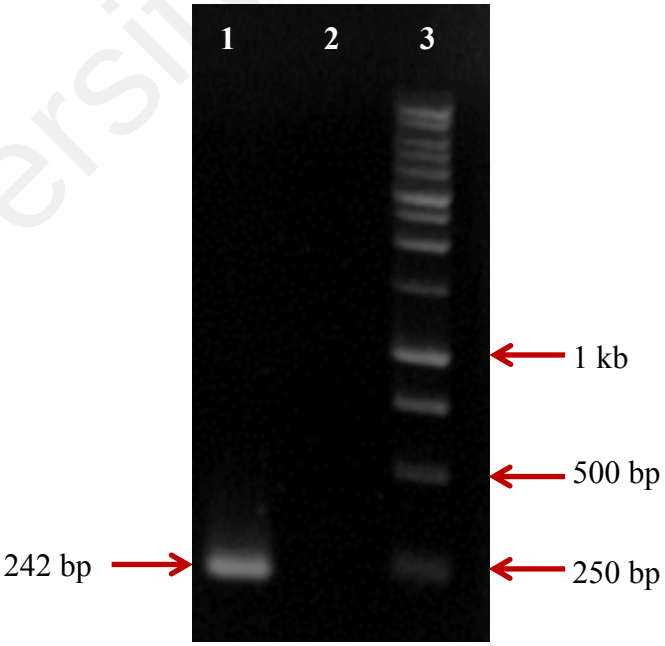

Figure 4.48: Amplification of FocR4-specific region using Foc4F and Foc4R primer pair. Amplified product is 242 bp in-length

Lane 1: FocR4-specific region
Lane 2: Negative control PCR
Lane 3: 1 kb DNA marker

4.9.2 Bioassay challenge of wild-type 'Berangan' plants against C1 HIR isolate

Wild-type _Berangan' plants infected with C1 HIR demonstrated external disease symptoms less than one week-post-inoculation. Streaking and yellowing symptoms initially started at the edges of lower leaves and later progressed to the mid rib area (Figure 4.49A). More severe infection was denoted when most or all of the leaves exhibited yellowing and wilting symptoms. Completely wilted leaves eventually abscised the petiole and led to plant's death (Figure 4.49B). Uninfected plants remained healthy throughout the bioassay experiment (Figure 4.49C).

Internally, plants started to display brownish to darkish discoloration at the junction of root and rhizome (Figure 4.50A). As hyphae advanced and colonized the vascular tissues, more discoloration was discovered especially in the stele region of the rhizome indicating a more severe disease incidence and ultimately contributed to the plant's death (Figure 4.50B). Rhizomes of uninfected plants showed no discoloration symptom (Figure 4.50C). Reference diagrams showing different degrees of infection on the leaves and rhizome with respective LSI and RDI scores were compiled to assist the evaluation process (Figure 4.51 and Figure 4.52, respectively). In the final disease scoring performed in the 5^{th} week, *M. acuminata* cv. _Berangan' was found to be highly susceptible to FocR4 (C1 HIR) isolate with LSI and RDI scores of 4.3 and 6.0, respectively (Appendix H).

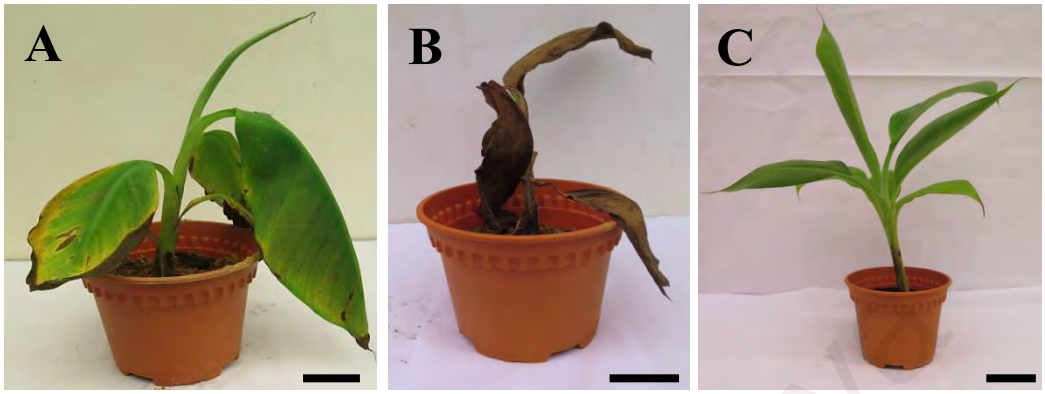

Figure 4.49: Representatives of C1 HIR-inoculated (A & B) and non-inoculated (C) 2-month-old wild-type _Berangan' plants for leaf symptoms. Plant A showed typical yellowing symptoms on the leaves as early as 6 days post-inoculation (dpi). As the disease progresses, more severe wilting symptoms that lead to plant's death (B) were observed at the end of the 5th week post-inoculation due to the advanced hyphae colonization of the vascular tissue. Non-inoculated plantlet (C) remained healthy throughout the bioassay experiment. Bar represents 5 cm

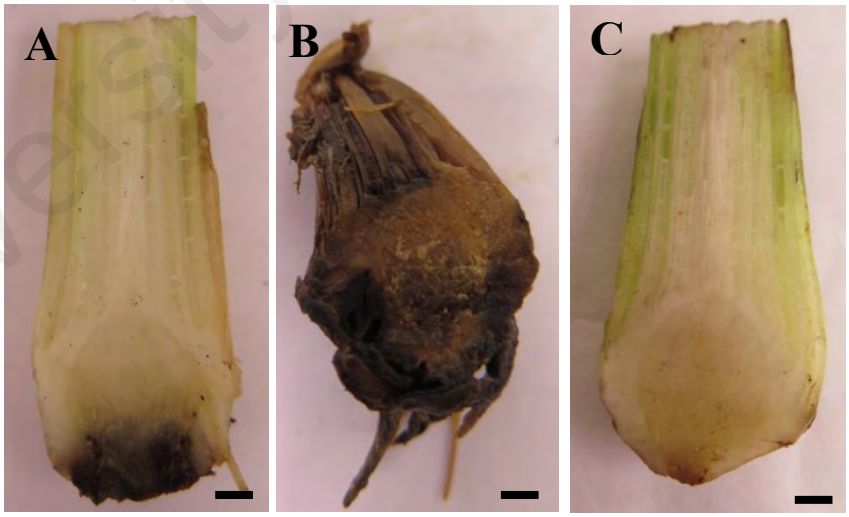

Figure 4.50: Representatives of C1 HIR-inoculated (A & B) and non-inoculated (C) 2-month-old wild-type _Berangan' plants for rhizome symptoms. Rhizomes of infected plants showed various degrees of discoloration that may start from the junction of root and rhizome (A) as early as 6-dpi advancing to the pseudostem and eventually led to the plant's death (B) at the end of the 5th week post-inoculation. Rhizome of non-inoculated wild-type _Berangan' (C) remained healthy throughout the bioassay experiment. Bar represents 0.5 cm

Figure 4.51: Reference diagrams for leaf symptoms index (LSI). LSI was divided into 5 scores to differentiate severity of disease symptoms ranging from healthy (lowest score) to dead plant (highest score)

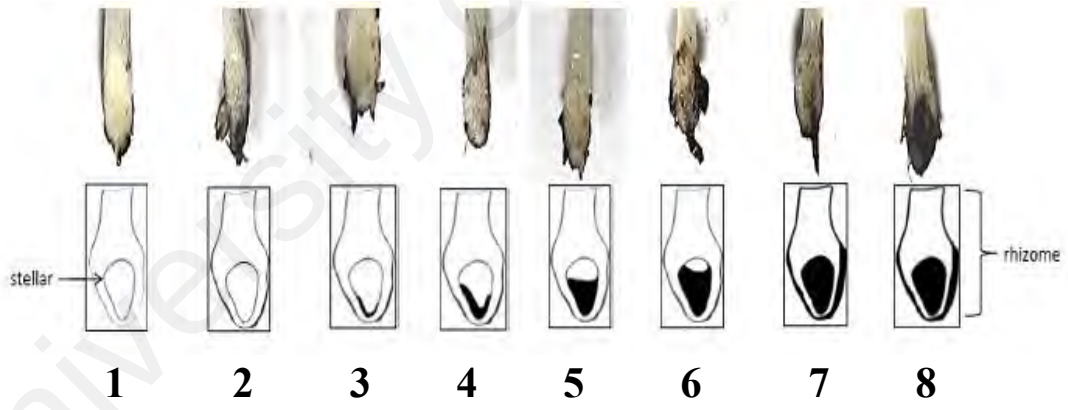

Figure 4.52: Reference diagrams for rhizome discoloration index (RDI). RDI was based on 8 different scores ranging from uninfected, healthy rhizome (lowest score) to complete discoloration (highest score) a) naked view b) schematic diagram

4.9.3 FESEM

FESEM examination on thin section of the infected rhizomes further confirmed the presence of typical Foc asexual spores, which were microconidia and chlamydospore, as early as 2 days-post-inoculation (Figure 4.53).

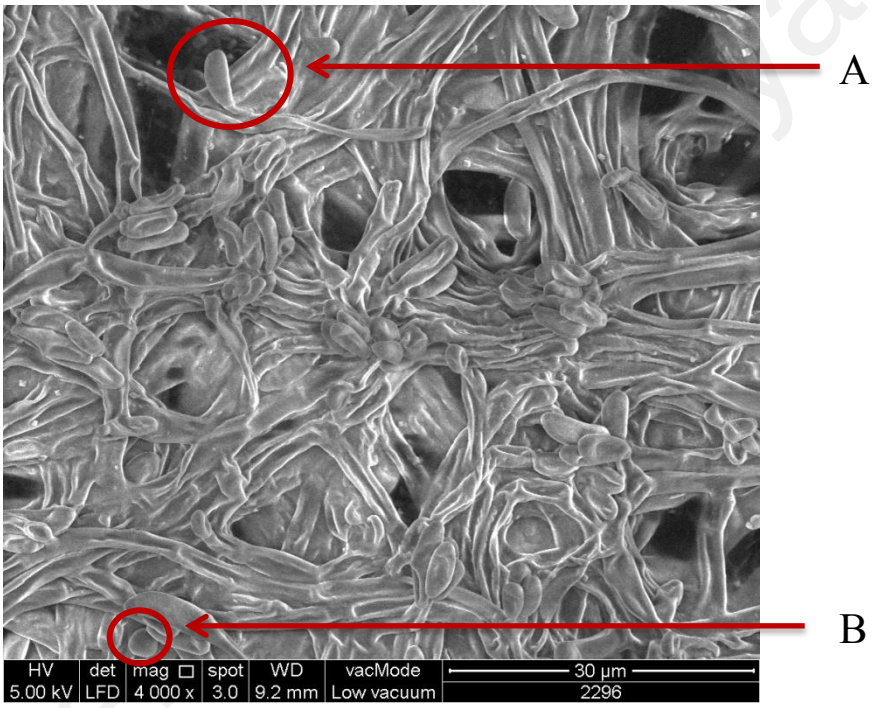

Figure 4.53: Field emission scanning electron microscope analysis of *F. oxysporum* f. sp. *cubense*. Showing two types of asexual spores: A) Microconidia growing from aerial mycelium and B) Chlamydospore under magnification of 4,000 X. Bar represents 30 µm

4.10 Analysis on the transgenic *MaPR-10* plants' response to C1 HIR infection

When grown at 29±1°C (16-8 hour photoperiod) for two months, about 75% (15 out of 20 plants including both the treated and untreated groups) of the transgenic plants depicted slower growth in comparison to the untransformed controls (Figure 4.54). At 6-dpi, infected untransformed plants started to show marked yellowing leaf symptoms (Figure 4.55). In comparison to the untransformed controls, about 30% (3 out of 10) of the transgenic plants underwent delayed disease symptoms which started to appear in the 2nd week post-inoculation (raw data is not available). At 20-dpi, the yellowing symptoms on the older leaves of the transgenic Line-19 plants became more visible initiated at the leaves' edges progressing to the midrib area while some of the leaves completely wilted (Figure 4.56). At the end of the 5th week, untreated controls remained healthy while infected plants demonstrated different degrees of disease symptoms. Treated Line-19 plants which showed delayed disease signs remain alive despite showing extensive wilting symptoms (Figure 4.57).

Internal observation on the rhizome of the survived transgenic plants showed incomplete discoloration in the stele region in which the infection did not proceed to the pseudostem. Rhizomes of more severely affected plants exhibited major to complete discoloration (Figure 4.58). In the final disease evaluation performed in the 5th week post-inoculation, transgenic Line-19 plants harboring *MaPR-10* was found to be highly susceptible to FocR4 (C1 HIR) isolate with LSI and RDI scores of 3.4 and 6.1, respectively. The LSI and RDI of treated untransformed controls were 4.5 and 6.3, respectively (Appendix I).

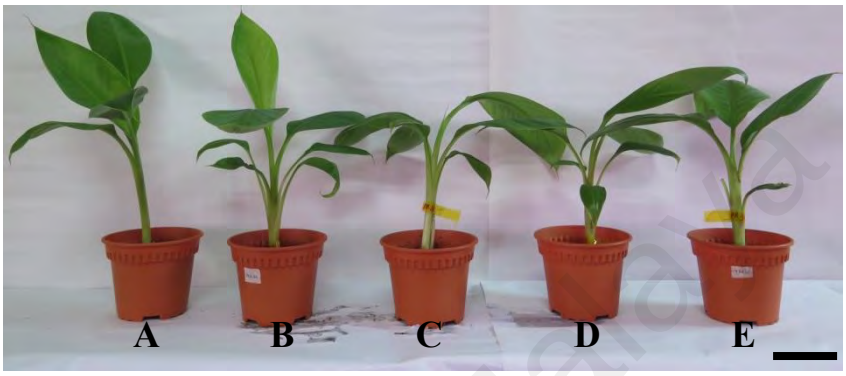

Figure 4.54: 0 day post-inoculation. Representatives shown were untransformed control (A) and Line-19 plants (B-E). A and B showed similar growth development which were faster than of C-E. Bar represents 8 cm

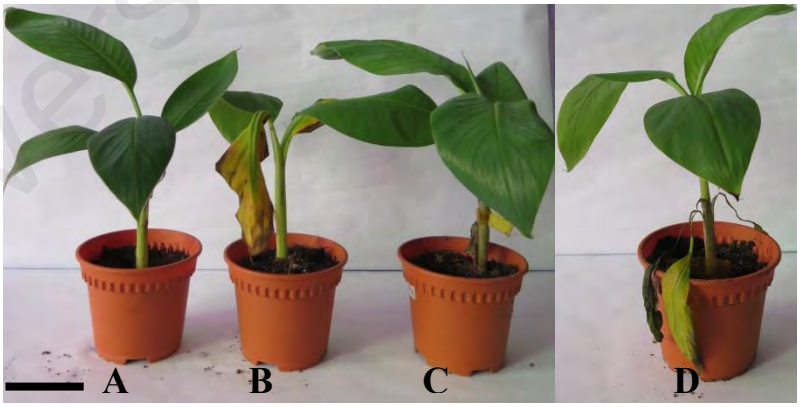

Figure 4.55: 6 days post-inoculation. Representatives shown were untreated control (A), treated untransformed control (B) and treated Line-19 plants (C & D). A and C remained healthy whereas D already exhibited minor yellowing symptoms. B started to show marked yellowing symptoms on the leaves. Bar represents 8 cm

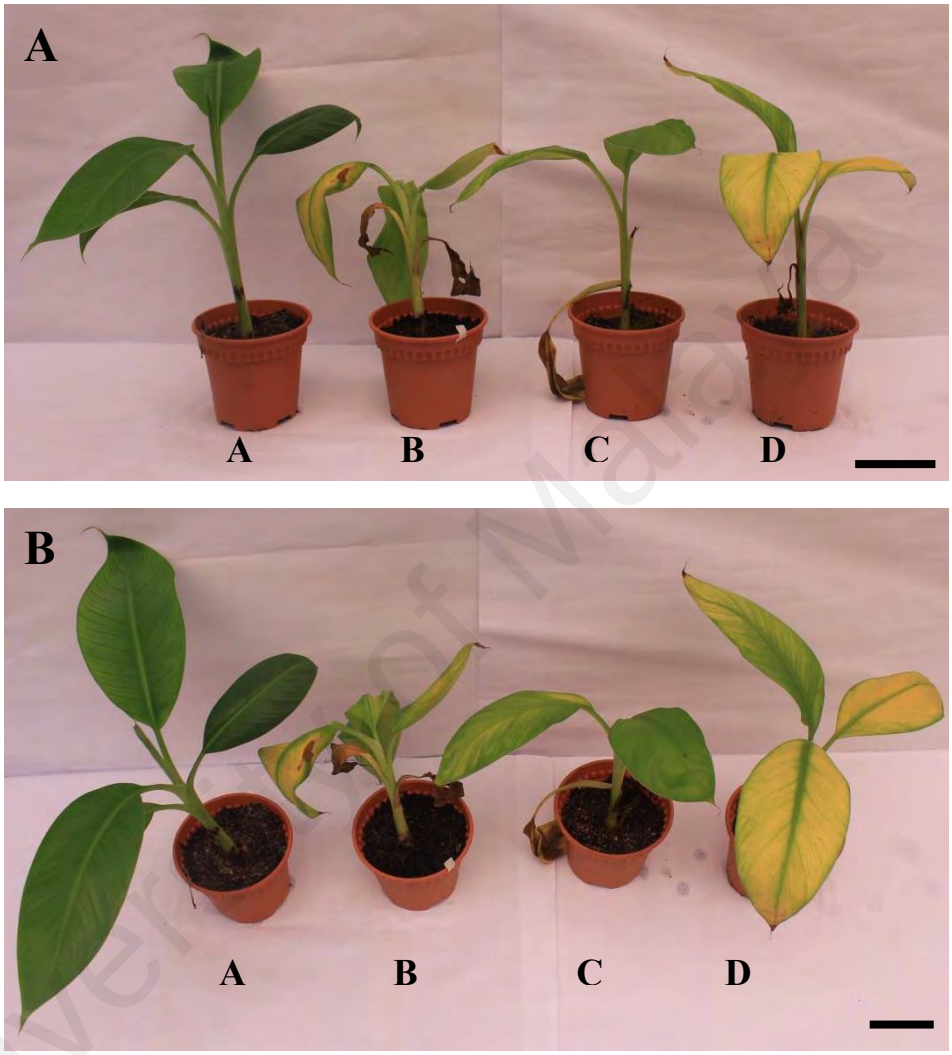

Figure 4.56: 20 days post-inoculation. Representatives shown were untreated control (A), treated untransformed control (B) and treated Line-19 plants (C & D) from front (A) and top (B) view. A remained healthy while B, C & D started to show marked yellowing and wilting leaf symptoms. Plantlet D displayed more extensive yellowing symptoms compared to C. Bar represents 8 cm

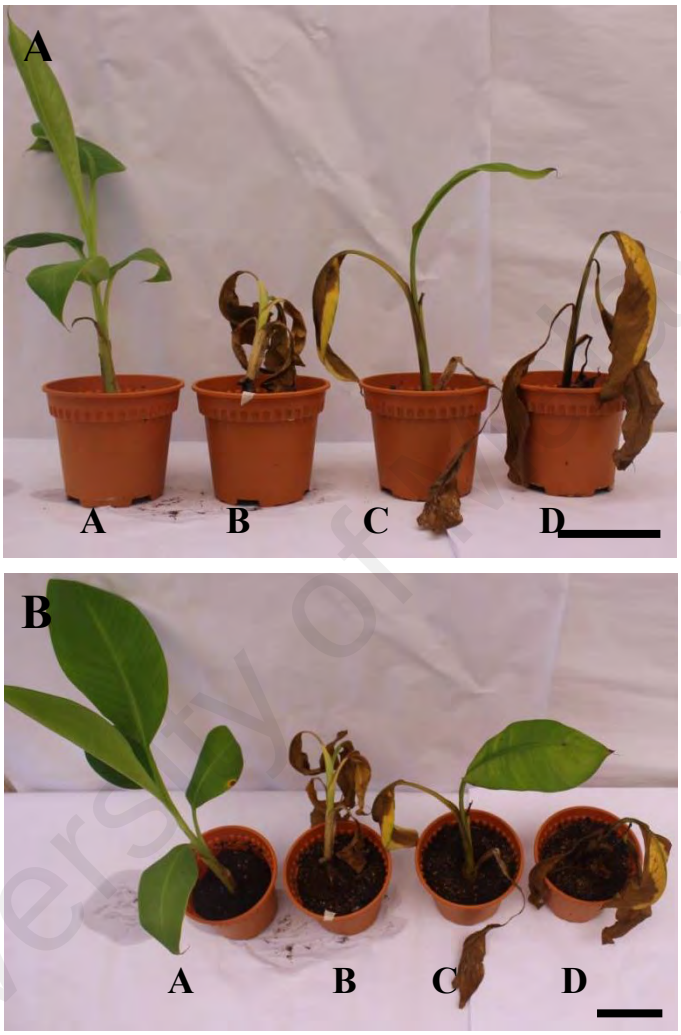

Figure 4.57: 35 days post-inoculation. Representatives shown were untreated control (A), treated untransformed control (B) and treated Line-19 plants (C & D) from front (A) and top (B) view. A remained healthy and showed steady growth while untransformed control B almost completely wilted out. The treated transgenic plants (C & D) showed variation in disease severity. C showed mixed yellowing and wilting leaf symptoms while D completely wilted out. Bar represents 8 cm

Figure 4.58: 35 days post-inoculation. Internal observation of the disease symptoms on the rhizomes of the plants. Representatives shown were untreated control (A), treated untransformed control (B) and treated Line-19 plants (C & D). Rhizome of untreated control (A) remained healthy without any discoloration while the untransformed control (B) showed extensive rhizome discoloration starting from the junction of root and rhizome as well as in the stele region expanding to the pseudostem. Treated transgenic plants (C & D) showed variation in disease severity. About 50% of the stele region of (C) showed discoloration that did not proceed to the pseudostem whilst more severe symptoms were detected in (D) comprising the stele region as well as the pseudostem. Bar represents 1 cm

CHAPTER 5: DISCUSSION

5.1 *MaPR-10*

Previously, a microarray study was conducted to examine the differential gene expression between susceptible *M. acuminata* cv. ‗Rastali' and tolerant *M. acuminata* cv. ‗Mutiara' banana following Foc infection (Lim, 2006). Several potential genes involved in the defense mechanism against Foc invasion have been identified including *PR-10*, which has become the basis of this current project. Moreover, the role of PR-10 as an anti-fungal protein has been widely investigated in many plants against various pathogens (Table 2.7). In this study, full-length *PR-10* cDNA was successfully isolated from *M. acuminata* ssp. malaccensis and designated as *MaPR-10*.

5.1.1 Analysis of full-length *MaPR-10* cDNA

A full-length cDNA encompasses sequences from 5'UTR to the polyA tail site of the gene which flank the coding sequence. In gene characterization studies, information provided by a full-length cDNA can be used to predict the exon-intron structure of a particular transcript (Kuroshu et al., 2010). However, the process to obtain a complete sequence can be quite challenging. Conventional cDNA amplification from expression libraries, two-hybrid or Genbank expressed sequence tags (EST) database likely resulted in partial transcript products (Zhang & Frohman, 1997). Consequently, the libraries had to be repeatedly screened hoping to get extended sequences that cover more towards the transcript's ends (Schaefer, 1995).

In this study, full-length *MaPR-10* cDNA has been obtained using a PCR-based method called RACE which extends the transcript amplification until 3' and 5' ends when only partial sequences are available (Frohman et al., 1988; Zhang, 2003). RACE is preferable because it is faster and could be performed on presumably any cDNA

transcripts including those that undergo alternative splicing or located upstream of rarely used promoters (Zhang & Frohman, 1997). The full-length *MaPR-10* cDNA encodes 773 bp nucleotides. Other studies recounted variation in the size of full-length *PR-10* cDNA from other plant species that could either be smaller (Chen et al., 2006; Lebel et al., 2010; Xie et al., 2010) or larger (Chadha & Das, 2006; Jwa et al., 2001; Kim et al., 2014; Liu et al., 2006; Park et al., 2004; Pulla et al., 2010; Xu et al., 2014) than that of *MaPR-10*.

The full length sequence of *MaPR-10* cDNA comprises of a 176-bp-intron spanned in between two exons with 62-bp 5'UTR and 228-bp 3'UTR, respectively. The intron region was determined by comparing the size of the full-length *MaPR-10* DNA and cDNA while the coding sequence of *MaPR-10* was predicted using protein translation tools (EMBOSS Transeq) as well as the alignment of *PR*-10 cds from other plant species (Appendix F10). The information was then utilized to determine the unstranlated regions and the exon spans of *MaPR-10*. Consistently, most *PR-10s* of other plants also consist of two exons and one intron. The length of the intron varies from 76 to 431 bp (Lebel et al., 2010; Liu & Ekramoddoullah, 2006). Lebel and co-workers (2010) isolated 17 *PR-10* sequences from *Vitis vinifera* and found that the exons were intruded by a single intron at a fixed position with varied intron length (102 bp to 431 bp). Intriguingly, a few of *Mal d 1* (*PR-10*) isoforms from *Malus domestica* were intronless while others had an intron of various sizes (Gao et al., 2005). Intronless transcript was also illustrated in *GmPR10* isolated from *Glycine max* (Xu et al., 2014) (Table 5.1).

While it is fairly easy to identify the 3' end with its polyA tail characteristic, confirmation on the 5' end proved to be a more challenging task. Classical RACE principles failed to select the transcripts with the actual 5' end. In other words, all cDNAs can be amplified regardless whether they were complete or truncated sequences

(Zhang & Frohman, 1997). Modifications in the RACE protocols have been adopted to allow a precise 5‘ end recognition using RNA ligase-mediated and oligo-capping approaches (Kazuo & Sumio, 1994; Schaefer, 1995). In the present study, treatment of mRNA isolated from *M. acuminata* ssp. malaccensis with calf intestinal phosphatase (CIP) removed the 5‘ phosphates from truncated or non-mRNA transcripts without eliminating the full-length, capped sequences. The later incubation with tobacco acid pyrophosphatase (TAP) eliminated the 5‘ cap structure from intact, full-length mRNA leaving a 5‘ phosphate needed for subsequent adapter ligation. These enzymatic treatments ensured only full-length mRNA was amplified in the actual RACE steps. The completion of full-length *MaPR-10* was verified by identifying the polyA tail and adapter sequences at 3‘ and 5‘ ends, respectively.

Amplification of non-specific products is one of the major drawbacks of RACE (Chenchik et al., 1996). In this experiment, the undesired background fragment from 3‘ RACE was eliminated by secondary PCR step using nested primers yielding one specific band which corresponded to *MaPR-10*. Other parameters concerning RACE experiment must also be taken into account including the quality of mRNA used, optimization of PCR conditions, primer design as well as selection of reverse transcriptase (Chen et al., 2005). Improved PCR-based methods for amplification of full-length cDNA sequences are continuously being elucidated such as inverse PCR (Huang, 1997), 3-step 5‘ RACE (Dallmeier & Neyts, 2013), targeted RACE (T-RACE) (Bower & Johnston, 2010) and thermal asymmetric interlaced PCR (TAIL PCR) (Liu & Chen, 2007).

5.1.2 Analysis of MaPR-10 protein

PR-10 is a member of huge PR families and has been characterized in many plant species including monocots, dicots and conifers (Chadha & Das, 2006; El-kereamy et al., 2009; McGee et al., 2001; Park et al., 2004; Soh et al., 2012; Swoboda et al., 1995; Xie et al., 2010). However, to date, isolation and characterization of *PR-10* from *M. acuminata* ssp. malaccensis (wild-type banana) (*MaPR-10*) has not been published. *MaPR-10* encodes for 160-amino-acid protein with a predicted molecular mass of 17.46 kDa and isoelectric point of 5.42. Portrayed as small protein with acidic property, these values are in accordance with the characteristics of PR-10 unraveled in other species (Dubos & Plomion, 2001; Soh et al., 2012; Zhang et al., 2010). Strikingly similar molecular mass and IE values were predicted from PR-10 of hot pepper at 17.3 kDa and 5.2, respectively (Park et al., 2004). Although the IE values of most PR-10s are in the range of 5 to 6 (Chadha & Das, 2006; Jain et al., 2012; Jwa et al., 2001; Liu et al., 2006; Xie et al., 2010; Xu et al., 2014; Zhang et al., 2010), PgPR10-2 from *Panax ginseng* C.A. Meyer has lower IE value (ie more acidic), which was about 4.56 (Pulla et al., 2010) (Table 5.1).

Isoforms from the same species also demonstrate wide range of IE values. In *Vitis vinifera*, for instance, the expected IE values of 17 VvPR10 isoforms (VvPR10.1-VvPR10.17) are in between 4.7 to 6.3 (Lebel et al., 2010). Similarly, the lowest and highest IE values of nine *Lilium regale* Wilson PR10s (LrPR10-1 to LrPR10-9) are 5.26 and 6.21, respectively (He et al., 2014) (Table 5.1).

In terms of size, the molecular masses of PR-10 proteins are consistently in the range of 15-18 kDa (Bahramnejad et al., 2010; Chadha & Das, 2006; Hashimoto et al., 2004; He et al., 2014; Jain et al., 2012; Kim et al., 2014; Liu et al., 2006; Park et al., 2004; Pulla et al., 2010; Soh et al., 2012; Zhang et al., 2010) although slightly higher

molecular weight is shown in some of the isoforms from *V. vinifera* (Lebel et al., 2010) and *Pinus monticola* (Liu & Ekramoddoullah, 2004) (Table 5.1).

In the absence of signal peptide, Ma-PR10 fits in the role of PR-10 that is believed to be the first intracellular PR protein involves in plants' response during pathogen invasion and is localized in cytosol (Yan et al., 2008). Even though *M. acuminata* is a monocot plant, MaPR-10 showed higher similarity with PR-10 isolated from conifers such as *Pinus monticola* (38%) and *Picea glauca* (37%) as well as eudicots like *Solanum virginianum* (37%). Nevertheless, phyologenetic analysis of various PR-10s disclosed that MaPR-10 is more closely related to other monocots such as *Oryza sativa* (OsPR-10), *Zea mays* (ZmPR10a) and *Hordeum vulgare* (HvPR10). *Pinus monticola* (PmPR10), on the other hand, was shown to be more similar to another group of monocots that include *Hyacinthus orientalis* (HoPR10), *Triticum aestivum* (TaPR10), *Elaeis guineensis* (EgPR10), *Zea mays* (ZmPR10b) and *Oryza sativa* (JIOsPR10) (Figure 4.25). Low amino acid similarity between PR-10s of different plant species was also observed. In *Zea mays*, while sharing 81.4 and 85.6% identity with PR-10 from sorghum and rice, respectively, ZmPR-10 showed lower amino acid similarity (≈30-35%) to a pathogenesis-related protein from white lupin (AJ000108) and a ribonuclease from ginseng (P80890) (Chen et al., 2006). Similarly, amino acid identity of AhPR-10 from peanut was less similar to GaPR10 (*Gossypium arboreum)* [AF416652] (38%), PANGIRNase2 (*Panax ginseng*) [P80890] (42%), CaPR10 (*Capsicum annuum*) [AF244121] (43%) and Betv1 allergen (*Betula pendula*) [Z72431] (44%) than Ll PR10.1B (*Lupinus luteus*) [X79975] (63%) (Chadha & Das, 2006).

Such variation did not only occur in PR-10 of different species, but rather in between isoforms of the same species. Among 17 VvPR10 isoforms (VvPR10.1-VvPR10.17) from *V. vinifera* (Lebel et al., 2010), the lowest and highest nucleotide similarity were

Table 5.1: Summary of *PR-10* sequence analysis from selected plant species including *M. acuminata* ssp. malaccensis (*MaPR-10*)

Plant species	**Nomenclature**	**Full-length cDNA (bp)**	**Open reading frames including stop codon (bp)**	**5' and 3' UTR (bp)**	**Exon and intron**	**Amino acid length (a.a.)**	**Protein size (kDa)**	**Isoelectric point**	**Reference**
Hot pepper (*Capsicum annuum*)	*CaPR-10*	797	480	N/A	N/A	159	17.30	5.20	Park et al. (2004)
Maize (*Zea mays*)	*pr-10*	620	483	N/A	1 intron (93 bp)	160	16.90	5.38	Chen et al. (2006)
Cotton (*Gossypium arboreum*)	*GaPR-10*	806*	480	N/A	N/A	159	17.30	4.95	Zhou et al. (2002)
Peanut (*Arachis hypogaea)*	*AhPR10*	847	453	5': 84 3': 310	N/A	150	16.20	5.30	Chadha & Das (2006)
Yellow-fruit nightshade (*Solanum surattense*)	*SsPR10*	844	483	5': 88 3': 273	N/A	160	17.58	5.29	Liu et al. (2006)
Wheat (*Triticum aestivum)*	*TaPR10*	567	483	N/A	2 exons and 1 intron (84 bp)	160	17.06	5.19	Zhang et al. (2010)
Ginseng (*Panax ginseng*)	*PgPR10-2*	885	468	5': 109 3': 138	2 exons and 1 intron (171 bp)	155	16.60	4.56	Pulla et al. (2010)

Ginseng (*Panax ginseng*)	*PgPR10-4*	778	477	N/A	N/A	158	17.70	5.72	Kim et al. (2014)
Banana *(Musa acuminata* ssp. malaccensis)	*MaPR-10*	773	483	5‘: 62 3‘: 228	2 exons and 1 intron (176 bp)	160	17.46	5.42	N/A
Common grape vine (*Vitis vinifera*)	*VvPR10.1-VvPR10.17*	769-1061**	204-486 (including pseudogenes)	5‘: 77-83** 3‘: 78-373**	2 exons and 1 intron (102-431 bp)	158-160	17.10-18.40	4.70-6.30	Lebel et al. (2010)
Apple (*Malus domestica*)	*Mal d 1 (PR-10)*	N/A	477-480	N/A	1 or 2 exons;1 intron or intronless	158-159	17.30-17.60	5.10-6.20	Gao et al. (2005)
Soybean (*Glycine max*)	*GmPR10*	831	474	5‘: 134 3‘: 223	1 exon, intronless	157	17.00	5.22	Xu et al. (2014)

*Not confirmed as full-length cDNA

**Selected isoforms only

Table 5.1 continued

48% and ≥92%, respectively making them an exciting subject for intraspecific variation research in PR-10. Amino acid sequences of PgPR10-4 from *Panax ginseng* were revealed to be 52, 47 and 49% similar to the previously isolated PgPR10-1 (ADW93867), PgPR10-2 (ADW93869) and PgPR10-3 (ADW93868), respectively (Kim et al., 2014; Lee et al., 2012a; Lee et al., 2012b; Pulla et al., 2010). On the same note, PgPR10-4 showed the highest sequence similarity (53% identity with 70% similarity) with AcBetV1 (CAM31908) demonstrating high interspecies identity (Kim et al., 2014). In this study, however, only one PR-10 homolog was identified using PR10 cds F/PR10 cds R primer pair. Thus, the intraspecies variation among PR-10 isoforms in *M. acuminata* ssp. malaccensis could not be inferred. PR-10 was once identified as a potential phylogenetic marker due to its low intraspecific and high interspecific variation (Finkler et al., 2005; Lebel et al., 2010; Schenk et al., 2009; Wen et al., 1997). However, as the researches progress, more compelling disparity has been recognized in either intraspecies or interspecies studies of PR-10.

Alignment between MaPR-10 and PR-10 of selected plant species, mainly of monocots taxa, showed 12 highly conserved amino acid residues, mostly of glycine. In GaPR-10 (cotton), nine amino acid residues were highly conserved when the protein sequences were aligned with PR-10 from different plants such as *Asparagus officinalis*, *Betula pendula* (Betv1), *Sorghum bicolor*, *Phaseous vulgaris* and *Petroselinum crispum* (Zhou et al., 2002). In the sequence alignments of Ypr10 (bean) (Walter et al., 1996) and RSOsPR10 (rice) (Hashimoto et al., 2004) with other plants, 12 and 13 highly conserved amino acid residues were identified, respectively.

While most PR-10s exhibit distinct GxGGxG P-loop motif (Chadha & Das, 2006; Hashimoto et al., 2004; Jain et al., 2012; Kim et al., 2014; Lebel et al., 2010; Lee et al., 2012b; Liu et al., 2006; Park et al., 2004; Soh et al., 2012; Xu et al., 2014), MaPR-10 possesses GxGxxG motif which is similar to JIOsPR10 (Jwa et al., 2001). Most of

LrPR10 isoforms in lily also retain the GxGGxG motif except for LrPR10-3 and LrPR10-8 isoforms that have the same GxGxxG motif with MaPR-10 (He et al., 2014). In *Lilium longiflorum*, another form of P-loop motif was observed in all seven PR-10 isoforms (PR10-1 to PR10-7) which is GxxGxxGG (He et al., 2014). P-loop is commonly recognized in phosphate-binding kinases as well as in nucleotide-binding proteins suggesting that a phosphate binding site could probably be the target site for an RNA phosphate group potentially associated with ribonucleolytic activity (Bantignies et al., 2000; Hoffmann-Sommergruber et al., 1997).

Besides P-loop motif, three highly conserved amino acid residues E103 (glutamate or Glu), E150 and Y152 (tyrosine or Tyr) (as positioned in PmPR10) have also been linked to the ribonuclease activity of PR-10 protein (Liu & Ekramoddoullah, 2006). This was further supported by the study of 3D structures of birch Bet v 1 and yellow lupine. In these two plants, E96, E148 and Y150 (as indicated in Bet v 1) were located near to each other and therefore were projected to be the active site for ribonuclease activity (Liu & Ekramoddoullah, 2006). In MaPR-10, those three residues were detected as E102, E149 & Y151. Wu and co-workers (2003) further extended the study by performing site-directed mutagenesis of SPE-16 with E95A, E147A and Y149A only to find out the ribonuclease activity of the protein had been abolished. By having P-loop motif and those three conserved residues, MaPR-10 could also be associated with ribonuclease activity though this prediction can only be strengthened by performing RNA degradation assay or RNase activity assay.

Predicted MaPR-10 has a functional domain called Betv1-like with glycine rich loop and hydrophobic ligand binding-site which were also consistently identified in other PR-10 proteins (Chadha & Das, 2006; Kim et al., 2014; Park et al., 2004; Yan et al., 2008; Zhou et al., 2002). The domain‘s name was derived from pollen antigen of white birch (*Betula verrucosa*), which is a family of protein allergens causing type I allergies

detected mainly in Europe and North America (Breiteneder et al., 1989; Chakraborty & Dutta, 2011; Faber et al., 1996). Exist in all three domains of life, Betv1-like structure can be further grouped into ten different families such as PR10-like, steroidogenic acute regulatory protein-related lipid transfer (START) and RHO α-subunit catalytic domain. Betv1 domain of MaPR-10, in this case, is probably a constituent of a superfamily domain called SRPBCC (START/RHOalphaC/PITP/Bet v1/CoxG/CalC) that includes StAR-related lipid-transfer (START) domain (Marchler-Bauer et al., 2014).

Alongside Bet v 1 family signature, four additional motifs found in MaPR-10 including four N-myristoylation sites (4-9 a.a, 51-56 a.a, 89-94 a.a and 109-114 a.a), one N-glycosylation site (13-16 a.a), three protein kinase C phosphorylation sites (52-54 a.a, 81-83 a.a and 95-97 a.a) and four casein kinase II phosphorylation sites (108-111 a.a, 117-120 a.a, 119-122 a.a and 123-126 a.a) provide a constructive postulation on the biological roles of the protein as well as its post-translational modification pathways (He et al., 2014; Xu et al., 2014). Identical extra motifs were also predicted in TaPR-10 (Zhang et al., 2010) and LrPR10 (He et al., 2014) with a small variation in terms of the site numbers and positions. All MaPR-10, TaPR10 and LrPR10 were predicted to have only one N-glycosylation site but several numbers of N-myristoylation sites. MaPR-10 has the highest number of protein kinase C phosphorylation and casein kinase II phosphorylation sites (three and four, respectively) in comparison to TaPR-10 and LrPR10 with only one or two sites.

Some plants showed changes in *PR-10* expression upon pathogen attacks, including by fungus. Transcript levels of *PR-10* in plum, maize and peppers increased after being infected with *Monilinia fructicola* (El-kereamy et al., 2009), *Aspergillus flavus* (Chen et al., 2006) and *Colletotrichum acutatum* (Soh et al., 2012), respectively. The function of PR-10 in fungal resistance has been associated with its RNase activity. Using RNA degradation assay, Xu et al. (2014) showed that dialytically renatured GmPR10 protein

was able to degrade *Glycine max* RNA. Growing interest in PR-10 studies help to inaugurate a closer association between RNase activity of PR-10 proteins.

5.2 Development of transgenic *M. acuminata* cv. 'Berangan' harboring *MaPR-10*

Successful plant regeneration mainly from two recognized pathways, which are meristematic tissues and embryogenic cells suspension (ECS), provides alternative starting materials for genetic engineering of banana (Remakanthan, Menon & Soniya, 2014). As a result, non-classical genetic modification strategies such as transformation with gene(s) of agronomic importance become conceivable. In this study, transgenic *M. acuminata* cv. _Berangan' clone over-expressing *MaPR-10* was produced via co-cultivation of ECS and *A. tumefaciens*.

5.2.1 Cultivar selection

_Berangan' is selected as the target cultivar for this study due to its popularity as a highly-demanded banana among Malaysians. Alongside Cavendish, *M. acuminata* cv. _Berangan' covers almost half of the total banana plantation area in Malaysia and is preferred by farmers as well as consumers for its flavor and good storage qualities (Jamaluddin et al., 2001; Mak et al., 2004). On top of that, _Berangan' is susceptible to Foc race 4 as found in a few locations in Perak, Malaysia (personal communication Prof. Dr. Baharuddin Salleh). High local market value and disease susceptibility are among the most common basis for cultivar selection in a *Fusarium*-resistance transgenic research. Ghag and co-workers (2014b), for example, chose *M. acuminata* cv. _Rasthali' (AAB), a top Indian cultivar that is prone to Foc race 1 for their study. Furenzhi banana

(AA) was used in another transgenic research against Foc race 4 due to its popularity among consumers in Yunnan province, China (Hu et al., 2013).

5.2.2 Choice of explant

Banana ECS has been initiated from various sources such as shoot-tip cultures (Dhed'a et al., 1991; Schoofs, 1997), immature zygotic embryos (Cronauer-Mitra & Krikorian, 1988; Escalant & Teisson, 1989; Marroquin et al., 1993), rhizome slices and leaf sheaths (Novak et al., 1989), scalps (proliferating meristem culture) (Huang et al., 2000; Strosse et al., 2006), and immature male/female inflorescence (Escalant et al., 1994; Grapin et al., 1996; Huang et al., 2000; Jalil et al., 2003; Khalil et al., 2002). ECS used in this study was initiated from male inflorescence of *M. acuminata* cv. _Berangan' (AAA) (Chin et al., 2014). Male inflorescence is recognized as the most responsive explant for initiation of somatic embryogenesis in banana (Khanna et al., 2004). Plantlets regenerated from ECS were reported to be of single cell origin thus minimizing the chimerism issue that is a common problem in transformation using other explants (Ghosh et al., 2009; Roux et al., 2004). No chimerism was observed in other transformation studies involving ECS (Ghag, Shekhawat & Ganapathi, 2012; Hu et al., 2013; Mohandas et al., 2013). The transgene screening has indirectly become easier since the DNA can be extracted from any parts of the plants.

However, initiation and maintenance of ECS is generally tedious and a long process. Cells were reported to have low generation rate and risk of losing totipotency over a certain period (Jain, 2008). Alternatively, Yip and co-workers (2011) successfully developed transgenic *M. acuminata* cv. _Pei Chiao' and _Gros Michel' (AAA) using multiple bud clumps (MBC). Although the process to establish MBC cultures was much shorter and easier compared to ECS, chimeric banana plants have been produced

probably as a result of MBC transformation system. Chimerism has also been described in the transformation of other meristematic tissues (Maziah, Sariah & Sreeramanan, 2007; Sreeramanan, Mahmood & Xavier, 2009).

5.2.3 Gene selection

To our knowledge, over-expression of *MaPR-10* gene for regeneration of *Fusarium*-tolerant banana has never been reported. *MaPR-10* was selected in this study due to its potential as an anti-fungal gene as described previously (section 2.4.5 and section 5.1.2). In addition, since *MaPR-10* was derived from banana, the product could minimize the biosafety issues and improve the public acceptance (Jacobsen & Schouten, 2007). To date, most of the genes used in *Fusarium* wilt-resistant studies were isolated from plants such as onion (Mohandas et al., 2013), ornamental flower (Ghag, Shekhawat & Ganapathi, 2012), rice (Mahdavi, Sariah & Maziah, 2012) and banana (Ghag, Shekhawat & Ganapathi, 2014a). In some cases, non-plant-derived genes from chicken, *C. elegans* and human were also integrated into banana genome (Paul et al., 2011; Pei et al., 2005). Significant number of reports utilizing native genes from banana have been published in the recent years probably owing to the completion of banana genome sequencing project (D'Hont et al., 2012) and/or the growing popularity of cisgenics approach (Negi, Tak & Ganapathi, 2014; Shekhawat & Ganapathi, 2014; Shekhawat, Ganapathi & Srinivas, 2011; Shekhawat, Srinivas & Ganapathi, 2011; Sreedharan, Shekhawat & Ganapathi, 2012; Sreedharan, Shekhawat & Ganapathi, 2013).

5.2.4 Vector construct

MaPR-10 was successfully inserted in between NcoI and BglII restriction sites in the T-DNA region of pCAMBIA1304 vector yielding pMaPR-10 construct. The insertion was verified by sequencing of *hptII* gene as well as upstream and downstream GOI regions (CaMV35 promoter-*MaPR-10* and *MaPR-10*-mgfp5, respectively). The expression of *MaPR-10* in this construct was driven by double-enhancer CaMV35S promoters and terminated by nopaline synthase (nos) polyA signal. The construct allows kanamycin and hygromycin selection in the bacteria and banana host cells, respectively.

pCAMBIA vectors contain pVS1 replicon for high stability in *Agrobacterium* cells and high copy number in *E. coli* (CAMBIA, n.d.; Slamet-Leodin et al., 1997). pCAMBIA has been extensively used as expression vector in many plants for diverse purposes including gene characterization (Hu et al., 2008), plant development study (Fan et al, 2013), transgenic experiments involving biotic (Arthikala et al., 2014; Lipsky et al., 2014) and abiotic stress (Wang et al., 2013). In banana, successful transgene expression using pCAMBIA has been exemplified in various cultivars including ‗Lady Finger' (AAB) (Khanna et al., 2004), ‗Mas' (AA) (Huang et al., 2007), Cavendish (AAA) (Ghosh et al, 2009; Khanna et al., 2004), ‗Rasthali' (AAB) (Sreedharan, Shekhawat & Ganapathi, 2015; Subramanyam et al., 2011) and ‗Nangka' (AAB) (Mahdavi, Sariah & Maziah, 2012).

5.2.5 Transformation method

As verified by PCR colony, *MaPR-10* was successfully introduced into *A. tumefaciens* LBA4404 using a freeze-thaw method (Go, 2013). In this study, *Agrobacterium*-mediated transformation was chosen since the transgene is likely to be integrated into transcriptional active region of the host genome at low copy number

(Koncz et al., 1989). While most banana studies also utilized this technique (Elayabalan et al., 2013; Hu et al., 2013; Mohandas et al., 2013; Namukwaya et al., 2012; Yip et al., 2011), Sreeramanan et al. (2006) preferred particle bombardment for co-transformation of β-1,3- glucanase and chitinase. Interestingly, Pei and co-workers (2005) combined these two methods and were also able to generate transgenic ‗Taijiao' (AAA) expressing a human lysozyme gene.

5.2.6 Co-cultivation and regeneration process

Co-cultivation of *MaPR-10*-transformed *A. tumefaciens* with *M. acuminata* cv. ‗Berangan' ECS for 30 minutes followed by 4-day-incubation in the dark at low shaking speed supports the attachment of *Agrobacterium* cells and transfer of T-DNA into banana cells which was similar to *M. acuminata* cv. ‗Mas' (Wong, 2007). With a combination of 50 µg/ml cefotaxime and shaking speed around 90-100 rpm during co-cultivation, the growth of *A. tumefaciens* was hampered. The speed was deemed appropriate since *A. tumefaciens* has been shown to grow markedly at lower speed (less than 40 rpm) while *M. acuminata* cv. ‗Mas' ECS demonstrated positive growth at 100 rpm (Huang et al., 2007).

The hygromycin concentration used in this study was lower (0.2 mg/L-5 mg/L) than other banana ECS transformation experiments (6.25 mg/L-10 mg/L). Effective antibiotic concentrations are influenced by several transformation parameters and thus are usually optimized independently by each study. In this study, positive control cells responded better and faster in 2 mg/L-hygromycin-supplemented medium and started showing protruding transparent cells as early as four weeks in M3 culture, which was about a week faster than that of *MaPR-10*-transformed cells. Although somatic embryos of *MaPR-10*-transformed cells were first formed in 5 mg/L-hygromycin-supplemented

M3 medium, overall somatic embryo induction was more prominent in 0.2 mg/L followed by 5 mg/L and 2 mg/L. The concentration of hygromycin was decreased to 0.2 mg/L in M8B and M4B medium (or maintained in the case of 0.2 mg/L M3 culture) before it was completely removed in M2B to control toxicity to the plantlets. Several effects of antibiotic toxicity have been exemplified previously that include necrosis on chimeric progenies, growth abnormalities and low survival rate (Maziah, Sariah & Sreeramanan, 2007; Vishnevetsky et al., 2011). Complete removal of antibiotic selection in the final shoot regeneration medium was also enlightened by other studies (Das & Rahman, 2012; Hu et al., 2013).

The BAP concentrations were reduced in each M4 medium starting from 8 mg/L to 4 mg/L and finally 2 mg/L depending on developmental stages. Gradual decline of BAP concentration has been described earlier (Mahdavi, Sariah & Maziah, 2012; Maziah, Sariah & Sreeramanan, 2007). High BAP concentration was initially needed to maximize the shoots induction and multiplication. Once the shoots were stably regenerated, the BAP concentration was lessened to minimize the external phytohormone effect imposed on the plants that could possibly disrupt the pattern of gene expression. In contrast to this study, Ghag and co-workers (2014b) first used reduced BAP concentration in embryo germination medium (0.5 mg/L) before increasing it to 2 mg/L in multiple shoot regeneration medium.

Using liquid cultivation medium with continuous hygromycin selection and cefotaxime supplement at 50 µg/ml, putative *in-vitro* transgenic ‗Berangan' plantlets harboring *MaPR-10* were regenerated within 6-7 months, which was about a month later than that of positive control plantlets with the pCAMBIA1304 construct. Slower regeneration process of some transgenic lines was observed in comparison to the positive controls as reported by Ghag and co-workers (2014). This phenomenon is deemed possible due to the ‗position effect' or preferential integration of T-DNA region

into host genome which might affect the expression of neighboring genes leading to changes in overall plant growth regulation process (Shekhawat, Srinivas & Ganapathi, 2011; Singer, Liu & Cox, 2012). Regeneration period of putative transgenic ‗Berangan' plantlets harboring *MaPR-10* from ECS was comparable to other ECS-derived transformation experiments (Ghag, Shekhawat & Ganapathi, 2012; Hu et al., 2013). The process was in general longer than meristamatic-derived transformation experiments such as using multiple bud clumps (Mahdavi, Sariah & Maziah, 2012; Pei et al., 2005; Yip et al., 2011).

Regeneration efficiency obtained in this study was fairly low, which was around 1.6%. This might be attributed to the age of the ECS used which has been established for more than six months. The risk of losing embryogenic potential increases over time as the culture undergoes numerous subculturing cycles (Jain et al., 2008). A contrasting result was shown by Mohandas and co-workers (2013) who used 2- to 3-month-old Rasthali ECS and was able to achieve higher regeneration efficiency at 5.1%.

5.3 Analysis of putative putative transgenic *M. acuminata* cv. 'Berangan' plants harboring *MaPR-10* cds

Out of 17 putative transgenic lines regenerated, 11 of them were verified positive by the amplification of *hptII* and gene-specific regions. Gene-specific primers for transgene screening were designed as such to amplify partial regulatory region on the vector as well as the gene of interest (GOI) itself to avoid getting false positive results from endogenous *PR-10* amplification. In addition, T-DNA integration was also verified by amplification of *hptII* sequences, which is also a part of T-DNA region presumably transferred into the host genome. Due to positive transgene insertion, vigorous growth and rapid shoot development on hygromycin-supplemented medium, Line-19 plantlets were chosen for further analysis. Six lines were most probably escapes as neither band

was detected. Escapes phenomenon has been shown in other studies either involving meristematic- (Guo et al., 2013; Hou et al., 2015; Huang et al., 2013) or somatic embryogenic-derived transformations (Kovács et al, 2013; Vishnevetsky et al., 2011).

5.4 Validation of infection parameters for Foc C1 HIR bioassay

Despite the long history of *Fusarium* wilt, no standardized bioassay for Foc challenge has been established owing partly to the wide variety of isolates and banana cultivars present. Thus, validation of the infection parameters is deemed essential prior to each bioassay experiment involving different Foc isolates and banana cultivars. In the current study, the bioassay parameters developed by Mak and his co-workers (2004) have been validated for FocR4 C1 HIR infection. This assay specifically serves to determine the baseline response of wild-type ‗Berangan' plants against C1 HIR isolate. The parameters of this assay can also be used as guidelines for future bioassay experiments to ensure reproducible and comparable results possibly across cultivars and test isolates.

5.4.1 Validation of Foc isolate

Foc is widely recognized as diverse species with huge evolutionary potential (section 2.2). Without proper molecular validation, the identity of an Foc isolate can be easily mistaken since morphological differentiation can only provide superficial information. Besides, Foc identification is also critical in ensuring the consistency of the isolate used in every experiment. Despite the importance, pathogen validation is often not properly addressed in the protocols of bioassay development (Mahdavi, Sariah & Maziah et al., 2012; Pei et al., 2005; Yip et al., 2011). Using Foc4F/Foc4R specific primers, C1 HIR has been verified as a Foc race 4 isolate based on the presence of a 242-bp band (Lin et

al., 2009). In comparison to other conventional applications like electrophoretic karyotyping or volatile compound production, PCR is a more common technique used in Foc identification for its simplicity, high accuracy and reproducibility than other methods reported (Table 2.3). Particularly for FocR4, a few other PCR-based identifications have been published with different product sizes. Dita and co-workers (2010) as well as Li and colleagues (2013) reported amplification of unique FocR4 band of 463 bp and 364 bp respectively. Foc4F/Foc4R primers (Lin et al., 2009) were chosen in the current study since amplification of smaller amplicon is more efficient and easier to manipulate.

5.4.2 Inoculum concentration

Previously, it was found that lower inoculum concentration failed to produce consistent disease symptoms in banana plants following Foc infection (Javed, Chai & Othman, 2004; Mak et al., 2004). According to Mak and his co-workers (2004), inoculum concentration of 1 x 10^6 spores/ml was found to be optimal in eliciting reliable Foc symptoms development in *M. acuminata* cv. ‗Berangan'. Thus, the same inoculum concentration was used in this study. Wide range of inoculum concentration has been found to be effective for Foc infection in banana (Table 5.2). Even though the general inoculum concentration in practice was in the range of 10^5-10^7 spores/ml, validation of the effectiveness of the selected inoculum concentration was inarguably a good pre-requisite for Foc bioassay as different banana cultivars would show diverse response to different isolates. A validated and consistent spore concentration should also be used in all bioassay experiments to ensure similar surface area to volume ratio for inoculum contact and to avoid discrepancies in the disease scores.

5.4.3 Host plant

Mak and co-workers (2004) showed that young plants (about 2-3 weeks old and 5-7 cm in height) were not eligible in producing steady disease progression probably due to imperfect vascular tissue development. Since the root system of the host plants must be fully developed for Foc penetration to take place, 2-month-old healthy plants with the following requirements were selected for the current bioassay experiment: 1) minimum of 3-5 green leaves, 2) vigorous white roots of at least 5 cm in length, and 3) sturdy stem with about 0.5 to 1.0 cm in diameter. These criteria were selected by taking into consideration the two essential indicators of Foc infection which are the leaves and rhizomes on top of the general physiological condition of the host plant.

5.4.4 Inoculation procedure

A root-dipping technique was chosen for this bioassay as it is simple, reliable and suitable for 2-month-old plants. Using this method, the inoculum concentration can be controlled while making sure all roots are in contact with the spore suspension. Another common technique used in Foc bioassay was inoculation of planting medium with the test isolate. While it is agreeable that this technique more similarly mimic the natural infection condition, the growth and reproduction rate of the spores in each potting mixture cannot be fully monitored which may affect the degree of infection. Masheva and Todorova (2013) in their study with strawberry and pepper plants, supported the idea that the root dipping method was able to facilitate direct contact between the roots and the inoculum. They found the index of infestation was higher in the root dipping technique compared to that recorded in non-infected plant material planted in infected soil.

Different inoculation period has been used for Foc infection (Table 5.2). For FocR4 C1 HIR, an exposure time of two hours can consistently produce the expected disease progression and response in susceptible *M. acuminata* cv. ‗Berangan' plants which has also previously been shown by Mak and his co-workers (2004). Besides root-dipping and incubation with infested growth substrate, other inoculation techniques such as direct injection into rhizome have also been applied (Daly et al, 2006).

Table 5.2: Different paramaters involved in Foc bioassay studies

Reference	Inoculum concentration	Inoculation period	Plant age and size	Disease evaluation (phenotypic description)	Post-challenge analysis	Disposal protocols	Remarks
Mak et al., 2004	10^6 spores/ml	2 hours	2-month-old plants, 10-15 cm tall	Based on Brake et al., 1995, Javed, Chai & Othman, 2004	Not applicable	Contaminated water was treated with sodium hychlorite overnight before disposal	Double-tray set up
Li et al., 2012	5,000 conidia/g soil	Not applicable. Plants were sampled at 48 and 96 hours after inoculation	4-5 leaves, 30 cm tall	Not applicable	Transcriptome analysis	No information	Suspension was added to planting medium (three parts of vermiculite, one part of peat, 0.5 part coconut coir to obtain soil concentration of 5,000 conidia/g soil
Javed, Chai & Othman, 2004	10^6 spores/ml	3 hours	4-5 weeks	Brake et al., 1995	Random amplified polymorphic DNA (RAPD)	Mohamed et al., 1999	Double-tray set up
Subramaniam et al., 2006	2×10^4, 2×10^6 and 2×10^8 spores/ml	1 hour. Additional 1 ml of different spore concentration was applied	1 week	Leaf chlorosis	Hydrogen peroxide, phenylalanine ammonia lyase, polyphenol oxidase,	No information	Double container

					peroxidase, chitinase and β-1,3-glucanase assays		
Mahdavi, Sariah & Maziah, 2012	2 x 10^8 spores/ml,	1 hour. Additional 1 ml of different spore concentration was applied	2-month-old	Leaf chlorosis	Not applicable	No information	
Paul et al., 2011	10^5 spores/ml	48 hours	2 weeks	Based on Smith et al., 2008	TUNEL assay	No information	*In-vitro*
	2 x 10^8 spores	In soil mix	2-month-old		Not applicable		Small bioassay (ex-vivo)
Mohandas et al., 2013	2.8 x 10^6 spores/ml	In 150 g sterilized sorghum grain	2-month-old, 7-8 leaf stage	Based on (a) Leaves: Nasir et al., (2003); (b) Corms: Ojeda et al., 1998	Western blot, ELISA	No information (except for corm sterilization)	
Pérez-Vicente et al., 2014	10^6 spores/ml	30 minutes	45-day-old, 15-25 cm tall, no signs of nutrient deficiency	Both external and internal symptoms were recorded	Not applicable	Available	Double pot

Table 5.2 continued

5.4.5 Disease rating scale

In order to evaluate the susceptibility of test plants towards a particular infection, a standardized and reliable disease rating scale is very much needed. To date, no standardized RDI and LSI had been widely used. Externally, yellowing and wilting of leaves were generally used as standards for evaluation (Javed, Chai & Othman, 2004; Mahdavi, Sariah & Maziah, 2012; Mak et al., 2004; Mohandas et al., 2013; Subramaniam et al., 2006). In green-house trials using tissue-cultured plants, rhizome discoloration of the infected plants after 6-8 weeks of inoculation would commonly be recorded for internal examination (Ghag, Shekhawat & Ganapathi, 2012; Javed, Chai & Othman, 2004; Mak et al., 2004; Paul et al., 2011).

A number of protocols distinguished both external and internal scoring systems (Javed, Chai & Othman, 2004; Mak et al., 2004; Paul et al., 2011); nevertheless, overall disease responses had also been used (Ghag, Shekhawat & Ganapathi, 2012; Mahdavi, Sariah & Maziah, 2012; Maziah, Sariah & Sreeramanan, 2007; Pei et al., 2005). Since the external and internal scales are often not linked with each other, inconsistent results could have been observed. For instance, infected plants may have fairly good rhizome discoloration score while displaying severe yellowing symptoms probably caused by external factors such as nutrient deficiency or unfavorable growth parameters. In this case, relying only on the symptoms superficially may have led to inaccurate disease estimation. The scoring methods used in this study established distinct scales for each leaf symptoms index (LSI) and rhizome discoloration index (RDI). The DSI values calculated from each LSI and RDI will be translated based on the standardized range to eventually determine the susceptibility or resistance level of the infected variety against test isolate. By correlating both external and internal scores, the observation will be less prone to bias allowing a just final disease evaluation.

5.4.6 Precautionary measures

Besides that, precautionary measures for pre- and post-bioassay experiment were frequently not mentioned thus providing us with very little information on the proper soil preparation and clean-up procedures (Table 5.2).

In this study, the sterilized soil was aerated for at least 72 hours prior to plant hardening to release any gas formed during sterilization. Phytotoxic effect on test plants has been illustrated previously instigated by improper soil aeration after autoclaving (Pérez-Vicente et al., 2014).

‗Double tray' method used in the current study provides a safe and systematized Foc bioassay challenge (Mak et al., 2004). The protocols offer controlled environment for testing the plants' responses against the pathogen with minimal space requirement. In this set-up, contamined water was drained into the lower tray prior to the treatment with sodium hypochlorite. Portable trays were washed with the same solution while all the plant materials were autoclaved before they were discarded. Pérez-Vicente and co-workers (2014) suggested all the materials to be autocvaled twice with a 24-hour-interval as a part of post-sterillization process.

5.5 Analysis of transgenic Line-19 plantlets harboring *MaPR-10*

5.5.1 Molecular analysis

Using qPCR analysis, putative transgenic Line-19 plantlets were estimated to contain a single copy of *MaPR-10* transgene. Traditionally, prediction of the transgene copy number has been done mostly using Southern blot. This technique provides considerably good estimation on the number of integration sites and introgression stability. However, it is also time consuming and lab-laborious while requiring high amount of starting DNA (Chen & Lin, 2010). qPCR analysis is an alternative method

used in estimating the gene copy number which has also been demonstrated in banana (Sreedharan, Shekhawat & Ganapathi, 2012) and some other plants including maize (Song et al., 2002), wheat (Li et al., 2004), tomato (Wang et al., 2013), cotton (Yi et al., 2008), rice (Qian et al., 2014; Yang et al., 2005) and grapevines (Dhekney, Li & Gray, 2011). In contrast to the conventional method, qPCR delivers more sensitive assay with less laborious work and shorter analysis period (reviewed by D'haene, Vandesompele & Hellemans, 2010).

Prior to actual analysis, a few optimizations on the dilution range, primer as well as template concentrations were performed to fulfill the criteria of a good standard curve (Figure 4.39). The information obtained from the standard curve was useful in estimating the PCR efficiency and linearity across the whole dilutions, which were deduced by the value of the slope (m) and correlation coefficient (r^2), respectively. Good r^2 and m values of 0.9983 and -3.63, respectively were produced from the standard curve. Even though the m-value was slightly higher than the recommended range (-3.3 to -3.6) (D'haene, Vandesompele & Hellemans, 2010), the amplification efficiency of the primers is sufficely good as it was obtained through series of optimization (data not shown). The assay was also not affected by the primer dimer or non-specific artifacts as shown by the single peak product generated from the dissociation curve.

Randomly selected Line-19 plantlets depicted considerable variations of *MaPR-10* transcript expression in between approximately 8- to 63-fold change with respect to untransformed control after normalization with *RPS2* gene. Expression variation in clonal plants is rarely discussed. In this experiment, all plants were maintained under controlled conditions with uniform growth supplements. The leaves were also harvested at the same time and processed using identical procedures prior to analysis. However, since the transgene is endogenous to banana and was proven to be actively responsive to

various stresses (Table 2.7), even slight changes in the environment or extra physical exert may have caused the fluctuations in the transcript expression. Furthermore, the plantlets may have undergone somaclonal variation during *in-vitro* and acclimatization stages.

In other studies with similar transformation method employing co-cultivation of ECS and transformed *A. tumefaciens*, low insertion numbers (1-4 copies) were also commonly obtained and regarded desirable (Shekhawat & Ganapathi, 2014; Shekhawat, Srinivas & Ganapathi, 2011; Sreedharan, Shekhawat & Ganapathi, 2012; Sreedharan, Shekhawat & Ganapathi, 2015). However, some transgenic lines have also been shown to carry considerably high number of transgene copies (Negi, Tak & Ganapathi, 2014; Sreedharan, Shekhawat & Ganapathi, 2012). Often, direct correlation between transgene copy number and level of gene expression could not be concluded (Namukwaya et al., 2012; Shekhawat, Srinivas & Ganapathi, 2011; Sreedharan, Shekhawat & Ganapathi, 2012). In this case, analysis on more transgenic lines is needed to gain more information on the integration pattern as well as to establish correlation between transgene copy number and its expression level.

5.5.2 Disease response against C1 HIR infection

Throughout the bioassay study, about 30% (3 out of 10) of Line-19 plants displayed a delay in disease progression (raw data is not available). Sturdy plants displayed delayed yellowing and wilting leaf symptoms which only appeared in the 2nd week post-inoculation. Diverse response against C1 HIR infection was probably implicated by different developmental stages among plants. During hardening, variation in plant height, shoot developments and thickness of the pseudostem was generally detected among Line-19 plants. About 75% the plants (15 out of 20) depicted slower growth in terms of height and pseudostem in comparison to the controls (raw data is not

available). These transgenic plants either failed or were barely able to fulfill the minimum requirements for bioassay experiment (section 3.1). Similarly, Mao and co-workers (2014) also spotted tardy growth of transgenic rice seedlings co-expressing chitinase (*RCH10*) and β-1,3-glucanase (*AGLU1*) genes under greenhouse conditions in comparison to the wild-types. Accordingly, the final disease score for transgenic Line-19 plants response against C1 HIR was also affected.

As discussed briefly, somaclonal variation may have affected the expression level of *MaPR-10* in Line-19 plants as well as the plants' development during acclimatization. Somaclonal variation is defined as genetic variability undergone by tissue-culture generated plantlets (Filipecki & Malepszy, 2006; Lee & Phillips, 1988). Somaclonal variation instigated by *in-vitro* micropropagation is commonly known as tissue-induced variation (Kaeppler, Kaeppler & Rhee, 2000) and it occurs as a result of stress response (Larkin & Scowcroft, 1981). Thus, the possibility of somaclonal variation inflicting the plants' development and consequently their disease response could not be entirely disregarded. Chimerism could also be another contributing factor to the variation observed in the plants' growth and their response to C1 HIR infection. However, no conclusion can be inferred from this postulation due to lack of data regarding the plants' chimerism. Although Line-19 *M. acuminata* cv. _Berangan' plants harboring *MaPR-10* did not confer complete resistance, they certainly had shown delayed disease progression against C1 HIR infection. Infection studies of other *MaPR-10* over-expressing lines will enrich the understanding about the capability of these transgenic plants in combating Foc infection.

Kovács and co-workers (2013) observed various degrees of infection among 17 transgenic _Gros Michel' lines carrying rice chitinase gene tested in leaf disk bioassay against *Mycosphaerella fijiensis*. Whereas it was not significantly different from the control line, almost half of the transgenic lines displayed less necrosis symptoms at

early time points but were later categorized under susceptible group as the disease progressed. While some lines like GM.RCG3.32 and GM.RCG3.10 definitely exhibited stronger resistance throughout the bioassay, other line such as GM.RCG3 was initially regarded as resistant but finally fell into susceptible group due to more severe necrosis development towards the end of the assay. In another bioassay study of transgenic melon expressing anti-fungal protein and chitinase against *R. solani*, mixed level of disease resistance was also elucidated (Bezirganoglu et al., 2013). In control plants, early necrosis symptoms were observed on the leaves that led to plants' death as early as 5 days post-inoculation. In the transgenic lines, some stronger lines survived more than two weeks wherein more susceptible lines succumbed to death at least 3-5 days later than of controls. These observations were also applicable to the current studies.

A few factors may have influenced the severity of disease development such as developmental stages of the plants, temperature changes or the gene construct (Bezirganoglu et al., 2013). Since this experiment has been optimized under controlled conditions, changes in the test environment may impose more minimal effects on the disease progression rather than the physical condition of the plants. Since this is the first study of *MaPR-10* over-expression in banana against Foc, the effectiveness of the gene construct in conferring disease resistance cannot be compared due to lack of information.

5.6 Future recommendations

5.6.1 Improved quality of ECS

In the previous discussion, low regeneration efficiency of the transgenic plantlets has been linked to the age of the ECS used. Besides losing regeneration capability after prolonged culture, the potential problems of ECS also include long establishment period, exposure to contamination as well as somaclonal variation. To address these

issues, a few cryopreservation protocols for different banana germplasms derived from various starting materials including ECS have been established (Panis, 1995; Panis et al., 2001; Panis, Withers & De Langhe, 1990). Cryopreservation allows deposition of biological materials at very low temperature, usually that of liquid nitrogen (-196°C) (Engelmann, 2004). Storage at cryogenic temperature freezes all cellular division and metabolic process, eliminates differentiated cells thus improving the culture's homogeneity (Georget et al., 2009). To improve the regeneration potential of ECS, lines producing high regeneration efficiency could be cryopreserved as a source of target cells for gene transfer. Since the technique is relatively cheap and only requires a small volume of suspension culture with minimal maintenance, it could serve as an efficient preservation method for maintaining ECS.

5.6.2 Cisgenics approach

In cisgenics plants' development, researchers must comply to a few strict requirements such as the use of identical native copy of target gene including its promoter, intron and terminator in sense orientation. Cisgenics plants are more desirable because not only the biosafety issues and linkage drag problem are minimized, the use of gene-specific promoter/terminator could also potentially improve the gene expression performance (Holme, Wendt & Holm, 2013). When this study was initially conducted, the banana genome project was still in progress and thus the full length *MaPR-10* cDNA had to be predicted by RACE. With the completion of whole genome sequences (D'Hont et al., 2012), emphasis on cisgenics banana studies is growing as massive information can be easily retrieved from the database.

5.6.3 Gene stacking

PR-10 is associated with various signaling pathways. Thus, the use of more than one defense-related genes or gene stacking can be considered to produce clones with higher level of resistance against Foc infection. For example, the gene can be co-expressed with R-genes, transcription factors or other PR genes to enhance the expression and overall tolerance effect. A few studies involving gene stacking in combating fungal diseases have been demonstrated such as co-expression of anti-fungal protein (AFP) and chitinase (CHI) in oriental melon (Bezirganoglu et al., 2013), chitinase (RCH10) and β-1,3-glucanase (AGLU1) in rice (Mao et al., 2014) as well as dermaseptine, osmotine and lysozyme in potato (Rivero et al., 2012).

5.6.4 Infection data of other transgenic *MaPR-10* lines and field trial

Additional data is needed from infection studies of other lines to further investigate the resistance capability of *MaPR-10* transgenic plants. For that reason, the current plantlets must be well-maintained and propagated. From the infection studies, various downstream analyses can be performed concerning the interaction between the host and the invading pathogen. These include transcriptome analysis of infected transgenic lines or quick qPCR analysis which focuses on a few selected genes following Foc infection.

Best performing *MaPR-10* lines from the bioassay experiments can be further tested on the Foc-infested field to monitor the consistency of disease response. Prior to this evaluation, proper field trial guidelines must be developed to minimize biosafety issues. A few factors can be taken into considerations which include location of the field, safety distance between test plants, buffer zone from other neighboring plants, water irrigation, as well as personnel and materials movement from and to the trial site.

CHAPTER 6: CONCLUSION

In this study, full-length cDNA sequences of *PR-10* gene from wild-type banana, *M. acuminata* ssp. malaccensis (*MaPR-10*) have been successfully isolated using RACE technique. *MaPR-10* encodes for 773 bp nucleotides and comprises of two exons spanned in between an intron. MaPR-10 is predicted to be a small acidic protein (160 a.a.) with predicted molecular weight and IE value of 17.46 kDa and 5.42, respectively. The protein contains Betv1-like functional domain, P-loop motif (GxGxxG) and three highly conserved residues (E102, E149 & Y151) which are postulated to be responsible for RNase activity.

Coding sequences of *MaPR-10* have been integrated into a commercial banana cultivar, _Berangan' via co-cultivation of transformed *A. tumefaciens* and ECS. Under continuous hygromycin selection for 6-7 months, 17 putative transgenic lines have been successfully established in which 11 of them showed positive transgene integration as verified by PCR. Line-19 plantlets, which have been chosen for further analysis for their vigorous shoot propagation, possibly harboring one transgene copy and have demonstrated over-expression of *MaPR-10*.

In this study, inoculation of two-month-old _Berangan' plants in 1 x 10^6 spores/ml of FocR4 C1 HIR suspension for two hours using _double-tray' set-up have been selected as the infection parameters for all bioassay experiments. The parameters were adopted from Mak and his co-workers (2004) and have been validated to test the baseline response of wild-type *M. acuminata* cv. _Berangan' plants against FocR4 C1 HIR infection. In wild-type *M. acuminata* cv. _Berangan' plants, external disease symptoms such as yellowing and wilting on the leaves can be visualized in the 1st week-post-inoculation. More severe infection was demonstrated by plants' death. Rhizome of the infected plants also demonstrated brownish to darkish discoloration which may spread

to the pseudostem. Wild-type ‗Berangan' plants were found to be highly susceptible to C1 HIR infection with LSI and RDI scores of 4.3 and 6.0, respectively.

During acclimatization under greenhouse conditions, about 75% (15 out of 20 plants, including both the treated and untreated groups) of Line-19 plants displayed slower growth in comparison to the untransformed controls. About 30% (3 out of 10 plants) of the infected Line-19 plants demonstrated a delay in disease progression and started to produce the common yellowing leaf symptoms in the 2nd week-post-inoculation. In contrast, untransformed control plants started developing the same disease symptoms as early as one-week-post inoculation. Somaclonal variation has been suggested as one of the factors affecting the transgenic plantlets' development. As a result, plants with weaker physical conditions were more prone to the infection and thus inflicting the final disease score. After five weeks of evaluation, transgenic Line-19 plants were found to be highly suspectible to C1 HIR with LSI and RDI scores of 3.4 and 6.1, respectively. The LSI and RDI scores for untransformed controls turned out to be 4.5 and 6.3, respectively.

To our knowledge, this is the first report of *MaPR-10* over-expression in *M. acuminata* cv. ‗Berangan' banana. The use of native banana gene reduces the issues concerning biosafety. Line-19 plants showed at least one-week-delay in disease progression when compared to the untransformed controls. This present study built a basis for studying *MaPR-10* and its over-expression effect on the banana's tolerance against *Fusarium* infection. The validated inoculation conditions can also be used as references for future bioassay experiments involving other Foc isolates and banana cultivars. The information obtained from this study would be useful for future genetic improvement of banana, particularly in *Fusarium*-host interaction studies.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

Publications:

1. Standardized bioassays for studying *Fusarium oxysporum* f. sp. *cubense* race 4 (FOCR4) pathogen stress response in *Musa acuminata* cv. ‗Berangan' (submitted)
2. Development of transgenic *Musa acuminata* cv. ‗Berangan' over-expressing pathogenesis-related (PR) 10 gene for *Fusarium* wilt tolerance (submitted)
3. Isolation of full-length *MaPR-10* cDNA sequences from *Musa acuminata* ssp. malaccensis) and *in silico* analysis of three *Musa* PR-10 proteins (submitted)

Conferences:

Nadiya Akmal Baharum, Wan Muhammad Farhan Syafiq Wan Mohd Nor, Nazia Abdul Majid, Yusmin Mohd. Yusuf, Rofina Yasmin Othman and Norzulaani Khalid. Isolation and expression analysis of putative pathogenesis related (PR) 10 from *Musa acuminata* cv. ‗Berangan' in response to exogenous salicylic acid supply. Poster presenter (2nd runner-up for Most Popular Poster) at 20th Society for Molecular Biology and Biotechnology (MSMBB) Scientific Meeting, 26th-27th June, 2013, Research Management and Innovation Complex, University of Malaya, Kuala Lumpur

Nadiya Akmal Baharum, Yusmin Mohd Yusuf, Rofina Yasmin Othman and Norzulaani Khalid. Integration of pathogenesis-related (PR) 10 gene from *Musa acuminata* ssp. malaccensis into commercial banana cv. ‗Berangan'. Oral presenter at International Conference on Crop Improvement: Issues and Prospects for Biotechnology Intervention (ICCI 2013), 25th-26th November, 2013, Equatorial Hotel Bangi, Selangor, Malaysia

Nadiya Akmal Baharum, Rofina Yasmin Othman, Yusmin Mohd Yusuf, Boon Chin Tan, Kamilatulhusna Zaidi and Norzulaani Khalid. Over-expression of pathogenesis-related (PR) 10 gene in *Musa acuminata* cv. _Berangan' for *Fusarium* wilt tolerance. Oral presenter at the 2nd International Conference on Crop Improvement (ICCI 2015), 2nd-3rd December, 2015, Engineering Auditorium, Faculty of Engineering, Universiti Putra Malaysia

Nadiya Akmal Baharum, Rofina Yasmin Othman, Yusmin Mohd Yusuf and Norzulaani Khalid. Pathogenesis-related (PR) 10 as a potential anti-fungal protein for the development of transgenic *Musa acuminata* cv. _Berangan' against *Fusarium* wilt. Oral presenter at the 20th Biological Sciences Graduate Congress, 9-11th December 2015, Chulalongkorn University, Bangkok, Thailand