# OVER-EXPRESSION OF TWO PUTATIVE DISEASE RESISTANT GENES *NBS-TYPE RGC* AND *WRKY* AGAINST *Fusarium oxysporum* f. sp. *cubense* IN PLANTS

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**FACULTY OF SCIENCE**
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**SITI NUR AKMAR BINTI MAZLIN**

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**OVER-EXPRESSION OF TWO PUTATIVE DISEASE RESISTANT GENES *NBS-TYPE RGC* AND *WRKY* AGAINST *Fusarium oxysporum* f. sp. *cubense* IN PLANTS**

## ABSTRACT

The widespread global distribution of *Fusarium* wilt disease caused by *Fusarium oxysporum* f. sp. *cubense* (Foc) has affected the production of banana fruits in almost all edible banana cultivars. This is due to lack of disease resistance banana, parthenocarpic nature of bananas and sterile fruits. This has limited the conventional breeding program to develop *Fusarium* wilt-resistant line. Strategies to control the *Fusarium* wilt disease are also ineffective due to polycyclic nature of the disease. Therefore, development of *Fusarium*-resistant banana through genetic engineering is of significant importance. In the past, two candidate genes, namely *nucleotide binding site* (*NBS*) *type* of *resistance gene candidate* (*RGC*) and *WRKY* transcription factor, showed up-regulation expression during Foc infection in banana. Therefore, in this study, *NBS-type RGC* and *WRKY* isolated from banana were molecularly characterized and expressed in banana and tobacco, respectively. *NBS-type RGC* was successfully isolated from *Musa acuminata* ssp. *malaccensis* (*MAN-RF*) with cDNA length of 1,160 bp and cloned. The constructed binary vector pCAMBIA1304-MAN-RF was transformed into embryogenic cells of *Musa acuminata* cv. 'Berangan' and leaf disc of tobacco, *Nicotiana tabacum* L. cv. SR1 via *Agrobacterium*-mediated transformation. The PCR analysis of the putative transgenic of *Musa acuminata* cv. 'Berangan' plantlets confirmed the stable integration into the genome. The transcript levels of *MAN-RF* were determined using $2^{-\Delta\Delta Ct}$ method in transgenic lines of T7, T13 and T14 resulted in 2.47, 3.29 and 4.69-fold changes compared to the untransformed plant, respectively. Unfortunately, the transformation of tobacco was unsuccessful. Therefore, the expression analysis of *MAN-RF* in tobacco

was unable to perform. While in WRKY transcription factor study, full-length WRKY gDNA and cDNA were successfully isolated from *Musa acuminata* ssp. *malaccensis* (*MamWRKY*) by using 'rapid amplification of cDNA ends' (RACE) with 1,414 bp and 1,224 bp length, respectively. The full-length *MamWRKY* cDNA contains 861 bp of coding sequences (CDS), which encodes 286 amino acids with the structural features of group IIe in the WRKY proteins family and predicted to have a molecular mass of 30.16 kDa and a theoretical p*I* of 5.05. The constructed binary vector pCAMBIA1304-MamWRKY CDS was introduced into leaf disc *Nicotiana tabacum* L. cv. SR1 via *Agrobacterium*-mediated transformation. The transcript levels were determined using $2^{-\Delta\Delta Ct}$ method in transgenic lines of W1, W2 and W4 resulted in 809.002, 739.804 and 1153.659-fold changes compared to the untransformed plant, respectively. A functional study of *MamWRKY* was carried out in tobacco with *PR1a* as our reference gene. *PR1a* is one of a pathogen-responsive gene, which salicylic acid-inducible defense gene of tobacco. The elevated expression of *PR1a* in transgenic lines, W1, W2 and W4 was at 968.763, 23.984 and 6812.648-fold changes, respectively, higher than untransformed plants. This finding suggests that *MamWKRY* might function as a transcriptional regulator upstream of defense signaling pathways. This study has laid the foundation for further study the role of *NBS-type RGC* and *MamWRKY* in the plant's defense mechanism as the candidate genes that might facilitate banana improvement programs.

**Keywords**: *Fusarium oxysporum* f. sp. *cubense*, *Musa acuminata* cv. 'Berangan', *WRKY*, *NBS-type RGC*, *PR1a*

**EKSPRESI LEBIH TINGGI OLEH DUA CALON GEN *NBS-TYPE RGC* DAN *WRKY* YANG RINTANG PENYAKIT TERHADAP *Fusarium oxysporum* f. sp. *cubense* DI DALAM TUMBUHAN**

## ABSTRAK

Penyebaran meluas penyakit kelayuan *Fusarium* secara global yang disebabkan oleh *Fusarium oxysporum* f. sp. *cubense* (Foc) telah menjejaskan penghasilan buah pisang di hampir semua kultivar pisang yang boleh dimakan. Ini berpunca daripada kurangnya daya tahan pisang terhadap penyakit, keadaan semula jadi pisang yang parthenocarpic dan buah yang mandul. Ini telah membataskan pembiakan pisang secara konvensional bagi menghasilkan pisang yang tahan penyakit kelayuan *Fusarium*. Strategi untuk mengawal penyakit kelayuan *Fusarium* ini juga tidak efektif kerana keadaan semulajadi penyakit ini yang banyak kitaran. Terdahulu, dua gen yang dikenali sebagai '*nucleotide binding site*' (*NBS*) dalam *calon gen rintangan* (*RGC*) dan faktor transkripsi *WRKY* menunjukkan peningkatan ekspresi regulasi semasa jangkitan Foc pada pisang. Oleh yang demikian, dalam kajian ini, *NBS-type RGC* dan *WRKY* yang dipencilkan daripada pisang telah menjalani pencirian molekular dan diekspreskan di dalam tumbuhan tembakau dan pisang. *NBS-type RGC* telah berjaya dipencilkan daripada *Musa acuminata* ssp. *malaccensis* (*MAN-RF*) dengan 1,160 bp panjang jujukan cDNA dan diklonkan. Konstruk binari vektor pCAMBIA1304-MAN-RF telah ditransformasikan ke dalam sel-sel embriogenik *Musa acuminata* cv. 'Berangan' dan cebisan daun tembakau, *Nicotiana tabacum* L. cv. SR1 melalui kaedah transformasi *Agrobacterium*-pengantara. Analisa PCR terhadap anak pokok *Musa acuminata* cv. 'Berangan' yang berkemungkinan transgenik telah dapat memastikan integrasi gen yang stabil di dalam genom pisang. Tahap transkrip gen yang ditentukan dengan kaedah $2^{-\Delta\Delta Ct}$ pada jaluran pokok transgenik, T7, T13 dan T14 menunjukkan ekspresi 2.47, 3.29 dan 4.69 kali

ganda lebih tinggi daripada anak pokok yang tidak ditransformasikan. Malangnya, transformasi tembakau tidak berjaya. Oleh itu, ekspresi analisis *MAN-RF* di dalam tembakau tidak dapat dilakukaan. Sementara itu, dalam kajian faktor transkripsi WRKY, jujukan lengkap gDNA dan cDNA telah berjaya dipencilkan daripada *Musa acuminata* ssp. *malaccensis* (*MamWRKY*) dengan meggunakan 'rapid amplification of cDNA ends' (RACE) yang masing-masing menghasilkan jujukan sepanjang 1,414 bp dan 1,224 bp. Jujukan lengkap *MamWRKY* cDNA mengandungi 861 bp jujukan pengkodan (CDS) yang mengkodkan 286 asid amino dengan ciri-ciri struktur grup IIe dalam kumpulan besar WRKY protin dan dijangka mempunyai jisim molekul 30.16 kDa dan p*I* teori 5.05. Binari vektor pCAMBIA1304-MamWRKY yang dikonstruk telah dimasukkan ke dalam *Nicotiana tabacum* L. cv. SR1 melalui kaedah transformasi *Agrobacterium*-pengantara. Tahap transkrip yang ditentukan dengan kaedah $2^{-\Delta\Delta Ct}$ pada jaluran pokok transgenik, W1, W2 dan W4 masing-masing menunjukkan 809.002, 739.804 dan 1153.659 kali ganda ekspresi lebih tinggi daripada anak pokok yang tidak ditransformasi. Walaubagaimanapun, pokok pisang transgenik *MamWRKY* tidak dapat dihasilkan kerana penjanaan semula kalus yang rendah daripada embriogenik sel. Satu kajian fungsi *MamWRKY* telah dilakukan dalam tembakau dengan *PR1a* sebagai gen rujukan. Gen yang responsif terhadap patogen, *PR1a*, adalah gen pertahanan yang terinduksi oleh asid salisilik. Ekspresi tinggi yang ditunjukkan oleh gen responsif kepada patogen, *PR1a* pada pokok yang ditransformasi, W1, W2 dan W4 ialah masing-masing 968.763, 23.984 dan 6812.648 kali ekspresi lebih tinggi berbanding anak pokok yang tidak ditransformasi. Kajian ini telah meletakkan asas untuk kajian selanjutnya mengenai peranan *NBS-type RGC* dan *MamWRKY* dalam mekanisma pertahanan tumbuhan sebagai calon gen yang dapat membantu program penambahbaikan pisang.

**Kata kunci**: *Fusarium oxysporum* f. sp. *cubense*, *Musa acuminata* cv. 'Berangan', *WRKY*, *NBS-type RGC*, *PR1a*

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## LIST OF SYMBOLS AND ABBREVIATIONS

| | | |
|---|---|---|
| ºC | : | degree Celsius |
| x *g* | : | times gravity (G-force) |
| % | : | percent |
| µg | : | microgram |
| µL | : | microliter |
| µm | : | micromolar |
| ATP | : | adenosine triphosphate |
| BAP | : | 6-benzylamonopurine |
| bp | : | base pair |
| BSA | : | bovine serum albumin |
| $CaCl_2$ | : | calcium chloride |
| cDNA | : | complementary deoxyribonucleic acid |
| CDS | : | coding sequences |
| CIP | : | calf intestine alkaline phosphatase |
| cm | : | centimeter |
| Ct | : | threshold cycle |
| CTAB | : | cetyltrimethylammonium bromide |
| cv | : | cultivar |
| DNA | : | deoxyribonucleic acid |
| DNase | : | deoxyribonuclease |
| DNTP | : | deoxynucleotide |
| DTT | : | dithiothreitol |
| *E. coli* | : | *Escherichia coli* |
| ECS | : | embryogenic cell suspension |

| | | |
|---|---|---|
| EDTA | : | ethylenediaminetetra acetic acid |
| EtBr | : | ethidium bromide |
| FAO | : | Food and Agriculture Organization |
| Foc | : | *Fusarium oxysporum* f. sp. *cubense* |
| g | : | gram |
| gDNA | : | genomic DNA |
| GTP | : | guanosine triphosphate |
| GUS | : | ß-glucuronidase |
| $H_2O$ | : | water |
| HCl | : | hydrogen chloride |
| kb | : | kilobase |
| kDa | : | kiloDalton |
| L | : | liter |
| LB | : | Luria-Bertani |
| LRR | : | leucine rich repeat |
| M | : | molar |
| mg | : | milligram |
| $MgCl_2$ | : | magnesium chloride |
| mL | : | mililiter |
| mM | : | milimolar |
| n | : | haploid number |
| NAA | : | naphthalene acetic acid |
| NaCl | : | sodium chloride |
| NaOAc | : | sodium acetate |
| NaOH | : | sodium hydroxide |
| NBS | : | nucleotide binding site |

| | | |
|---|---|---|
| NCBI | : | National Center for Biotechnology Information |
| nm | : | nanometer |
| OD | : | optimal density |
| PCR | : | polymerase chain reaction |
| pH | : | potential of hydrogen |
| p*I* | : | isoelectric point |
| PR | : | pathogenesis-related |
| psi | : | pounds per square inch |
| PVD | : | polyvinylpyrrolidone |
| qPCR | : | quantitative polymerase chain reaction |
| RACE | : | rapid amplification of cDNA ends |
| RE | : | restriction enzyme |
| RGC | : | resistance gene candidate |
| RNA | : | ribonucleic acid |
| RNase | : | ribonuclease |
| RT | : | reverse transcriptase |
| SDS | : | sodium dodecyl sulphate |
| T-DNA | : | transfer DNA |
| TAP | : | tobacco acid pyrophosphatase |
| TBE | : | Tris-Borate-EDTA |
| TMV | : | tobacco mosaic virus |
| TRM | : | tobacco root multiplication |
| TSM | : | tobacco shoot multiplication |
| U | : | unit |
| USD | : | United States Dollar |
| UTR | : | Untranslated region |

| | | |
|---|---|---|
| UV | : | Ultraviolet |
| V | : | volt |
| v/v | : | volume/volume |
| w/v | : | weight/volume |

## LIST OF APPENDICES

# CHAPTER 1: INTRODUCTION

## 1.1 General introduction

Banana and plantains (*Musa* spp.) are large monocotyledonous herbs cultivated mainly in the tropical and subtropical countries with high annual production for trade or local consumption. The world banana production is mostly produced by small-holder farmers for local markets which cover 85% of the production and 15% entering international trade with a total trade value of USD 9 billion (Food and Agriculture Organization of the United Nations (FAO, 2013). Despite of their importance and popularity, the productivity was hampered by abiotic and biotic stress primarily imparted by numerous diseases (Bakry, 2009) which hampered the export and production of smallholder. One of the primarily global constraints is the emerging of tropical race 4 of *Fusarium* wilt, over the impact of black leaf streak diseases (BLSD) throughout Asian, African, and American production. This disease has spread to none of the banana growing regions, Jordan and Mozambique (Butler, 2013).

*Fusarium* wilt, a destructive vascular fungal disease of banana plants is caused by soil-borne fungal pathogen known as *Fusarium oxysporum* f. sp. *cubense* (Foc) with four recognized races on host susceptibility. Initially, banana breeding was implemented to overcome this disease by crossing Gros Michel cultivar with disease-resistant diploids to generate hybrids resistant to *Fusarium* wilt and Black Sigatoka (Smith et al., 2005; Pillay & Tripathi, 2007). However, only limited number of these hybrids banana have been released for field trial because of slow progress of banana breeding due to polyploidy characteristics, parthenocarpic fruit development, low female fertility and prolonged life cycle (Sipen, 2011). Despite possibility of selection of resistant cultivars to Foc in banana breeding, genomic approaches offered a reliable strategy for genetic improvement that focused on identifying the genes through their characteristics and functional role in the defense response mechanism. This strategy

provides knowledge on candidate genes to be selected for genetic transformation of banana involving the introduction of target genes into plant cells to generate *Fusarium* wilt-resistant transgenic line. Foc infects the plant through roots by penetration of root cortex, proliferation of the hyphal in xylem vessels followed by phytotoxins secretion that blocks the vascular system and decrease the function of plant cells (Dong, 2012). Upon the pathogen attack, plant cell receptors will detect the invading pathogens which will activate various signal transduction mechanisms that results in the transcription of defense genes (Chen et al., 2012). The plant defense mechanism showed that the resistant genes can arrest the development of pathogen but require a particular transcription factor to interact with the promoter of the gene before it can be regulated.

Our study generally looked at two different genes, the nucleotide binding site-type resistance gene candidate (*NBS-type RGC*) and *WRKY* transcription factor that showed up-regulation expression during Foc infection in banana (Peraza-Echeverria et al., 2008; Lim, 2006). *NBS-encoding resistance* genes play an important role in the responses of plants to various pathogens. WRKY transcription factor is a transcriptional activators or repressors of genes involved in differential responses to abiotic or biotic stresses in plants. In this study, the expression of these two genes was further analyzed in separate experiment.

In the previous study by Dumin (2007), the highly conserved motifs of *Resistance* gene (*R*), the NBS-type domain sequences have been isolated and characterized in the wild type and susceptible banana cultivar to Foc. Although studies have shown the incapability of any *R* gene to confer resistance towards TR4 Foc, but contrary results was shown in the wild diploid banana, *Musa acuminata* ssp. *malaccensis* (Peraza-Echeverria et al., 2008). These plants were resistant to TR4 Foc when grown for field trials (Ploetz & Pegg, 2000). In this study, NBS-type sequence from *Musa acuminata* spp. *malaccensis* isolated by Dumin (2007) designated *MAN-RF*, underwent cloning

and introduced into embryogenic cell suspension of banana and tobacco leaf explants. Gene expression analysis was carried out via qPCR.

While, in the previous study by Lim (2006), WRKY transcription factor was identified via cDNA microarray where its expression was up-regulated in *Musa acuminata* x *balbisiana* cv. 'Mutiara' (banana Mutiara, AAB) during a challenge experiment involving the colonization of Foc in the roots. *Musa* ssp. cv. 'Mutiara' is a Foc tolerant-banana cultivar which was obtained through somaclonal variation. As the partial gene of *WRKY* from *Musa* ssp. 'Mutiara' showed similarity with the partial gene of WRKY from *Musa acuminata* ssp. *malaccensis*, a wild type banana resistant to Foc Race 4, isolation and characterization of full length WRKY gene from *Musa acuminata* ssp. *malaccensis* (designated *MamWRKY*) was conducted in the present study. The coding sequence of *MamWRKY* gene was cloned and introduced into embryogenic cells of *Musa acuminata* cv. 'Berangan' and leaf disc of *Nicotiana tabacum* L. cv. SR1 driven by a constitutive promoter CaMV35S via *Agrobacterium*-mediated transformation. Gene expression in the transformed plants was characterized by real-time quantitative PCR (qPCR). A functional study of WRKY was carried out in tobacco with *PR1a* as our reference gene. *PR1a* is one of a pathogen-responsive gene, which salicylic acid-inducible defense gene of tobacco. Previously, the first report of *WRKY* transcription factor gene from banana, *MusaWRKY71* has been characterized in detail for its involvement in abiotic and biotic stress response pathways but *MusaWRKY71* overexpressing plants were found to be susceptible to Foc (Shekhawat, 2011; Shekhawat, 2013).

## 1.2 Objectives

The specific objectives of this study are as follows:

i. To generate full length cDNA of *WRKY* gene and characterized it sequences via *in silico.*

ii. To construct two gene expression cassettes based on plant expression vector, pCAMBIA1304 with *NBS-type RGC* partial gene and *WRKY* transcription factor gene.

iii. To individually transform *NBS-type RGC* and *WRKY* transcription factor gene into leaf discs of *Nicotiana tabacum* L. cv. SR1 and embryogenic cells suspension (ECS) of *Musa acuminata* cv. 'Berangan' via *Agrobacterium*-mediated transformation.

iv. To determine the expression levels of these two genes of interest in the transformed plants via qPCR.

# CHAPTER 2: LITERATURE REVIEW

## 2.1 Introduction to bananas

Bananas are large monocotyledonous herbs that belong to the family of Musaceae under *Musa* genus. Banana plant grows from corm and its trunk is not a woody trunk but is a "false stem" or pseudostem that consists of leaves and their fused petiole bases (Nelson et al., 2006). The bananas fruits develop from each flower cluster are known as hands and individual fruits are known as fingers (Nelson et al., 2006).

### 2.1.1 Origin and taxonomy of bananas

Bananas (*Musa* spp.) were recorded to be earliest cultivated food crops about 7000 years ago from New Guinea (Denham et al., 2004). It was located from South Asia across to South East Asia which primarily distribution from India to Polynesia (Simmonds, 1962). Meanwhile, this diversity has been found in Malaysia or Indonesia (Daniells, 2001).

*Musa* spp. comprised of four genomes which correspond to the genetics composition of four wild Eumusa species *Musa acuminata* (A-genome, 2n = 2x = 22), *Musa balbisiana* (B-genome, 2n = 2x = 22), *Musa schizocarpa* (S genome, 2n = 2x = 22) and the Australimusa species (T genome, 2n = 2x = 20) (Davey et al., 2013). *M. acuminata* evolved in tropical rainforest of Southeast Asia and *M. balbisiana* originated from monsoon areas in northern Southeast Asia and Southern Asia (Ploetz et al., 2007).

Cultivated bananas resulted primarily from hybridizations between wild diploid *M. acuminata* and *M. balbisiana* species. Intraspecific hybridizations within *M. acuminata,* and interspecific hybridizations between *M. acuminata* and *M. balbisiana* have resulted in various A- and B –genomes combinations. The majority of diploid and triploid edible bananas are classified in the groups AA, AAA (dessert banana), AAB (plantains) and

ABB (cooking banana), according to the relative participation of the respective genomes in the genotype (Simmonds & Shepherd, 1955). The diversity of *M. acuminata* and its derivative hybrids are from Malaysia and Indonesia (Asif et al., 2001). Edible bananas are parthenocarpic, generally seedless and vegetative propagated hybrids.

### 2.1.2 Importance of bananas

Bananas and plantains are the most exported fruit in the world and also the fifth most produced food crop in least-developed countries (FAO, 2016). India is the biggest producer of bananas with an annual production of 30 million tons. Meanwhile China is the world's largest producer of Cavendish bananas, produced approximately 11 million tons annually (FAO, 2012). The coverage of banana in humid tropical areas worldwide spanned over 5.1 million, with total production of approximately 107 million tons (FAO, 2015). Other major banana producers in Asia include the Philippines (8.6 million tons) and Indonesia (5.4 million tons) (Mostert et al., 2017). About 85% of domestic banana made up of many different cultivars, Cavendish, Lakatan, Rasthali, Gros Michel and Sukali Ndizi. However, 15% of export bananas come from only one dessert cultivar, Cavendish (Dale, 2017).

Bananas grown in Asia made up 45% of bananas grown worldwide and comprised of more than 150 varieties that are produced for domestic consumption and export (Lescot, 2015). Bananas productions domestically are significant staple foods in Africa, Asia and tropical America, where varieties of cultivars are eaten raw as dessert fruit, cooked and brewed. Banana has progressed from snacks into vital food security to peoples in developed countries as source of calories in undertaking malnutrition, poverty alleviation and sustainability. Banana improvement of cultivated and commercially

important varieties is important to reduce losses of banana production due to diseases and contribute to increase nutritional quality for food security.

### 2.1.3 Diseases in bananas

There are diverse abiotic and biotic factors influencing production of banana, namely soil and plant nutrition, drainage and irrigation, type of cultivars, planting densities and disease management. Diseases are among the most important reasons for reduced banana productivity including those caused by fungi, bacteria, viruses, and nematodes. The most prevalent fungal diseases are *Fusarium* wilt caused by *Fusarium oxysporum* f. sp. *cubense* and Black Sigatoka caused by *Mycosphaerella fijiensis*. Other diseases such as bacterial wilt caused by *Xanthomonas*, viral diseases caused by banana bunchy top virus (BBTV), banana streak virus (BSV), banana bract mosaic virus (BBMV) and nematode infestation by root-knot nematode *Meloidogyne* spp. (Ghag & Ganapathi, 2017).

## 2.2 Banana *Fusarium* wilt disease

### 2.2.1 History and distribution

Banana *Fusarium* wilt is a vascular wilt disease caused by the soil-borne fungus, *Fusarium oxysporum* f. sp. *cubense* (Foc). This disease was first discovered in Australia in 1876 (Bancroft, 1876) and caused great damage in the export plantations of western tropics before 1860 (Ploetz, 2006). *Fusarium* wilt was then known as Panama disease in 1890 when it developed major epidemics in Panama and continued to destroy Gros Michel cultivar in Costa Rica (Ploetz, 1994). This disease became less of a concern as a problem for the trades when Gros Michel was replaced with Cavendish cultivars

resistant to Foc race 1 for the American and African market (Buddenhagen, 1990; Stover, 1962). However in 1990s, Cavendish cultivars were also found to be susceptible to a new race of Foc, tropical race 4 (TR4) and it rapidly spread, first in the subtropics region of Southeast Asia and then in the tropics region of Africa and Western Asia (Ploetz, 2005; Ploetz, 2015a).

There are four recognized races of Foc which are separated based on host susceptibility (Daly & Walduck, 2006). Race 1, which was responsible for the epidemics in 'Gros Michel' plantations, also attacks 'Lady Finger' (AAB) and 'Silk' (AAB) varieties. Race 2 affects cooking bananas such as 'Bluggoe' (ABB) and race 3 affects *Heliconia* spp., a close relative of banana, and is not considered to be a banana pathogen. Race 4 is capable of attacking 'Cavendish' (AAA) as well as the other varieties susceptible to races 1 and 2 and was further divided into 'subtropical' and 'tropical' strains. 'Tropical' race 4 is a more virulent form of the pathogen and is capable of causing disease in 'Cavendish' growing under any conditions, whereas 'subtropical' race 4 generally only causes disease in plants growing sub-optimally (cool temperatures, water stress and poor soil). The 'tropical race 4' (TR4) has caused widespread devastation in the production regions of the Philippines, Indonesia, Taiwan, Malaysia and in the southern provinces of China and 'subtropical race 4' (STR4) strain of Foc causes losses of Cavendish cultivars in the subtropical regions of the Canary Islands, South Africa, Australia and Taiwan (Ploetz, 2006). Recently, TR4 has been reported to spread to Africa (Mozambique) and some countries of the Middle East (FAO, 2016).

### 2.2.2 Mechanism of infection by *Fusarium oxysporum* f. sp. *cubense* (Foc)

*Fusarium* wilt is a classic vascular disease causing disruption of water translocation, systemic foliar symptoms and plant collapse (Jeger et al., 1995). Systemic infection of the pseudostem does not occur in tolerant cultivars due to the rapid production of tyloses, gums, and gels in xylem lumena in response to infection causing obstruction of the invading pathogen compared to susceptible cultivars (Ploetz, 2015a). In susceptible cultivars, the germinated hyphae of Foc will infect the roots and ascend through the xylem causing vascular brownness discoloration of the rhizome and wilting of leaves with bright yellow color (Figure 2.1). This wilting and leaf chlorosis is caused by phytotoxin, a fusaric acid (FA) that is produced by Foc (Dong et al., 2012). The resting spores formed in the wilting plant and repeatedly germinate in the soil. Once the area is infected, Foc will remain as chlamydospores in the soil for more than 30 years (Stover, 1962).

### 2.2.3 Management of disease

At present, there are limited effective, economical, environmentally safe, and curative management options available for managing *Fusarium* wilt of banana. Once established in a field, the fungus could survive in soil for a long period of time and could not be controlled chemically by fungicides, soil fumigants or cultural practices such as rotations or soil amendment (Hwang & Ko, 2004; Daly & Walduck, 2006). Banana-free rotations were ineffective for managing the spread of *Fusarium* wilt in most soils because the "polycyclic" nature of the disease where multiple cycles of infection could occur by even very small amounts of the pathogen infesting the banana growth area (Stover, 1962; Ploetz, 2015b).

(a)

(b)

(c)

**Figure 2:1:** Symptoms of banana plants susceptible to *Fusarium* wilt.

The symptoms are (a) wilting of leaves with bright yellow color; (b)-(c) rhizome and pseudostem with vascular brownness discoloration (FAO, 2016; Daly & Walduck, 2006).

Currently, there are several strategies that have been implemented to control the disease, such as eradication of Foc-infected bananas, circumvention of Foc-contaminated areas and the breeding of *Fusarium* wilt-resistant lines (Lin et al., 2016). The implementations of eradicating and avoiding the pathogen are time consuming

where diagnostic methods for the identification of the pathogen are laborious and unspecific. Breeding for resistance is generally complicated due to the lack of resistance cultivar, parthenocarpic nature of bananas and sterile fruits (Lin & Lin, 2016; Ploetz, 2015b). Alternatively, for continuous banana production, resistant cultivars using genetic transformation of highly desirable cultivars should be developed (Moran, 2013).

## 2.3 Natural defense mechanism

Plants differ from mammals as they lack mobile immune cells to defend themselves from several of pathogenic microorganisms upon infection. Thus they rely on the innate immunity of each cells and systemic signals to detect foreign entities. The plant's innate immune system consists of primary immune response, pathogen-associated molecular patterns (PAMP)-triggered immunity (PTI) and followed by effector-triggered immunity (ETI) (Jones & Dangl, 2006) that activates various transcriptional reprogramming to suppress the development of the pathogen (Figure 2.2). The PTI is activated when pathogen/microbe-associated molecular patterns (PAMPs/MAMPs) such as flagellin, chitin, glycoproteins and lipopolysaccharides are detected by pattern-recognition receptors (PRRs) at the plant cell surface (Spoel & Dong, 2012). During this initial infection, phytopathogens released proteins called effectors, encoded by *Avr* (avirulence) genes into the host cell to suppress PTI and promote virulence of the pathogen. In turn, plants have developed immune receptors called resistance (R) proteins that can specifically detect pathogen *Avr* gene and triggered ETI. The activation of ETI is followed by burst of reactive oxygen species and hypersensitive response (HR), a rapid programmed cell death at the site of pathogen invasion that prevent damage at the other parts of plants (De Wit, 1997).

Pathogen attacking plants to gain access to nutrients that abundantly contained within host plants via several lifestyle strategies of biotrophy, necrotrophy or hemibiotrophy (Collinge et al., 2016). In case of Foc, it utilizes necrotrophic lifestyle as its kill the infected plant part by secreting toxins and hydrolytic enzymes and then consume the nutrients released from the dead cells (Chen et al., 2012; Collinge et al., 2016). Meanwhile, biotrophic lifestyle is employed by pathogens that parasitize the living host cells to acquire nutrition from living cells (Chen et al., 2012) and hemibiotrophy lifestyle is apparently pathogen that starts as biotrophy lifestyle and predominantly exhibiting necrotrophy lifestyle with toxin production to complete infection.

The development of transgenic disease resistance plants is commonly based on first generation strategies that introduced single gene encoding directly antimicrobial proteins such as chitinases or pathogenesis-related proteins as opposed to second generation strategies that are based on the manipulation of signal-transduction pathways such as hormones, defence modulators and transcription factors to induce defenses against pathogens (Chen et al., 2012). The signaling pathways of SAR (systemic acquired resistance) against biotrophic pathogens and ISR (induced systemic resistance) against nectrotrophic pathogens form a complex regulatory network which is modulated by hormones and modulator (Grant & Jones 2009; Pieterse, 2009; Robert-Seilaniantz et al., 2011). Particularly, abscisic acid (ABA) is the main regulator of signaling molecules salicylic acid (SA) and ethylene with jasmonic acid (Et/JA) in SAR and ISR pathways, respectively. *NPR1* as the example of a modulator is required as a switch between the signaling pathways involving ET/JA (ISR) and salicylic pathway (SAR) to induce defense mechanisms (Kim & Delaney, 2002). Therefore, manipulation of ABA levels and *NPR1* gene is a potential target for developing transgenic disease resistance via SAR and ISR. The manipulation of plant transcription factors' expression, NAC, WRKY and ERF provide a potential strategy for engineering broad-spectrum disease

resistance in crop plants. Any gene in response to environmental stimulus requires a specific transcription factor and may facilitate manipulation of native plant defense response pathways in a pathogen non-specific manner.

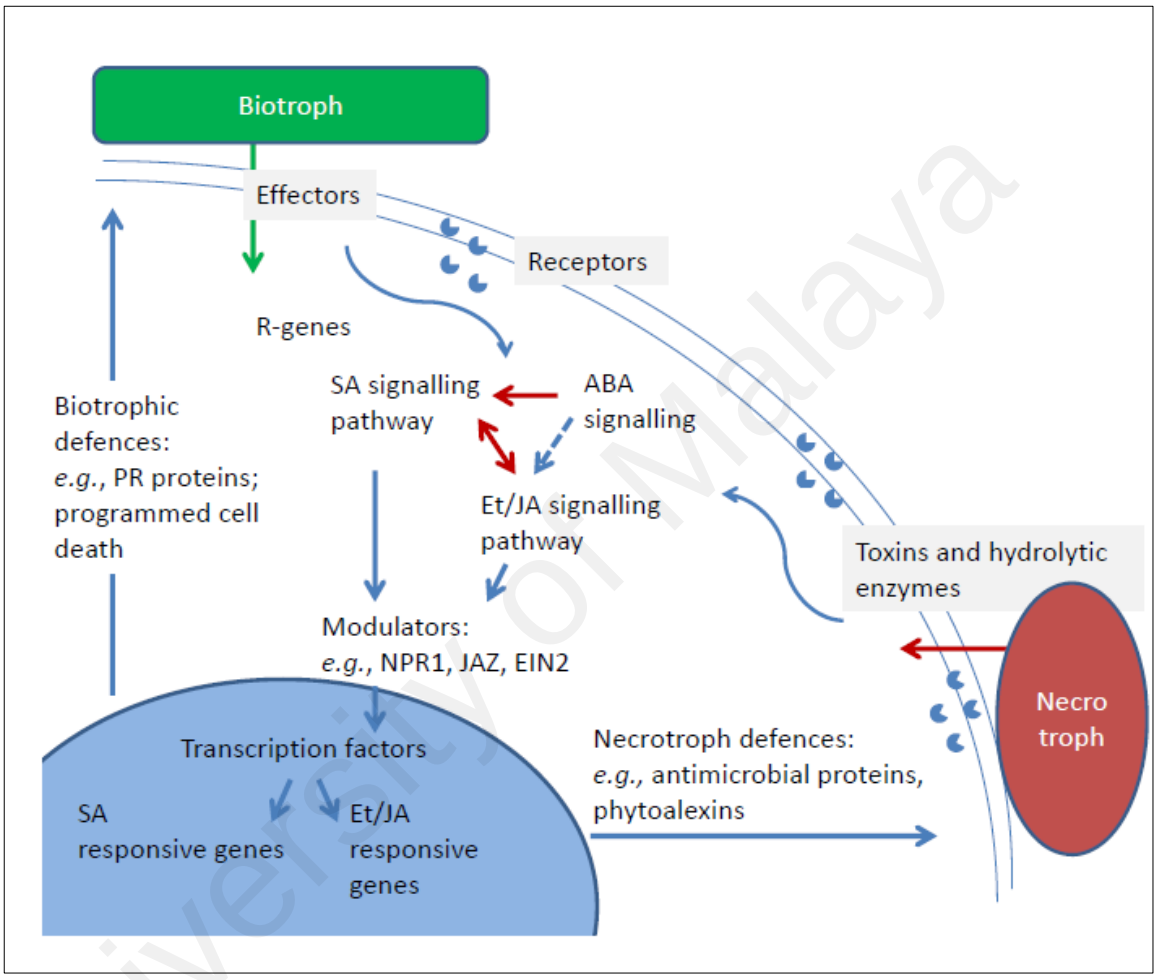



**Figure 2:2:** The process of plant's defence mechanisms. Image reproduced from Chen et al. (2012), with permission from John Wiley and Sons.

## 2.4 Establishment of disease resistant bananas

Globally, the productivity and yield of bananas was reduced due *Fusarium* wilt, BBTV, Sigatoka disease and Moko disease (Jones, 1999; Ghag & Ganapathi, 2017; Ploezt et al., 2015). In order to control diseases in bananas and to ensure sustainable production, the establishment of disease resistant cultivars generated by conventional

breeding and genetic transformation is critical to develop resistant bananas that meet local and export market standards. However, the characteristics of bananas which produce less pollen, sterile, polyploidy and long generation time has limited the breeding of resistant varieties (Sagi et al., 1995). Genetic engineering is the most promising strategy to develop transgenic bananas with improved traits by transferring gene into banana embryogenic cell suspension cultures using efficient and reliable banana transformation protocols of *Agrobacterium tumefaciens*-mediated transformation and microprojectile bombardment protocol (Paul et al., 2011). D'hont et al. (2012) has sequenced the 523-megabase genome of DH-Pahang, the doubled haploid *M. acuminata* ssp. *malaccensis* (2n=22). This genome and transcriptome sequencing of banana provides valuable resource information for banana improvement on various investigations of thousands of genes, transcripts, proteins, and metabolites involved in biotic and abiotic stress response. In addition, induction of somaclonal variants through tissue culture technique could be used to develop disease resistant cultivars.

### 2.4.1 Somaclonal variation

Somaclonal variation is a phenomenon in plants cloning where certain regenerants are not identical the mother plants as a response to plant genome caused by mutagenic plant growth regulators, non-optimal culture duration for shoot regeneration tissue and tissue culture procedure. However, somaclonal variation could be useful for agricultural trait improvement. Hwang and Ko (2004) discovered resistant banana cultivars against Foc race 4 as a result of somaclonal variation induced by repeated micropropagation-selection cycles of Cavendish banana plantlets from suckers.

### 2.4.2 Genetic transformation of plants

Plant transformation is a significant tool for the experimental investigation of gene function and the improvement of plants either to enhance existing traits or to introduce new traits into totipotent plant cells which are able to regenerate into whole fertile plants (Twyman et al., 2002). DNA can be introduced into cells or tissues that can easily and reproducibly regenerate whole plants such as isolated cells or protoplasts, explanted tissues, callus or cell suspension cultures.

The gene transfer techniques in plant genetic transformation are broadly grouped into two categories, vector-mediated gene transfer and direct or vector less DNA transfer. Vector-mediated gene transfer is carried out by using *Agrobacterium*-mediated transformation or plant viruses as vectors. Direct or vector less DNA transfer is the introduction of naked DNA into plant cells via physical gene transfer methods of electroporation, particle bombardment, microinjection, liposome fusion, silicon carbide fibres and chemical gene transfer methods using poly-ethylene glycol (PEG)-mediated, diethyl amino ethyl (DEAE) dextran-mediated and calcium phosphate precipitation. The first transgenic tobacco plants expressing recombinant genes in integrated T-DNA sequences were reported in 1983 and since then, the *Agrobacterium*-mediated transformation has been developed to become a widely used strategy for gene transfer to plants (Twyman et al., 2002).

#### 2.4.2.1 *Agrobacterium tumefaciens*-mediated transformation

*Agrobacterium tumefaciens*-mediated transformation is one of the naturally occurring inter-kingdom gene transfers and widely used as tool for genetic transformation in plants to produce stably transformed plants (Pacurar et al., 2011). *Agrobacterium*-mediated transformation method provides small copy numbers of genes

transfer into plant chromosomes thus reducing frequency of gene silencing events caused by gene dosage (Curtis, 2010). The method also demonstrated stable transformation and expression of gene encoded by the T-DNA (transferred-DNA) into single cells of plant genome and inheritable in the progeny (Krenek et al., 2015). Other advantages of this method including the transfer of large segments of DNA with minimal rearrangement and it results in the high quality and fertility of both monocotyledonous and dicotyledonous transgenic plants (Komari & Kubo, 1999). In order to obtain a successful *Agrobacterium*-mediated transformation, the T-DNA and the vir region must be present in the bacterium. The introduction of recombinant T-DNA into *A. tumefaciens*, involved the insertion of gene of interest into an *Escherichia coli* for gene replication followed by integration into Ti plasmid to create an expression vector (Fraley et al., 1986). The plant genetic engineering via *Agrobacterium*-mediated transformation comprised of five essential procedures involving the induction of the bacterial virulence system, generation of T-DNA complex, transfer of T-DNA from *Agrobacterium* to the host cell nucleus, integration of T-DNA into the plant genome and expression of gene encoded by T-DNA (Pitzschke & Hirt, 2010; Gelvin, 2012). The induction of the bacterial virulence system initiated by the wounded plant cells excreted low molecular weight of phenolic compounds and promoted *Agrobacterium* to enter the plant cells thereby induce the *vir* gene expression following activation of T-DNA transfer.

There are various factors limit the efficiency of *Agrobacterium* mediated transformation (Ziemienowicz, 2001). They are including genotype of the plant, type of explant, plasmid vector, bacterial strain and composition of components in culture medium such as salt, sugars and growth regulators in culture medium. Others are the efficiency of various antibiotics like cefotaxime, carbenicillin, and kanamycin to eliminate *Agrobacterium* from the plant tissue and culture medium after the co-

cultivation of explants with *Agrobacterium*. The use of chemicals like acetosyringone, an imitation of natural phenolic compounds also contributes to enhance the *Agrobacterium*-mediated transformation. The actively dividing embryogenic cell of monocotyledons was co-cultivated with *Agrobacterium* in the presence of acetosyringone to help activate the virulence genes of *Agrobacterium* and induce DNA synthesis (Komari & Kubo, 1999). This is because monocotyledons do not exhibit active responses to wounding compared to dicotyledons that depend profoundly on the cell divisions that are induced by wounding. The *Agrobacterium*-mediated transformation through somatic embryogenic calli has been used for transformation of the cereals and other recalcitrant crops such as banana, grapevine, coffee, tea, cotton and sugarcane as the advantage of transformants are developed from a single cell that limits the occurrence of chimeras (Curtis, 2010; Curtis, 2004; Sagi, 2000).

## 2.5 Genetic transformation for disease resistant bananas

As pre-requisite to genetic engineering, embryogenic cell suspensions (ECS) cultures are the most suitable explant tissue for banana transformation because the chimerism can be eliminated by regeneration of single cells into whole plants (Huang et al., 2007). In some of banana cultivars, ECS cultures were initiated from immature male/female flower buds, scalps, shoot tip cultures, zygotic embryos, basal leaf sheaths and corm section explants (Ghosh et al., 2009). The cell suspension culture protocol has been established for genetic transformation of few banana cultivars (Ganapathi et al., 2001; Strosse et al., 2006; Khanna et al., 2007; Chong-Pérez et al., 2012) following the development of high yield regeneration and transformation method by Tripathi's research group (2015). A number of transgenic banana for *Fusarium* resistance have been developed using an effective ECS system with various defense related genes such

as β 1-3 endoglucanase, rice chitinase, anti-apoptopsis gene, petunia floral defensin and rice thaumatin like-protein which exhibited enhanced resistance (Maziah et al., 2007; Paul et al., 2011; Ghag et al., 2012; Mahdavi et al., 2012). Rice chitinases has been expressed in ECS to produce transgenic resistant-banana against Black Sigatoka which showed lesser necrotic leaf area compared to the untransformed susceptible controls (Kovács et al., 2013). In, transgenic banana plants expressing plant ferredoxin-like amphipathic protein (PFLP) or hypersensitive response-assisting protein (HRAP) from sweet pepper demonstrated resistance to banana bacterial wilt (BBW) caused by *Xanthomonas campestris* pv. *musacearum* infection (Tripathi et al., 2014). In addition, genes encoding for antiviral proteins, antiviral systemic signal inducers and viral genome specific ribozymes have been engineered to confer viral resistance in banana plants against BBTV, banana streak virus and banana bract mosaic virus (Gadani et al., 1990; Mandadi & Scholthof, 2013).

## 2.6 Candidate genes for *Fusarium* wilt resistant bananas

Various genes has been selected as candidate gene and transformed into different banana cultivars as an attempt to develop *Fusarium* wilt resistant cultivars. The candidate genes are included pathogen-related (*PR*) gene, genes coding for antimicrobial peptides, anti-apoptopsis genes, and transcription factor genes (Table 2.1). Upon Foc infection in banana, the first line of defense initiated by the activation of genes involved in phenyl propanoids pathway to strengthen the cell wall in blocking the Foc entry (Li et al., 2013). PR genes that encode for hydrolytic enzymes, chitinase and glucanase are important to hydrolyse the fungal cell wall of chitin and β-1, 3-glucan, respectively (Edreva, 2005). The thaumatin-like proteins (TLPs) or PR-5 proteins were functionally act to alter the fungal cell membrane which caused the inhibition of fungal

growth and spore lyses (Tobias et al., 2007). Plant defensins are among one of antimicrobial peptides having small basic proteins with 3- 4 disulfide linkages, form ion permeable channels which helps in destabilization of microbial membranes (Zasloff, 2002). Transgenic banana cultivars constitutively expressing *Ace-AMP1* (Mohandas et al., 2013), *Petunia* floral defensins (Ghag et al., 2012), magainin *MSI-99* (Chakrabarti et al., 2003) and *Sm-AMP-D1* (Ghag et al., 2014) have shown significant resistance against *Fusarium* wilt compared to untransformed control banana plants. The necrotrophy lifestyle of Foc which feeds the dead tissues to gain nutrient for survival has exhibited a programmed cell death, an apoptosis-like features in banana plants (Trusov et al., 2006). Hence, overexpressing of anti-apoptosis animal genes, namely *Bcl-xL*, *Ced-9* and *Bcl-2 3′* UTR in transgenic banana cultivar 'Lady Finger' have inhibited the apoptosis regulation when they activated Bax and Bak that preventing released of cytochrome C in the cytosol (Paul et al., 2011). Primarily in plant, the transcriptional reprogramming associated with the abiotic and biotic stress response requires the action of diverse transcription factors like ethylene-responsive element binding proteins, bZIP proteins and WRKY proteins (Eulgem, 2005; Amorim et al., 2017). *MusaWRKY71* overexpressing bananas were found to be susceptible to Foc infection, but PR protein genes and *chitinase* genes have shown significant differential regulation in qPCR analysis (Shekhawat & Ganapathi, 2013).

Plants are known to contain hundreds of *R* genes, but review on *R* genes that have been cloned to develop resistant banana to Foc has not been identified yet (Sanseverino et al., 2010; Swarupa et al., 2014).

**Table 2.1:** Candidate genes used for transgenic disease resistance banana against Foc.

| Type of candidate genes | Candidate gene | References |
|---|---|---|
| *PR* gene | *β-1,3 glucanase* (PR-2) | Maziah et al. (2007) |
| | *Thaumatin-like protein* (*tlp*) (PR-5) | Mahdavi et al. (2012) |
| | *Chit42* (PR-3) | Hu et al. (2013) |
| Gene encoding antimicrobial peptides | *Ace-AMP1* | Mohandas et al. (2013) |
| | Magainin *MSI-99* | Chakrabarti at al. (2003) |
| | *PhDef1*, *PhDef2* | Ghag et al. (2012) |
| | *Sm-AMP-D1* | Ghag et al. (2014) |
| Anti-apoptosis genes | *Bcl-xL*, *Ced-9* and *Bcl-2* 3′ UTR | Paul et al. (2011) |
| Transcription factor | *MusaWRKY71* | Shekhawat & Ganapathi (2013) |

### 2.6.1 *Nucleotide binding site (NBS) type of disease resistance gene candidate (RGC)*

#### 2.6.1.1 General background

Over the last decade, many disease resistance or *R* genes have been cloned but to date, there is no report on the capability of *R* genes to confer resistance to Foc race 4 in cultivated bananas. However, field trial has shown that wild type diploid *Musa acuminata* ssp. *malaccensis* conferred resistance to Australian Foc tropical race 4 and thus suggesting a single dominant gene was involved (Smith et al., 2005). Subsequently, Ploetz and Pegg (2000) reported that the potential source of resistance in the wild type bananas was '*R*' gene.

*R* genes in plants are able to recognize specific pathogens that carry Avirulence (*Avr*) gene followed by the activation of signaling pathways to confer resistance to a wide variety of pathogens and pests including viruses, bacteria, fungi, nematodes and insects (Dangl & Jones, 2001). Several classes of *R* genes have been divided according to their protein products. The most prevalent class encodes nucleotide binding site proteins (NBS-proteins) that is related to the mammalian caspase recruitment domain (CARD)/nucleotide-binding oligomerization domain (Nod) family and functionally act

in innate immunity (Inohara & Nunez, 2003). NBS domain is attached to leucine-rich repeat (LRR) region at the C terminus and the N terminus consist of Toll/Interleukin 1 receptor (TIR) or an alpha-helical coiled-coil (CC) domain (Noir et al., 2001) (Figure 2.3). The N terminus that has no TIR domain but CC domain is known as non-TIR containing groups. The NBS domain of plant non-TIR- and TIR-NBS-LRR *R* genes consist of eight conserved motifs including P-loop (phosphate-binding loop)/kinase-1 (GMGGVGKT) or Walker A motif, kinase-2 (VLDDVW) or Walker B motif, kinase-3a, kinase-3 or known as hydrophobic (GLPL), resistance NBS (RNBS) of RNBS-A-TIR, RNBS-D-TIR, RNBS-A-non-TIR and RNBS-D-non-TIR (Traut, 1994; Meyers et. al., 1999; Yue et al., 2012).

NBS domain is also known as NB-ARC (nucleotide binding adaptor shared by NOD-LRR proteins, APAF-1, R proteins and CED4) domain which contains several defined motifs characteristic of the 'signal transduction ATPases with numerous domains' (STAND) family of ATPases (McHale et al., 2006). STAND proteins function as molecular switches in disease signaling pathways and ATP hydrolysis is assumed to result in conformational changes that regulate downstream signaling (McHale et al., 2006). NBS sequences have been amplified from a variety of plant species using PCR with degenerate primers based on conserved motifs of the NBS domain sequences (Meyers et al., 1999).

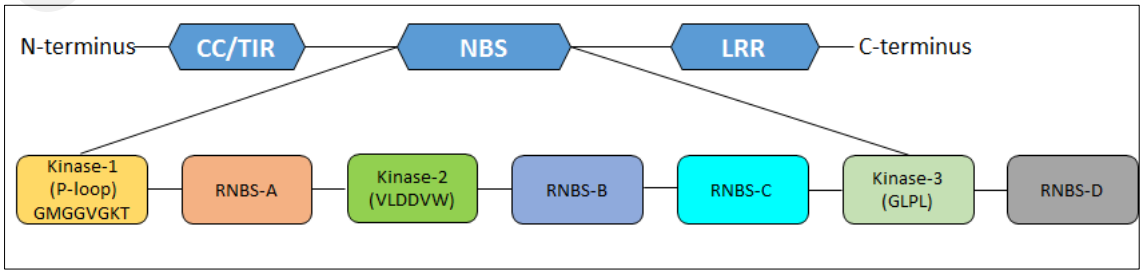



**Figure 2:3:** Structure of NBS-LRR plant resistance gene.

#### 2.6.1.2 Functional roles

In each NBS-LRR protein, conserved protein of NBS plays a key role for ATP binding and hydrolysis involving release of potential signaling upon plant pathogen attack (Tameling et al., 2002). The putative regulatory factors are required for activation of downstream signaling (Belkhadir et al., 2004) (Figure 2.4). The NBS domain motif, p-loop (kinase-1) is involved in ATP and GTP binding protein while the kinase-2 contains an aspartate critical for coordinating the metal ion ($Mg^{2+}$) required for phospho-transfer reactions (Traut, 1994). The amino-terminal domain of the LRR functions to modulate activation and specific residues that are located in the carboxy-terminal domain of the LRR shown to function in bacteria recognition which made the LRR region as an interaction platform for upstream activators.

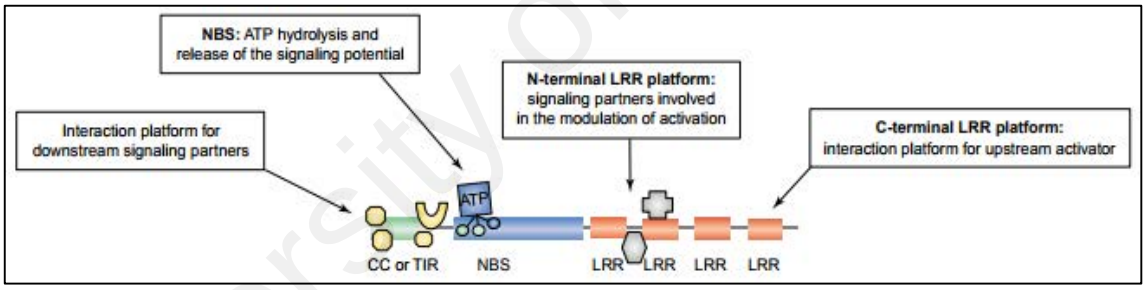



**Figure 2:4:** A schematic represent of various putative regulatory factors of NBS-LRR protein for signaling control. Image reproduced from Belkhadir et al. (2004), with permission from Elsevier.

### 2.6.2 WRKY transcription factor

#### 2.6.2.1 General background

The WRKY transcription factor family is among the ten largest families of transcription factors in higher plants and throughout the green lineage (green algae and land plants) (Ulker & Somssich, 2004). It comprises an integral part of signaling webs that modulates many plant processes such as plant developmental processes of

embryogenesis, seed coat and trichome development, regulation of biosynthetic pathways, hormone signaling and plant immune responses against abiotic and biotic stimuli either as positive or negative regulator of defense-related genes (Rushton et al., 2010; Agarwal et al., 2011). The characteristic of WRKY transcription is defined by its DNA-binding domain, known as the WRKY domain with a highly conserved peptide sequence (WRKYGQK) at its N-terminal and a novel zinc-finger motif at the C-terminal, structurally consist of either $Cx_{4-5}Cx_{22-23}HxH$ or $Cx_7Cx_{23}HxC$) (Rushton et al., 2010) (Figure 2.5). In the absence of a complete gene family from any plant species, it was divided into three groups based on the number of WRKY domains (two domains in Group I proteins and one in the others) and the structure of their zinc fingers ($C_2HC$ in Group III proteins) (Eulgem et al., 2000). Group II genes were further divided into IIa, IIb, IIc, IId and IIe based on the primary amino acid sequence. Yamasaki et al. (2005) reported that the WRKY domain consists of a four-stranded β-sheet, with the zinc coordinating Cys/His residues forming a zinc-binding pocket at C-terminus and WRKYGQK residues that corresponds to the most N-terminal β-strand. The conservation of the WRKY domain is mirrored by a remarkable conservation of the cognate *cis*-acting W box elements (TTGACC/T). These (TTGACC/T) sequence elements contain the invariant TGAC core, which is essential for WRKY binding and at the same time mediates transcriptional responses to pathogen-derived elicitors (Fukuda & Shinshi, 1994; Rushton et al., 1996).

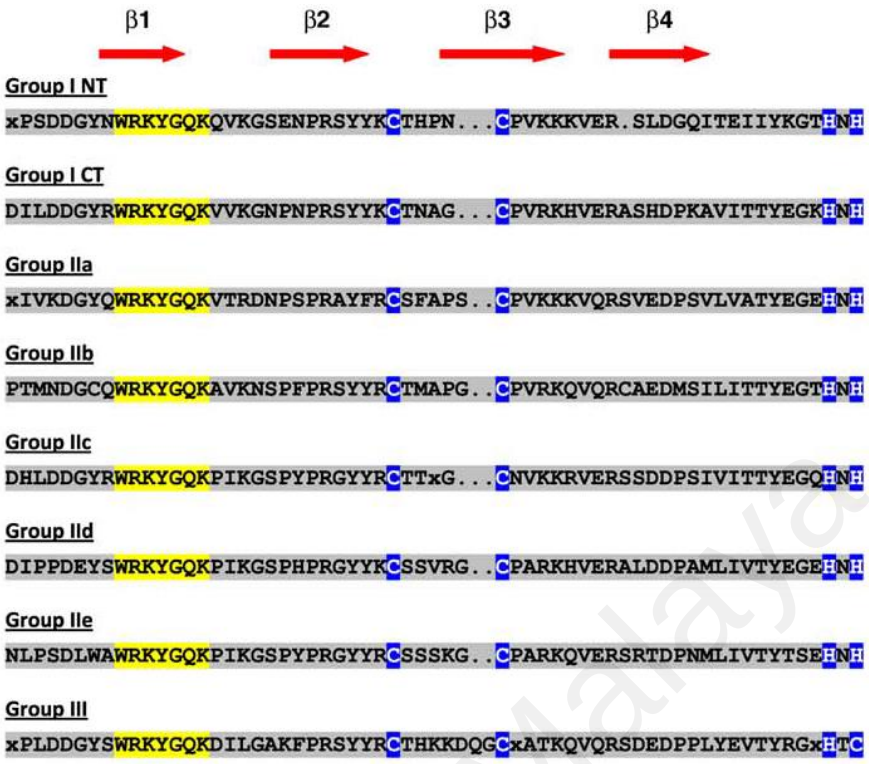



**Figure 2:5:** The WRKY domain for each of WRKY subfamily in higher plants.

The WRKY motif is highlighted in yellow and the cysteines and histidines that form the zinc finger are shown in blue. The four β-strands are shown in red. I CT and I NT denote the N-terminal and C-terminal WRKY domains from Group I WRKY proteins. Image reproduced from Rushton et al. (2010), with permission from Elsevier.

#### 2.6.2.2 Functional roles

Despite of WRKY protein function in regulation diverse plant processes, WRKY protein particularly function as key regulators of the two partly interconnected branches of plant innate immunity, microbe- or pathogen-associated molecular pattern-triggered immunity (MTI or PTI), effector-triggered immunity (ETI), basal defense and systemic acquired resistance (Eulgem & Somssich, 2007). The first evidence of WRKY proteins functionally act in regulating plant responses to pathogens was revealed and discovered a single WRKY protein might be involved in signaling mechanism via interactions with a diverse array of protein partners, including MAP kinases, MAP kinase kinases, 14-3-3 proteins, calmodulin, histone deacetylases, resistance proteins and other WRKY

transcription factors (Rushton et al., 2010). Numerous research has reported on the overexpression or knockdown of *WRKY* gene expression has subsequent effects on plant defense. Shen et al. (2007) reported that the MLA proteins that confer resistance against the powdery mildew fungus in barley has recognized fungal avirulence A10 effector and this event induces nuclear associations between the receptor and WRKY transcription factors. Figure 2.6 illustrates the WRKY transcription factor mediate immune responses in two pathways. The right pathway is in barley (*Hordeum vulgare*) involves the resistance protein MLA (mildew-resistance locus A) and left pathway is in *Arabidopsis*. In barley, the MAMP or PAMP invasion was detected by receptors that activate ETI and initiate signaling via intracellular MAPK cascades. This event has directly stimulates the induction of unknown WRKY transcriptional activators (red) and *HvWRKY1/2* repressors (blue). Auto repressed MLA receptors are folded by RAR1 (required for Mla12 resistance 1), SGT1 (suppressor of G-two allele of skp1) and cytosolic HSP90 (heat shock protein 90) and resulted in basal defense. An integrated MTI or PTI and MLA-triggered immune response is triggered by co-activation of one or several MAMP or PAMP receptors and MLA by cognate *Blumeria graminis* f. sp. *hordei* (powdery mildew) effectors ($AVR_A$). Activated MLA stimulates nuclear association with the *WRKY1/2* repressors, thereby de-repressing MAMP-triggered immunity that lead to expression of defense-related genes. In *Arabidopsis*, WRKY33 enhances the expression of *PAD3* for the synthesis of the antimicrobial camalexin (Qiu et al., 2008) as the MAMP or PAMP activated the MEKK1–MKK1/2–MPK4 module. This leads to nuclear dissociation of the MPK4–MKS1–WRKY33 complex and release of WRKY33 and MKS1. WRKY transcription factors frequently upregulate the expression of several pathogen-related genes and *NONEXPRESSOR OF PR1* (*NPR1*) by binding specifically to the W-box element in their promoter region in order to increase the defense response (Yang et al., 1999; Yu et al., 2001). Constitutive

overexpression of *WRKY70* results in increased resistance to pathogens and the constitutive expression of salicylic acid (SA)-induced *PR* genes (Yu et al., 2012). Meanwhile, the enhanced resistance of *CaWRKY1*-silenced chili pepper leaves to *Xanthomonas axonopodis* pv. *vesicatoria* suggests that *CaWRKY1* acts as a negative regulator of the defense response (Oh et al., 2008). The dual function of WRKY was shown when *WRKY41*-overexpressing *Arabidopsis* exhibited enhanced resistance to *Pseudomonas syringae* but increased susceptibility to *Erwinia carotovora* (Higashi et al., 2008).

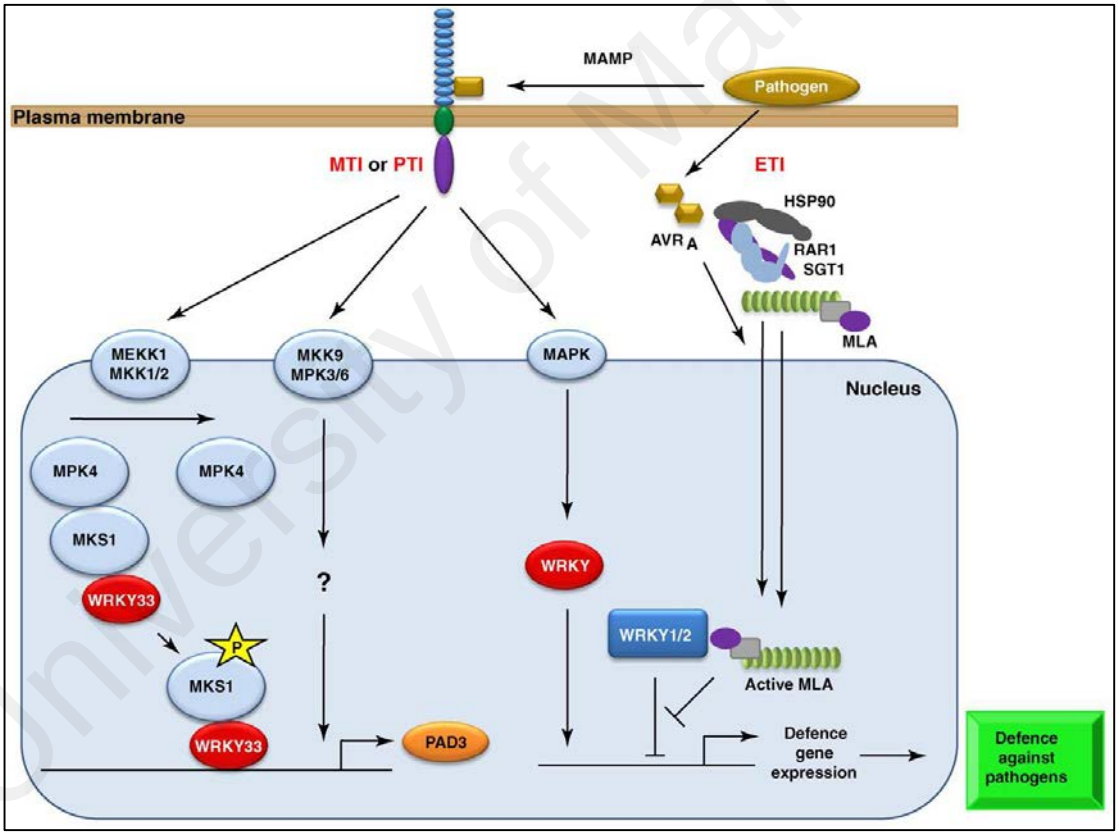



**Figure 2:6:** WRKY transcription factors mediate immune responses in two pathways in barley (*Hordeum vulgare*) (right) and pathway in *Arabidopsis* (left). Image reproduced from Rushton et al. (2010), with permission from Elsevier.

# CHAPTER 3: MATERIALS AND METHODS

## 3.1 Plant material

The wild type banana, *Musa acuminata* ssp. *malaccensis* (AA) was used as source of DNA for cloning of *NBS-type RGC* and *WRKY* transcription factor genes. Nucleic acid extraction was carried out from young banana leaves. The harvested young leaves were frozen in liquid nitrogen and stored at -80 ºC prior to use. This wild type banana was collected from PALAPES, University of Malaya (UM), Kuala Lumpur (voucher number: KLU48226).

## 3.2 Target tissue for genetic transformation

Embryogenic cells suspension of banana, *Musa acuminata* cv. 'Berangan' and leaf disc explants of tobacco, *Nicotiana tabacum* L. cv. SR1 were used as targets for genetic transformation. Gene expression of *NBS-type RGC* and *WRKY* were analyzed after being individually introduced into the plant cells via *Agrobacterium*-mediated transformation. Both cell suspension and tobacco plants were provided by Plant Biotechnology Research Laboratory, UM, Kuala Lumpur.

## 3.3 Gene of interest and vector

The full sequence of *NBS*-type gene and the partial *WRKY* gene sequence were provided by Dumin (2007) and Lim (2006), respectively. In the previous study, the latter was obtained from *Musa* ssp. 'Mutiara'. The cDNA of this partial *WRKY* gene was integrated in Uni-ZAP XR vector and cloned into *E. coli* host, XL1-Blue MRF'. Based on the partial sequence of WRKY, cDNA from *Musa acuminata* ssp. *malaccensis* (AA) was further amplified to obtain a full length gene.

In this study, pCAMBIA1304 (GenBank accession no.: AF234300.1) was used as a binary vector for the plasmid cassette backbone. The vector pCAMBIA1304 (Figure 3.1) was provided by Go (2013) and extracted from the glycerol stock.

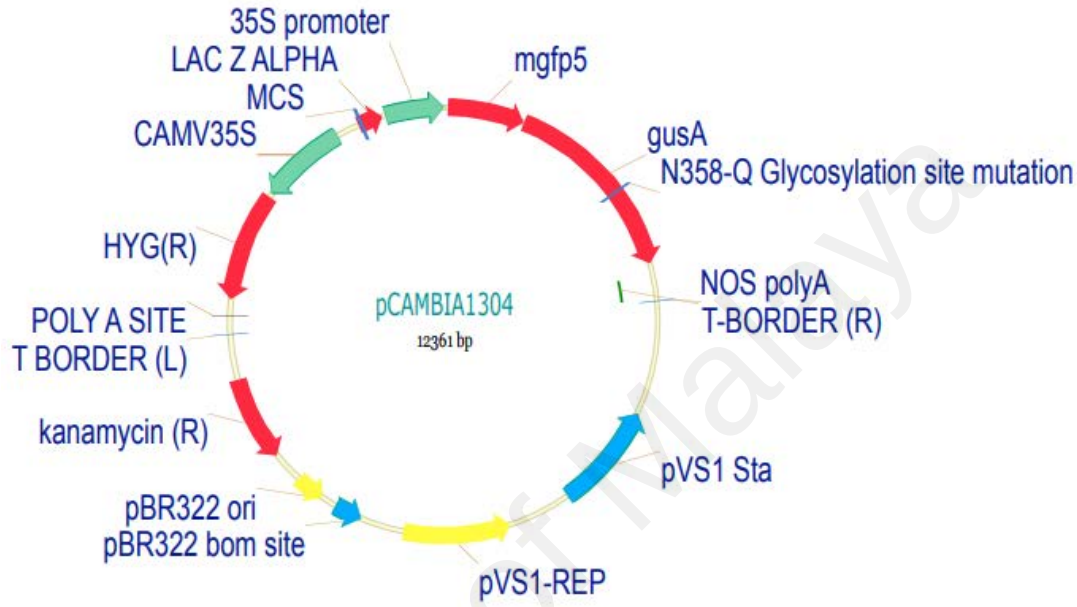



**Figure 3:1:** Map of pCAMBIA1304 (www.cambia.org).

### 3.4 RNA extraction

Total RNA was isolated from the young leaves of both varieties of banana (*Musa acuminata* ssp. *malaccensis* and *Musa acuminata* cv. 'Berangan') and tobacco using CTAB method (Al-Obaidi et al., 2010) with modifications. Prior to extraction, all solution were treated with diethyl pycarbonate (DEPC) (Appendix B1) and consumables were underwent sterilization for RNA work purpose (Appendix A). Approximately 0.5 g of banana or tobacco leaves were ground into fine powder using a mortar and pestle chilled with liquid nitrogen before added with 1 mL of pre-heated CTAB buffer [2% w/v CTAB, 2% w/v polyvinylpyrrolidone (PVP), 8% w/v NaCl, 5%

w/v EDTA (0.5 M, pH 8), 10% w/v Tris (1 M, pH 8.0) and DEPC-treated water] (Appendix B1) and 35 µL β-mecapethanol and incubated at 65 ºC for 10 minutes. An equal volume of chloroform: isoamylalcohol, $CHCl_3$:IAA (24:1) (Appendix B1) was then added to the mixture and vortexed before centrifuged at 9,391 x *g* for 15 minutes. The aqueous phase was transferred to a 1.5 mL tube. This step was repeated for three times. At the final centrifugation, 0.1 volume of 3 M sodium acetate (NaOAc) (Appendix B1) was added to the aqueous solution and fill up the tube with ice-cold absolute ethanol. Tubes were inverted for thorough mixing and precipitated at -80 °C for two days. After centrifugation at 9,391 x *g* for 30 minutes, the pellet was washed with 1 mL of 70% (v/v) ice-cold ethanol (Appendix B1). The mixture was centrifuged at 9,391 x *g* for 7 minutes at 4 °C. The pellet was then air-dried and re-suspended with 30 µL of DEPC-treated water. The RNA extract was kept at -80 ºC, 2-3 days prior to RNA precipitation.

RNA precipitation was carried out by centrifuge at 4 °C, 15,871 x *g* for 15 minutes. All liquid was discarded. The pellet was washed 2 times with 1000 µL of 70% (v/v) ethanol and centrifuged at 4 °C, 15,871 x *g* for 5 minutes. The pellet was air dried by short centrifugation and it was re-suspended in 15 µL of sterile DEPC-treated water. The RNA extract was stored at -80 °C prior to quantification and qualification of RNA integrity.

### 3.5 DNA extraction

DNA from banana (*Musa acuminata* ssp. *malaccensis* and *Musa acuminata* cv. 'Berangan') and tobacco were isolated using the same method as described in Section 3.4 without DEPC treatment. At the final step, the DNA extract dissolved in sterile

distilled water was treated with RNase A (20 mg/mL) (Invitrogen, U.S.A) for 10 minutes at 65 ºC.

### 3.6 Plasmid extraction

Plasmid was extracted according to Sambrook and Russell (2001) with modifications. A single colony of bacteria was cultured overnight at 37 °C in 10 mL LB broth supplemented with 50 µg/mL of kanamycin under shaking condition of 220 rpm. A total of 500 µL overnight culture was used to prepare a glycerol stock with final concentration of 50% (v/v) total and stored at -80 °C. The remaining culture was transferred to a 15 mL Falcon tube and centrifuged at 3,381 x *g* for 15 minutes at room temperature. The pellet was dissolved in 200 µL of ice-cold Solution I (Appendix B3). The mixture was then transferred to a 1.5 mL microcentrifuge tube before added with 200 µL freshly prepared Solution II (Appendix B3). After mixing, the mixture was left at room temperature for 4 minutes. An aliquot of 200 µL ice-cold Solution III (Appendix B3) was added into the mixture and mixed thoroughly by inversion before left on ice for 15 minutes. After centrifugation at 15,871 x *g* for 10 minutes, the supernatant was transferred to a new 1.5 mL microcentrifuge tube. A total of 1.5 µL of RNase A (20 mg/mL) (Invitrogen, U.S.A) was added and incubated at 37 °C in a water bath for 2 hours. Later, 600 µL of ice-cold phenol was added, the mixture was vortexed and centrifuged at 15,871 x *g* for 3 minutes. The aqueous phase was then transferred to a new 1.5 mL microcentrifuge tube. Chloroform (600 µL) was added, vortexed and centrifuged at 15,871 x *g* for 3 minutes. The aqueous phase was transferred into a new 1.5 mL microcentrifuge tube. An aliquot of 0.1 volume 3 M (NaOAc) and 2.5 volume of absolute ethanol were added. The mixture was left on ice for 20 minutes before centrifuged at 15,871 x *g* for 15 minutes. A total of 1 mL of 70% (v/v) ethanol was

added to the pellet and the mixture was inverted a few times prior to centrifuging at 15,871 x *g* for 5 minutes. The pellet was air-dried, dissolved with sterile distilled water (30-100 µL) and kept at -20 °C.

### 3.7 Quantification and qualification of RNA and DNA

The quantity and purity of RNA and DNA were determined using NanoDrop 2000 spectrophotometer (Thermo Scientific, U.S.A) by pipetting 1 µL DNA or RNA onto the detector. The purity of the sample was based on the ratio of the absorbance at 260 nm and 280 nm. An absorbance of 260 nm indicates the concentration of nucleic acid where 1 unit of $A_{260}$ reading is equivalent to 40 µg/mL of RNA and 50 µg/mL of DNA. An absorbance of 280 nm is used to check the purity of nucleic acid where the ratio value of $A_{260/280}$ at 1.8 to 2.0 indicated that pure RNA and DNA had been isolated. RNA integrity was determined by agarose gel electrophoreses. RNA mixture (0.1 µg total RNA extract, 1.5 µL of DEPC-treated water and 1 µL 2X RNA loading dye pre-boiled at 70 ºC for 10 minutes) was loaded onto 1% (v/v) agarose gel (diluted with 1X TBE buffer) (Appendix B4) and electrophoresed at 125 V for 25 minutes. The gel was stained in ethidium bromide solution for 30 seconds and de-stained in tap water for 10 minutes. The gel was visualized under ultra violet (UV) light to examine the integrity of RNA bands. The same procedure was carried out for DNA extract using 6X loading dye.

### 3.8 Complementary DNA (cDNA) synthesis

Prior to cDNA synthesis, DNA was denatured using DNase I kit (Thermo Scientific, U.S.A). About 1 µg of total RNA was mixed with 1 µL of 10X DNase I reaction buffer,

1 µL of DNase I and DEPC-treated water to a final volume of 10 µL. After incubation at 37 ºC for 30 minutes, 1 µL of 25 mM EDTA solution was added to the mixture and incubated at 65 ºC for 10 minutes to inactivate the DNase I activity. For the cDNA synthesis, RevertAid H Minus (Thermo Scientific, U.S.A) was used. A total of 12.5 µL mixture consisted of 1 µg of DNase-treated RNA, 1 µL of random hexamer and DEPC-treated water was mixed with 4 µL of 5X reaction buffer, 0.5 µL of RiboLock RNase inhibitor (Thermo Scientific, U.S.A), 2 µL of DNTP (10 mM dATP, dTTP, dGTP and dCTP) and 1 µL (200 U/µL) of RevertAid H Minus reverse transcriptase. The reaction mixture was first incubated at 25 ºC for 10 minutes and subsequently at 42 ºC for 60 minutes before inactivated at 70 ºC for 10 minutes. The product was kept at -20 ºC prior to use.

## 3.9 Polymerase chain reaction (PCR) and Rapid amplification of cDNA ends (RACE)

### 3.9.1 *NBS-type* of *resistance* gene

*NBS*-type of *resistance* gene from the cDNA of *Musa acuminata* ssp. *malaccensis* (*MAN-RF*) was amplified by PCR using GoTaq® DNA polymerase (Promega, U.S.A.) as follows:

**Table 3.1:** PCR reaction mixtures for amplification of *MAN-RF* cDNA.

| **Components** | **Volume** |
|---|---|
| 5X Green GoTaq® | 10 µL |
| DNTP (10 mM each dATP, dTTP, dGTP and dCTP) | 1 µL |
| Forward primer (NF) (10 µM) | 1 µL |
| Reverse primer (NR) (10 µM) | 1 µL |
| GoTaq® DNA Polymerase (5u/µL) | 0.25 µL |
| DNA template (<0.5 µg/ 50 µL) | 2 µL |
| Nuclease-free water | 19.75 µL |
| **Total reaction** | **50 µL** |

The primers are designed on the partial sequence of *MAN-RF* cDNA from Dumin (2007).

**Table 3.2:** Primers sequences for amplification of *MAN-RF* cDNA.

| Primer name | Primer sequence (5'→3') |
|---|---|
| NF | GCGAGATCTTCGAAGCCATGAAGGAT<br>*Bgl*II |
| NR | GACACTAGTTGGGTAGAGTCAATTCACG<br>*Spe*I |

The PCR cycles condition consists as follows:

**Table 3.3:** PCR cycle condition for amplification of *MAN-RF* cDNA.

| Stage | Condition | No. of cycle |
|---|---|---|
| Initial denaturation | 94 °C for 2 minutes | 1 |
| Denaturation<br>Annealing<br>Extension | 94 °C for 1 minute<br>59 °C for 20 seconds<br>72 °C for 1 minute | 35 |
| Final extension | 72 °C for 7 minutes | 1 |
| Cooling down | 25 °C for 5 minutes | 1 |

All PCR products were electrophoresed on 1% (w/v) agarose gel at 120 V for 30 minutes. PCR products were visualized under UV light (Vilber Lourmat), excised and purified using QIAquick Gel Extraction Kit (QIAGEN, Germany). The weight of the gel containing DNA fragment was measured (not exceed 0.50 g). After that, 3 volumes of Buffer QG were added to 1 volume of gel (if gel mass is 100 mg, 300 µL of buffer was added) and incubated at 50 °C for 10 minutes to dissolve the gel. Isopropanol was added to 1 gel volume (if gel mass is 100 mg, 300 µL of isopropanol was added), mixed and transferred to a QIAquick column. After centrifugation at 15,871 x *g* for 1 minute, the filtrate was discarded. Buffer QG (0.50 mL) was then added to remove all traces of agarose gel. The sample was then centrifuged at 15,871 x *g* for 1 minute. The filtrate

was discarded. Buffer PE at 0.75 mL was added to wash the sample and the column was left standing for 3 minutes before centrifuged at 15,871 x *g* for 1 minute. The filtrate was then discarded. Additional centrifugation was done to remove residual ethanol. After placing the column on a clean 1.5 mL microcentrifuge tube, 50 µL buffer EB was added to the mixture to elute DNA. The column was then stand for 5 minutes before centrifuge for 1 minute at 15,871 x *g*. The purified product was sent for sequencing (AITbiotech, Singapore) and kept at -20 °C.

### 3.9.2 WRKY transcription factor

#### 3.9.2.1 Partial gene

Partial cDNA of WRKY transcription factor obtained from *Musa acuminata* cv. 'Mutiara', previously inserted into Uni-ZAP XR vector, was amplified by PCR using GoTaq® DNA polymerase (Promega, U.S.A.). The PCR reaction mixture and cycles condition were performed according to the procedures described in Section 3.9.1 with the annealing temperature at 47 ºC. The primer pair used was as follow:

**Table 3.4:** Sequence of primers used to amplify partial cDNA of WRKY.

| **Primer name** | **Primer sequence (5'→3')** |
|---|---|
| T3 | AATTAACCCTCACTAAAGGG |
| T7 | TAATACGACTCACTATAGGG |

All PCR products were electrophoresed on 1% (w/v) agarose gel at 120 V for 30 minutes. PCR products were excised, purified and sequenced as described in Section 3.9.1.

#### 3.9.2.2 Rapid amplification of cDNA ends (RACE)

The amplification of 5' and 3' cDNA ends of WRKY transcription factor was performed using GeneRacer™ kit (Invitrogen, U.S.A.) according to the manufacturer's manual. In this experiment, 5' and 3' gene specific primers (designed based on the partial gene sequences obtained from *Musa* ssp. 'Mutiara' cDNA library) were used. Total RNA was obtained from *Musa acuminata* ssp. *malaccensis*. In isolation of full length cDNA, dephosphorylation reaction was first performed in a 10 µL mixture of 1 µg of total RNA, 1 µL of 10X Calf Intestine Alkaline Phosphatase (CIP) buffer, 1 µL of RNaseOut (40 U/µL), CIP (10U/µL) and DEPC-treated water. The mixture was mixed gently and incubated at 50 ºC for 1 hour. The RNA was precipitated (Section 3.4) and pellet was re-suspended in 7 µL of DEPC-treated water. The dephosphorylated RNA was mixed with 1 µL of 10X Tobacco Acid Pyrophosphatase (TAP) buffer, 1 µL of RNaseOut (40 U/µL) and 1 µL of TAP (0.5 U/µL) for de-capping reaction. After brief vortexing, the mixture was incubated at 37 ºC for 1 hour. The RNA was precipitated (Section 3.4) and pellet was re-suspended in 7 µL of DEPC-treated water. The resulted dephosphorylated and de-capped RNA was mixed and re-suspended with lyophilized GeneRacer™ RNA Oligo (0.25 µg). After incubation at 65 °C for 5 minutes, the RNA was incubated in a ligation reaction containing 1 µL of 10X ligase buffer, 1 µL of 10 mM ATP, 1 µL of RNaseOut (40 U/µL) and 1 µL of T4 RNA ligase (5 U/µL) at 37 ºC for 1 hour. The RNA was precipitated and pellet was re-suspended in 10 µL of DEPC-treated water. The RNA was reverse transcribed using SuperScript III Reverse Transcriptase provided in the GeneRacer kit. The ligated RNA was added with 1 µL of random primers, 1 µL of DNTP Mix (25 mM each nucleotides) and 1 µL of sterile distilled water and incubated at 65 ºC for 5 minutes before chilled on ice for 1 minute. The following reagents: 4 µL of 5X 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), were then added to

the mixture and incubated at 50 ºC for 1 hour. The reaction was inactivated at 70 ºC for 15 minutes followed by adding 1 µL of RNase H (2 U) and incubated at 37 ºC for 20 minutes. Amplification of 5' and 3' cDNA ends was carried out using HotStar Taq DNA Polymerase (QIAGEN, Germany) as follows:

**Table 3.5:** PCR mixture for 5' and 3' RACE of WRKY.

| **Components** | **Volume** |
|---|---|
| 10X PCR Buffer | 10 µL |
| DNTP (10 mM each dATP, dTTP, dGTP and dCTP) | 2 µL |
| GeneRacer 5'/ 3' primer (5RACE/ 3RACE) (10 µM) | 3 µL |
| 5'/ 3' outer primer (5'O/ 3'O) (10 µM) | 1 µL |
| HotStar Taq DNA Polymerase (5 U/µL) | 0.25 µL |
| DNA template (<0.5 µg/ 50 µL) | 2 µL |
| Nuclease-free water | 31.75 µL |
| **Total reaction** | **50 µL** |

**Table 3.6:** PCR mixture for 5' and 3' nested RACE of WRKY.

| **Components** | **Volume** |
|---|---|
| 10X PCR Buffer | 10 µL |
| DNTP (10 mM each dATP, dTTP, dGTP and dCTP) | 2 µL |
| GeneRacer 5'/ 3' nested primer (5nRACE/ 3nRACE) (10 µM) | 1 µL |
| 5'/ 3' inner primer (5'I/ 3'I) (10 µM) | 1 µL |
| HotStar Taq DNA Polymerase (5 U/µL) | 0.25 µL |
| 5'/ 3' RACE initial PCR product | 1 µL |
| Nuclease-free water | 34.75 µL |
| **Total reaction** | **50 µL** |

The primer pairs used were:

**Table 3.7:** Sequence of the primers used for RACE PCR for WRKY.

| **Primer name** | **Primer sequence (5'→3')** |
|---|---|
| 5RACE | CGACTGGAGCACGAGGACACTGA |
| 5'O | GTGGAGGCACCCAAAGAGTGTA |
| 5nRACE | GGACACTGACATGGACTGAAGGAGTA |
| 5'I | ATCTCGGATGCGATCTCGACCT |
| 3RACE | GCTGTCAACGATACGCTACGTAACG |
| 3'O | GAAGTTCTCCGACCTGATCACC |
| 3nRACE | CGCTACGTAACGGCATGACAGTG |
| 3'I | GACTCCGACGAGTTGCTCTA |

The PCR condition was as follows:

**Table 3.8:** PCR condition for RACE WRKY.

| Stage | Condition | No. of cycle |
|---|---|---|
| Initial denaturation | 94 °C for 3 minutes | 1 |
| Denaturation | 94 °C for 30 seconds | 35 |
| Annealing | 56-62 ºC for 5' RACE/Nested RACE<br>52-58 ºC for 3' RACE/Nested RACE<br>30 seconds | |
| Extension | 72 °C for 1 minute | |
| Final extension | 72 °C for 7 minutes | 1 |
| Cooling down | 25 °C for 5 minutes | 1 |

All PCR products were electrophoresed on 1% (w/v) agarose gel at 120 V for 30 minutes. PCR products were excised and purified as described in Section 3.9.1. The purified PCR products were then cloned into a vector using TOPO® TA Cloning® Kit (Invitrogen, U.S.A.) before sent for sequencing (AITbiotech, Singapore).

### 3.9.3 Full length cDNA and coding sequences (CDS)

The full length WRKY cDNA sequence of *Musa acuminata* ssp. *malaccensis* (*MamWRKY*) was obtained by aligning and merging the 3' and 5' RACE sequences with the partial sequence of WRKY. The sequence was then verified by PCR amplification. A pair of gene specific primers was designed from the putative 5'- and 3'-untranslated region (UTR) of the full length cDNA sequence. PCR was performed using Phusion High-Fidelity.

**Table 3.9:** PCR mixture for amplification of full length cDNA of *MamWRKY* and *MamWRKY* CDS.

| **Components** | **Volume** |
|---|---|
| 2X Phusion High-Fidelity PCR mixture (0.04 U/µl Phusion DNA Polymerase, 2x Phusion HF Buffer, 400 µM of each dNTP along with 1.5 mM $MgCl_2$) | 25.0 µL |
| Forward primer (WFL_F)/(WC_F) (10 µM) | 2.5 µL |
| Reverse primer (WFL_R))/(WC_R) (10 µM) | 2.5 µL |
| Nuclease-free water | 19.0 µL |
| DNA template/ Plasmid extract (<0.5 µg/ 50 µL) | 1 µL |
| **Total reaction** | **50 µL** |

The amplification of full length *MamWRKY* was performed in genomic DNA and cDNA of *Musa acuminata* ssp. *malaccensis*. Meanwhile *MamWRKY* CDS was amplified by using pCAMBIA1304-MamWRKY plasmid as the DNA template. The PCR condition was as follows:

**Table 3.10:** PCR conditions to amplify full length cDNA and CDS of *MamWRKY.*

| **Stage** | **Condition** | **Number of cycle** |
|---|---|---|
| Initial denaturation | 98 °C for 30 seconds | 1 |
| Denaturation<br>Annealing<br>Extension | 94 °C for 10 seconds<br>58 ºC for 30 seconds<br>72 °C for 45 seconds | 35 |
| Final extension | 72 °C for 10 minutes | 1 |

The primer pairs used were:

**Table 3.11:** Sequences of primers used to amplify full length cDNA and CDS of *MamWRKY.*

| **Primer name** | **Primer sequence (5'→3')** |
|---|---|
| WFL_F | CCGGCGGAAATGTGATTTTAATACGATGGATGGG |
| WFL_R | CCGGCGGGCTGAAGGTAAAAATAAAGAACCTAAAG |
| WC_F | TCCAGATCTATGAACGGGAGCTGCAGCAA (*Bgl*II) |
| WC_R | AATACTAGTTCACCCAGCGGTCCCCACCCA (*Spe*I) |

All PCR products were electrophoresed on 1% (w/v) agarose gel at 120 V for 30 minutes. PCR products were excised, purified and sequenced as described in Section 3.9.1.

## 3.10 Sequence analysis and primer design

All primers were designed using Primer 3 software (http://bioinfo.ut.ee/primer3-0.4.0/). All sequences were analyzed through NCBI using Basic Local Alignment Search Tool (BLAST) software (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the alignment of the sequences was performed through ClustalW2 using Multiple Sequence Alignment software (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Location of genes on banana genome was determined by BLAST tool on Banana Genome Hub (http://banana-genome.cirad.fr/blast). The deduced amino acid, theoretical isoelectric point (*p*I) and molecular weight (MW) were identified in ExPASy (http://web.expasy.org/translate/). Phylogenetic tree of the predicted proteins and similar proteins from other plant species were carried out using MEGA6 software by neighboring joining method (Tamura et al., 2013).

## 3.11 Construction of binary vector

### 3.11.1 Digestion and ligation

#### 3.11.1.1 *NBS-type RGC* (*MAN-RF*)

Binary vector of pCAMBIA1304 and *MAN-RF* were double digested with restriction enzyme (RE), *Bgl*II and *Spe*I (Thermo Scientific, U.S.A) to form sticky end. The reaction mixture as follows:

**Table 3.12:** Digestion reaction mixture of pCAMBIA1304 and *MAN-RF*.

| Components | Volume |
|---|---|
| Purified plasmid pCAMBIA1304/ Purified PCR product (1 µg) | 10 µL |
| 10X Tango digestion buffer | 5 µL |
| RE *Bgl*II (10 U/µg) | 1 µL |
| RE *Spe*I (10 U/µg) | 1 µL |
| Nuclease-free water | 3 µL |
| **Total reaction** | **20 µL** |

The digestion reaction was incubated at 37 ºC for 4 hours. After electrophoresed on 1% (w/v) agarose gel at 120 V for 30 minutes, the DNA fragment was excised, purified and sequenced as described in Section 3.9.1. The sticky-ended cDNA and DNA plasmid were ligated together using DNA ligase (Thermo Scientific, U.S.A) as follows:

**Table 3.13:** Ligation reaction mixture of pCAMBIA1304 and *MAN-RF*.

| Volume / Components | DNA : vector | |
|---|---|---|
| | 1:1 **(B)** | 3:2 µL **(C)** |
| MAN-RF purified digestion product (0.3 µg/µL) | 3 µL | 6 µL |
| pCAMBIA1304 purified digestion product (0.9 µg/µL) | 3 µL | 4 µL |
| T4 DNA ligase (5 U/µL) | 1 µL | 1 µL |
| 10X T4 DNA buffer | 1 µL | 1 µL |
| Nuclease-free $H_2O$ (up to 20 µL) | 12 µL | 8 µL |
| **Total reaction** | **20 µL** | **20 µL** |

The ligation mixture was incubated at 22 ºC for 1 hour and the product was kept at -20 ºC.

### 3.11.1.2 WRKY transcription factor (*MamWRKY*)

Binary vector of pCAMBIA1304, full cDNA *MamWRKY* and CDS *MamWRKY* were double digested with restriction enzymes (RE), namely *Bgl*II and *Spe*I (Thermo Scientific, U.S.A), to form sticky end. The reaction mixture as follows:

**Table 3.14:** Digestion reaction mixture of pCAMBIA1304 and *MamWRKY*.

| **Components** | **Volume (*MamWRKY*)** | **Volume (pCAMBIA1304)** |
|---|---|---|
| Purified DNA (1 µg) | 4 µL | 5 µL |
| RE 1 (*Bgl*II) (10 U/µg) | 1 µL | 1 µL |
| RE 2 (*Spe*I) (10 U/µg) | 1 µL | 1 µL |
| 10X buffer (buffer 2) | 5 µL | 5 µL |
| BSA (1:10) | 0.5 µL | 0.5 µL |
| Nuclease-free water | 38.5 µL | 37.5 µL |
| **Total** | **50 µL** | **50 µL** |

The digestion reaction was incubated at 37 ºC for 3 hours. After electrophoresed on 1% (w/v) agarose gel at 120 V for 30 minutes, the DNA fragment was excised, purified and sequenced as described in Section 3.9.1. The sticky-ended cDNA and DNA plasmid were ligated together using DNA ligase (Thermo Scientific, U.S.A.) as follows:

**Table 3.15:** Ligation reaction mixture of pCAMBIA1304 and *MamWRKY*.

| **Components** | **Amount** |
|---|---|
| *MamWRKY* purified digestion product (0.2 µg/µL) | 5 µL |
| pCAMBIA1304 purified digestion product (0.5 µg/µL) | 7 µL |
| T4 DNA ligase (5 U/µL) | 2 µL |
| 10X T4 DNA ligase buffer | 2 µL |
| Nuclease-free water | 4 µL |
| **Total reaction** | **20 µL** |

The ligation mixture was incubated at 4 ºC for overnight and the product was kept at -20 ºC.

## 3.12 Preparation of competent cells

### 3.12.1 *Escherichia coli* (*E. coli*)

A colony of *E. coli* (JM 109 or TOP 10 strains) was inoculated in 10 mL of LB broth and incubated overnight at 37 °C under a shaking condition of 220 rpm. The $OD_{600}$ of the overnight culture was determined. After that, 500 µL of the culture was inoculated in 50 mL of LB broth in a 250 mL conical flask and incubated at 37 °C for 2-3 hours with a shaking condition of 220 rpm until $OD_{600}$ reached 0.25-0.35. The ready bacterial culture was transferred into a 50 mL Falcon tube and kept on ice for 15 minutes. The bacterial culture was then centrifuged at 1,503 x *g* for 10 minutes at 4 °C. The tube (containing bacteria pellet) was dried on paper towel for 1 minute before re-suspended in 30-40 mL of ice-cold 0.1 M $CaCl_2$ (Appendix B2). After 30 minutes, the mixture was centrifuged at 1,503 x *g* for 10 minutes at 4 °C. The pellet was re-suspended in 3 mL ice-cold 0.1 M $CaCl_2$. Each 1 mL of this re-suspended culture was used to prepare a glycerol stock with final concentration of 15% (v/v) total and stored at -80 °C.

### 3.12.2 *Agrobacterium tumefaciens* (*A. tumefaciens*)

*A. tumefaciens* (LBA4404 strain) from the glycerol stock was streaked on LB plate supplemented with 50 µg/mL rifampicin and incubated at 28 °C for 2-3 days. Single colony was selected and inoculated in 5 mL of LB broth supplemented with 50 µg/mL rifampicin. After incubated overnight at 28 °C with 200 rpm shaking condition, 2 mL *Agrobacterium* cultures were transferred to 50 mL of fresh LB broth (without antibiotic) and incubated at 28 °C with 200 rpm shaking condition until their $OD_{600}$ reached about 0.50. After centrifugation at 2,348 x *g* for 10 minutes at 4 °C, the pellet was resuspended in 20 mL ice-cold 0.1 M $CaCl_2$. This step was repeated once. The pellet was then resuspended in 500 µL ice-cold 10% glycerol. After that, each 100 µL of this

resuspended culture was used to prepare a glycerol stock with final concentration of 15% (v/v) total. The microcentrifuge tubes were dipped in liquid nitrogen for a minute before stored at -80 ℃.

### 3.13 Transformation for *E. coli* competent cells

Each plasmid construct harbouring *MAN-RF* (pCAMBIA1304-MAN-RF) or *MamWRKY* CDS gene (pCAMBIA1304-MamWRKY) was transformed into *E. coli* (JM109) or TOP 10 competent cell (Invitrogen, U.S.A) using heat shock method. An aliquot of 5 µL ligation product was mixed with 100 µL competent *E. coli* and put on ice for 30 minutes before incubated at 42 ºC for 30 seconds. After 2 minutes on ice, 250 µL of LB broth was added into the culture. The culture was incubated at 37 ºC with shaking condition at 220 rpm for 90 minutes. Total of 50 µL and 100 µL of cultures were separately spread onto LB agar supplemented with 50 µg/mL of kanamycin and incubated overnight at 37 ºC. PCR colony was carried out to identify the colony with recombinant plasmid using Phusion High-Fidelity PCR master mix. The PCR reaction mixture and cycle condition were same as described in Section 3.9.2.3 using NF and NR primer pair for *MAN-RF* and WC_F and WC_R primer pair for *MamWRKY* CDS gene. The plasmid from the positive result of PCR colony was isolated, purified and sequenced as describe in Section 3.6.

### 3.14 Transformation into *A. tumefaciens* competent cells

Plasmid extracts from the *E. coli* harbouring pCAMBIA1304-*MAN-RF* or pCAMBIA1304-*MamWRKY* were transformed into *A. tumefaciens* LBA4404 by freeze thaw method. An aliquot of 5 µL of plasmid extract was mixed with 100 µL competent

cell and kept on ice for 30 minutes. After frozen in liquid nitrogen for a minute, the mixture was incubated at 37 °C for 4 minutes before immediately chilled on ice for a minute. After adding 900 µL LB broth, the mixture was incubated at 28 °C with shaking condition at 200 rpm for 2 hours. An aliquot of 100 µL of the culture was spread on LB agar supplemented with 50 µg/mL of rifampicin and 50 µg/mL of kanamycin and incubated at 28 °C in the dark for 2-3 days. PCR colony was performed to identify positive colonies using Phusion High-Fidelity PCR master mix. The PCR reaction mixture and cycle condition were according to the procedures described in Section 3.9.2.3 using NF and NR primer pair for *MAN-RF* and WC_F and WC_R primer pair for *MamWRKY* CDS gene. The bacteria colonies with positive result were kept in a glycerol stock.

### 3.15 Plant transformation and regeneration of transgenic *Nicotiana tabacum*

Plant transformation was carried out according to Wong (2008). The glycerol stock of *A. tumefaciens* harbouring pCAMBIA1304-MAN-RF or pCAMBIA1304-MamWRKY was inoculated in 3 mL LB broth supplemented with 50 mg/L kanamycin and 50 mg/L rifampicin. The culture was incubated overnight at 28 ºC with an orbital shaking at 200 rpm. Once the $OD_{600nm}$ reached ~1.0, *A. tumefaciens* culture were pelleted by centrifugation at 2,348 x *g* for 10 minutes at 4 °C. The pellet of bacterial culture was re-suspended in 10 mL of co-culture medium (Appendix B5). The tobacco leaf discs of *Nicotiana tabacum* L. cv. SR1 were cut into 1 $cm^2$ and infected by *A. tumefaciens* suspension culture for 10 minutes with gentle shaking. After 3 days of co-cultivation, *Agrobacterium* culture was pipetted out and the leaf discs were blot dried on a sterile filter paper. The dried leaf discs were transferred onto tobacco shoot multiplication (TSM) medium (Appendix B5) and incubated at 25 ºC in the dark. After

3 days of incubation, the leaf discs were washed with MS liquid media supplemented with cefotaxime (250 mg/mL) for three times for 10 minutes each. The leaf discs for pCAMBIA1304-MAN-RF, pCAMBIA1304-MamWRKY and pCAMBIA1304 constructs were then transferred to TSM selection medium supplemented with 250 mg/L cefotaxime and 25 mg/L hygromycin. The leaf discs did not undergo transformation were cultured on TSM with and without antibiotics. All leaf discs were incubated under 16 hours light/8 hours dark photoperiod. The leaf discs were sub-cultured onto fresh media every week until new shoots were formed. The regenerated shoots were excised and transferred to tobacco root multiplication (TRM) media (Appendix B5) with or without antibiotics before transferring to soil. GUS staining was performed according to Hull and Devic (1995). Leaf was harvested from each plantlet was placed in a 1.5 microcentrifuge tube containing the GUS assay solution (Appendix B5) and incubated overnight at room temperature in the dark. The leaf was placed in 70% ethanol for overnight and then placed in FAA solution (Appendix B5) before visualized under a light microscope Leica DM750 (Leica Microsystems, Germany). Rooted plantlets were transferred to soil in pots and acclimatized in a containment room at MB1 Laboratory, University of Malaya.

### 3.16 Plant transformation and regeneration of transgenic *Musa acuminata* cv. 'Berangan'

*A. tumefaciens* transformation for *Musa acuminata* cv. 'Berangan' was carried out according to Wong (2008). The glycerol stock of *A. tumefaciens* harbouring pCAMBIA1304-MAN-RF was inoculated in 3 mL LB broth containing 50 µg/mL kanamycin and 50 µg/mL rifampicin. After incubated at 28 °C, 200 rpm, the ready *Agrobacterium* culture ($OD_{600nm}$=~0.5) was centrifuged at 2,348 x *g* for 10 minutes at 4 °C. The pellet was resuspended into 30 mL M2 media (Appendix B5) with 100 µM

acetosyringone (Sigma Aldrich, Malaysia). The embryogenic cell suspension cultures of *Musa accuminata* were sieved through 425 µM mesh and the settled cells were adjusted to a ratio of 1:5 using M2 media. An aliquot of 500 µL suspended cells were dispersed into 10 mL M2 media with 1 mL *Agrobacterium* culture and shook at 250 rpm in dark, at 28 °C for 30 minutes. All liquid media was removed and 10 mL fresh M2 media was added to the mixture with 100 µM acetosyringone. The cultures were co-cultivated for 4 days in dark at 80 rpm shaking. After liquid media was removed, 10 mL fresh M2 media containing 50 µg/mL cefotaxime was added. The mixture was maintained for 48 hours. After liquid media was removed, 10 mL M3 media (Appendix B5) without antibiotic was added. The mixture was kept in the dark at 80 rpm shaking condition. The media was changed at 10 day-intervals. The embryogenic cells were transferred to M4 media (Appendix B5) (MS medium containing 8 mg/L BAP and supplemented with 50 µg/mL cefotaxime and 200 µg/L hygromycin) and incubated in the dark for 4 weeks. The sub-culturing was carried out at 2 week-intervals. The cells that were observed with extended shoots appearance were transferred to M4 media with reduced BAP (4 mg/L to 2 mg/L) in a white cap bottle or jar under 16 hours light/8 hours dark photoperiod until shoot formation. The generated shoots were transferred to M5 media (Appendix B5) for rooting.

## 3.17 Molecular analysis of the transformed plantlets

### 3.17.1 PCR analysis

PCR was carried out to determine the integration of hygromycin phosphotransferase gene, (*hptII*) in the genomic DNA of transformed tobacco and banana. Genomic DNA was isolated from leaves of hygromycin resistant-plantlets using DNeasy plant mini kit (QIAGEN, Germany). The extracted DNA was amplified using primers (Table 3.16)

targeting hygromycin phosphotransferase coding sequence for pCAMBIA1304-MAN-RF and pCAMBIA1304-MamWRKY binary vector. DNA from the untransformed plantlet was used as negative control whereas the plasmid DNAs from pCAMBIA1304-MAN-RF and pCAMBIA1304-MamWRKY were used as positive control.

**Table 3.16:** Sequence of primers used to amplify *hptII* in the transformed plantlets.

| Primer name | Primer sequence (5'→3') |
|---|---|
| HPT_F | ATGCGGAGCATATACGCCCGG |
| HPT_R | TTATCGGCACTTTGCATCGGC |

The PCR reaction mixture and cycles condition were carried out using Phusion High-Fidelity PCR master mix (Thermo Scientific, U.S.A) as described in Section 3.9.2.3 with an annealing temperature of 60 ºC. All PCR products were electrophoresed on 1% (w/v) agarose gel at 120 V for 30 minutes. The PCR products were excised, purified and sequenced as described in Section 3.9.1.

### 3.17.2 Isolation of RNA and real-time quantitative PCR (qPCR) analysis

Total RNA was isolated from the young leaf of putative transgenic tobacco and banana and untransformed plantlets using CTAB method (as describe in Section 3.4) or RNaeasy plant mini kit (QIAGEN, Germany). The DNase treatment and first strand cDNA synthesize were performed as described in Section 3.8.

The primer pairs used were as follows:

**Table 3.17:** Primers sequence for qPCR in transformed plantlets.

| Primer name | Primer sequence (5'→3') |
|---|---|
| 2F-NBS | CTTGTACTGCAGCGTCTTCC |
| 2R-NBS | CCTCACCCTTCCGAAGCTAT |
| W2_F | CGAAGCCCGTACACTCTTT |
| W2_R | CTCCAGGTCCACGAATCAGG |
| Tac9_F | CCTGAGGTCCTTTTCCAACCA |
| Tac9_R | CTCCAGGTCCACGAATCAGG |
| EF_F | TGAGATGCACCACGAAGCTC |
| EF_R | CCAACATTGTCACCAGGAAGTG |
| U6_F | ACAGAGAAGATTAGCATGGCC |
| U6_R | GACCAATTCTCGATTTGTGCG |
| RP_F | TAGGGATTCCGACGATTTGTTT |
| RP_R | TAGCGTCATCATTGGCTGGGA |
| PR-1a_F | GCATCCTCCATTGTTACACTGAAC |
| PR-1a_R | GCTTCCCAATTGGCTGCAG |

The qPCR analysis in transgenic banana, *Musa acuminata* cv. 'Berangan' was done to study the expression of *MAN-RF* mRNA transcript. A specific primers pair, 2F-NBS and 2R-NBS were used to amplify the specific region of *MAN-RF*. The expression of *MAN-RF* was normalized with *U6* (Luo et al., 2014) and *RPS2* (Ribosomal Protein S2) gene as endogenous control. The specific primer pairs for *U6* are U6_F and U6_R and for RPS are RP_F and RP_R (Table 3.17).

The qPCR analysis in transgenic tobacco, *Nicotiana tabacum* L. cv. SR1 was done to study the expression of *MamWRKY* mRNA transcript using W2_F and W2_R primer pairs and PR-1a mRNA transcripts using PR-1a_F and PR-1a_R primer pairs. The expression of *MamWRKY* and *PR1a* were normalized with Actin (*Tac9*) (GenBank accession no.: X69885) and elongation factor 1α (*EF-1α*) (GenBank accession no.: AF120093) as endogenous control (Schmidt & Delaney, 2010). The specific primers for Actin (*Tac9*) are Tac9_F and Tac9_R and for EF-1α are EF_F and EF_R (Table 3.17).

**Table 3.18:** qPCR reaction mixture.

| **Components** | **Amount** |
|---|---|
| SYBR Green PCR Master Mix | 10 µL |
| Forward primer (100 µM) | 1 µL |
| Reverse primer (100 µM) | 1 µL |
| Nuclease-free water | 7 µL |
| cDNA template (30 ng/µL) | 1 µL |
| **Total reaction** | **20 µL** |

**Table 3.19:** qPCR cycles condition.

| **Stage** | **Condition** | **Number of cycle** |
|---|---|---|
| Hold stage | 95 °C for 10 minutes | 1 |
| Denaturation<br>Annealing | 95 °C for 15 seconds<br>60 ºC for 1 minutes | 40 |
| Melting curve | 95 °C for 15 seconds<br>60 ºC for 1 minutes<br>95 °C for 15 seconds (Dissociation) | 1 |

The specificity of the primers was verified using melting curve analysis. Only primer sets producing a single sequence-specific peak in the dissociation curve were selected. The threshold cycle (Ct) value of the primary amplification curve was used as quantification results. The relative expression level was analyzed using the $2^{-\Delta\Delta Ct}$ method with normalization of two reference genes (Livak & Schmittgen, 2001; Riedel et al., 2014; Yang et al., 2012) using the formula:

$$\Delta\Delta Ct = (\Delta Ct, \text{transformed plant} - \Delta Ct, \text{untransformed control plant})$$

$$\Delta Ct = (Ct, \text{target gene} - Ct, \text{reference gene}) \text{ transformed/ untransformed control plant}$$

$$\text{Extended } \Delta Ct: Ct, \text{reference gene} = \frac{(Ct, \text{reference gene 1} + Ct, \text{reference gene 2})}{2}$$

Data were represented as means and standard errors of three replicates.

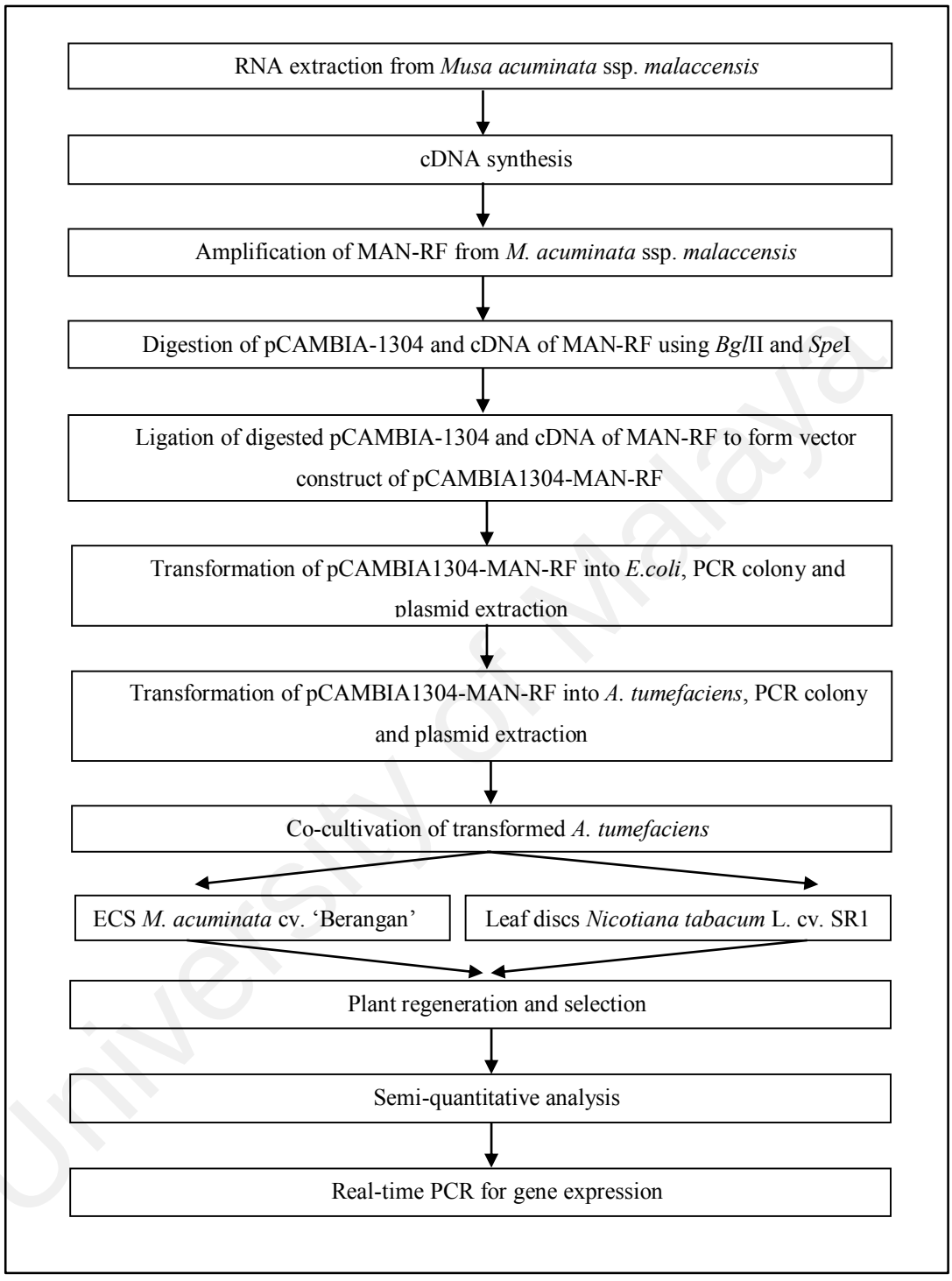



**Figure 3:2:** Workflow to develop transgenic plant expressing *MAN-RF* gene.

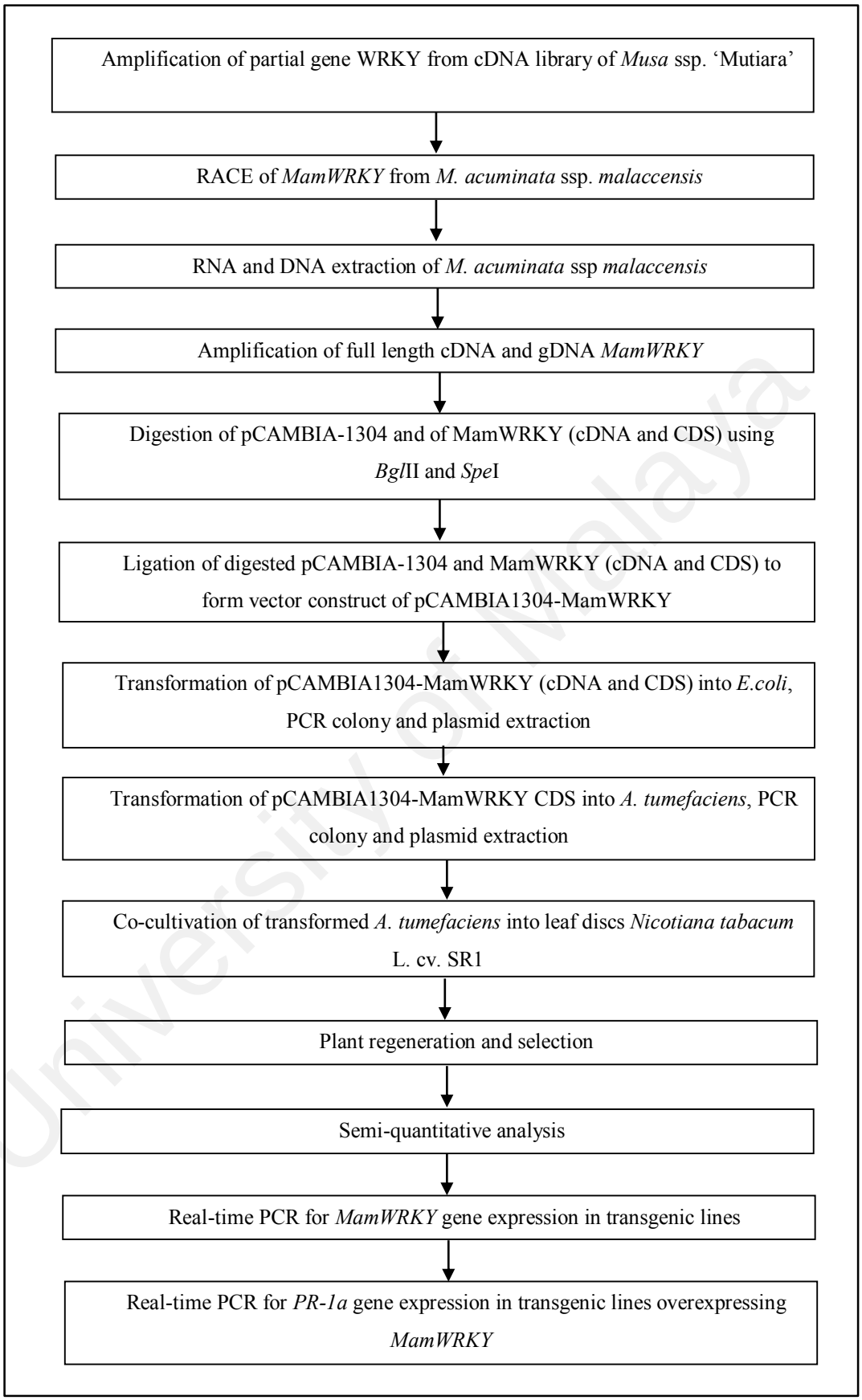



**Figure 3:3:** Workflow to develop transgenic plant expressing *MamWRKY* gene.

# CHAPTER 4: RESULTS

In this study, two genes (*MAN-RF* and *MamWRKY*) from *Musa acuminata* ssp. *malaccensis* were isolated and characterized prior to develop transgenic bananas.

## 4.1 Analyses of *MAN-RF* (NBS-type of resistance gene from *Musa acuminata* ssp. *malaccensis*)

### 4.1.1 Full sequence amplification

The RNA from young leaf of *Musa acuminata* ssp. *malaccensis* was isolated and served as a template for *MAN-RF* gene amplification. The RNA product was purified and separated by electrophoresis to confirm the presence of 26S and 18S RNAs (Figure 4.1).

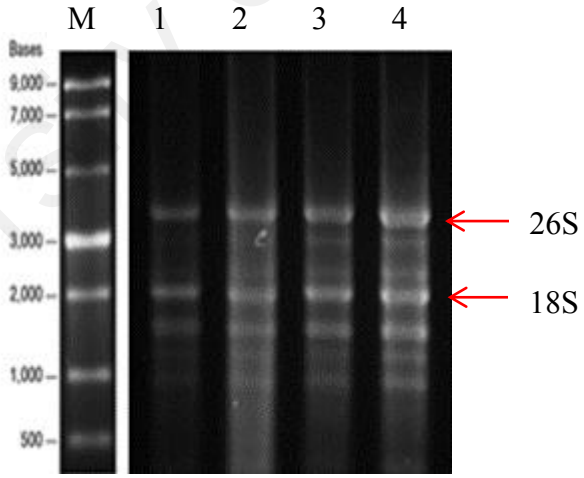



**Figure 4:1:** RNA extracts from young leaves *Musa acuminata* ssp. *malaccensis* after DNase treatment for *MAN-RF* gene isolation.

M : Marker; RNA ladder (NEB)
1-4 : DNase-treated RNA

The cDNA synthesized from total RNA was used as PCR template to isolate *MAN-RF* gene using gene specific primers, NF and NR. The cDNA of *MAN-RF* with a total size of 1,160 bp (Figure 4.2) was purified and cloned into a TOPO PCR cloning vector (Thermo Fisher Scientific, U. S. A) prior to sequencing using NF and NR primers pair. After BLAST analysis, 99% of the *MAN-RF* nucleotide sequence was found identical to *Musa acuminata* ssp. *malaccensis* with a coverage of 99% from the study carried out by Dumin (2007) (Figure 4.3). The conserved NBS domain motif of MAN-RF protein consisted of main motifs of p-loop, kinase-2 and kinase-3 (Figure 4.4). By referring to Banana Genome Hub, *MAN-RF* gene is located on chromosome 8 (Figure 4.5) with 100% of protein sequences (311 amino acids) matched putative disease resistance protein RPM1 of *Arabidopsis thaliana*.

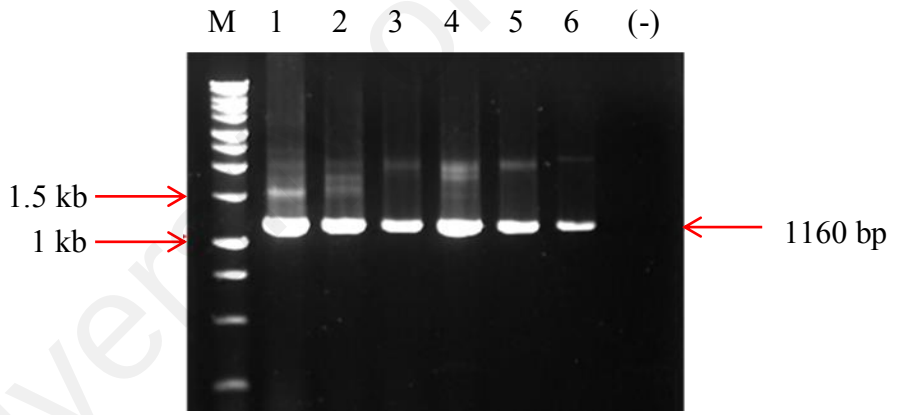
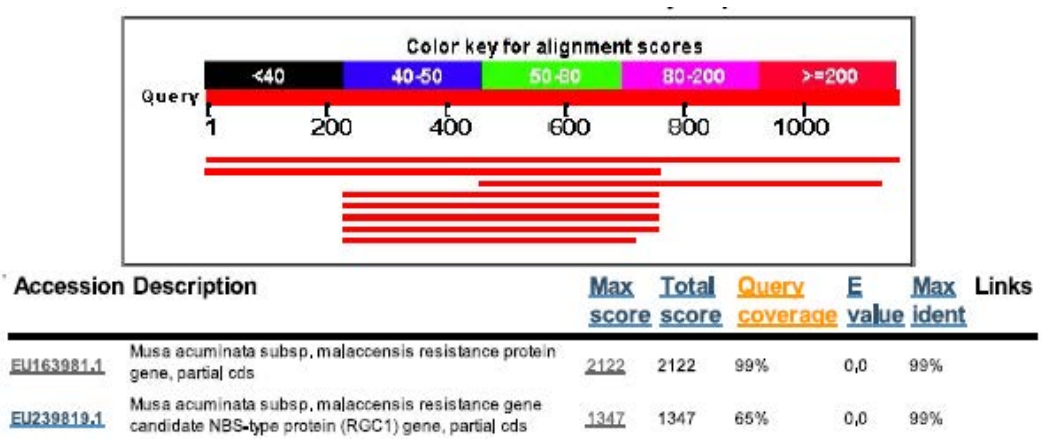



**Figure 4:2:** PCR screening of *MAN-RF* gene in *Musa acuminata* ssp. *malaccensis*.

M : Marker; 1 kb DNA Ladder (Fermentas)
1-6 : cDNA of *MAN-RF*
(-) : Negative control (PCR mixture without the DNA template)




| Accession | Description | Max score | Total score | Query coverage | E value | Max ident | Links |
|---|---|---|---|---|---|---|---|
| EU163981.1 | Musa acuminata subsp. malaccensis resistance protein gene, partial cds | 2122 | 2122 | 99% | 0.0 | 99% | |
| EU239819.1 | Musa acuminata subsp. malaccensis resistance gene candidate NBS-type protein (RGC1) gene, partial cds | 1347 | 1347 | 65% | 0.0 | 99% | |

**Figure 4:3:** BLAST analysis of *MAN-RF* with original nucleotide sequences from previous study.

### 4.1.2 Digestion and ligation of pCAMBIA1304 and *MAN-RF* fragment

The pCAMBIA1304 and *MAN-RF* digested with *Bgl*II and *Spe*I enzymes produced a single fragment size of ~12,000 bp and ~1,160 bp, respectively (Figure 4.6). These digested products were ligated with DNA ligase before cloned into *E. coli* to generate pCAMBIA1304 containing *MAN-RF*, kanamycin and hygromycin antibiotic genes (pCAMBIA1304-MAN-RF). The expression of *MAN-RF* was driven by CaMV35S (cauliflower mosaic virus) promoter (Figure 4.7).

### 4.1.3 Transformation of the constructed plasmid into *E. coli* competent cells

The pCAMBIA1304-MAN-RF was transformed into *E. coli* TOP 10 competent cells. The positive colonies were PCR amplified using NF and NR primer pairs to confirm the presence of pCAMBIA1304-MAN-RF. The extracted plasmids are shown in Figure 4.8. The $OD_{260/280}$ reading of plasmid extract was in the range of 1.90-2.20. As shown in Figure 4.9, the expected fragment size of 1,160 bp was observed among 12 randomly selected colonies. These colonies were cultured before plasmid extraction.

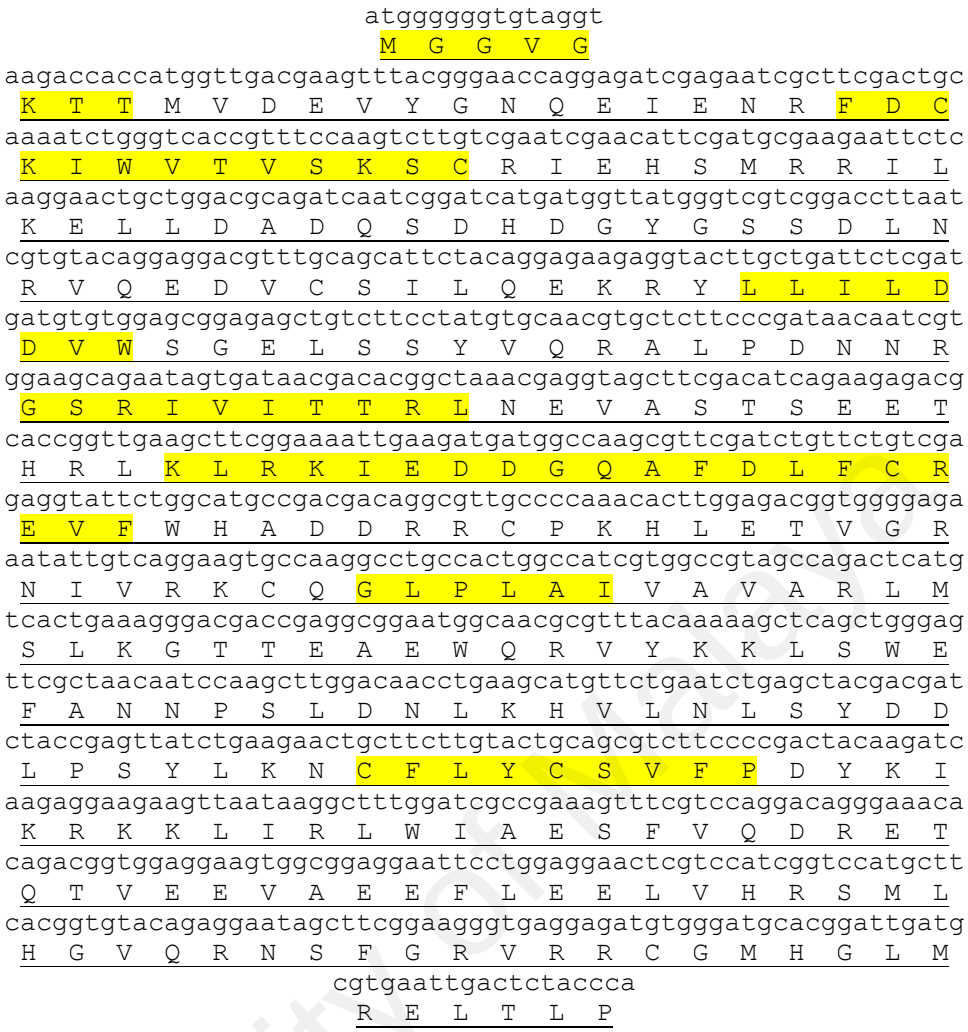



**Figure 4:4:** Translated amino acid of *MAN-RF*. The highlighted yellow texts are NBS domain.

The NBS domain are GMGGVGKTT (kinase-1/p-loop), FDCKIWVTVSKSC (RNBS-A), LLILDDVW (kinase-2), GSRIVITTRL (kinase-3a), KLRKIEDDGQAFDLFCREVF (RNBS-C), GLPLAI (kinase-3), CFLYCSVFPD (RNBS-D).

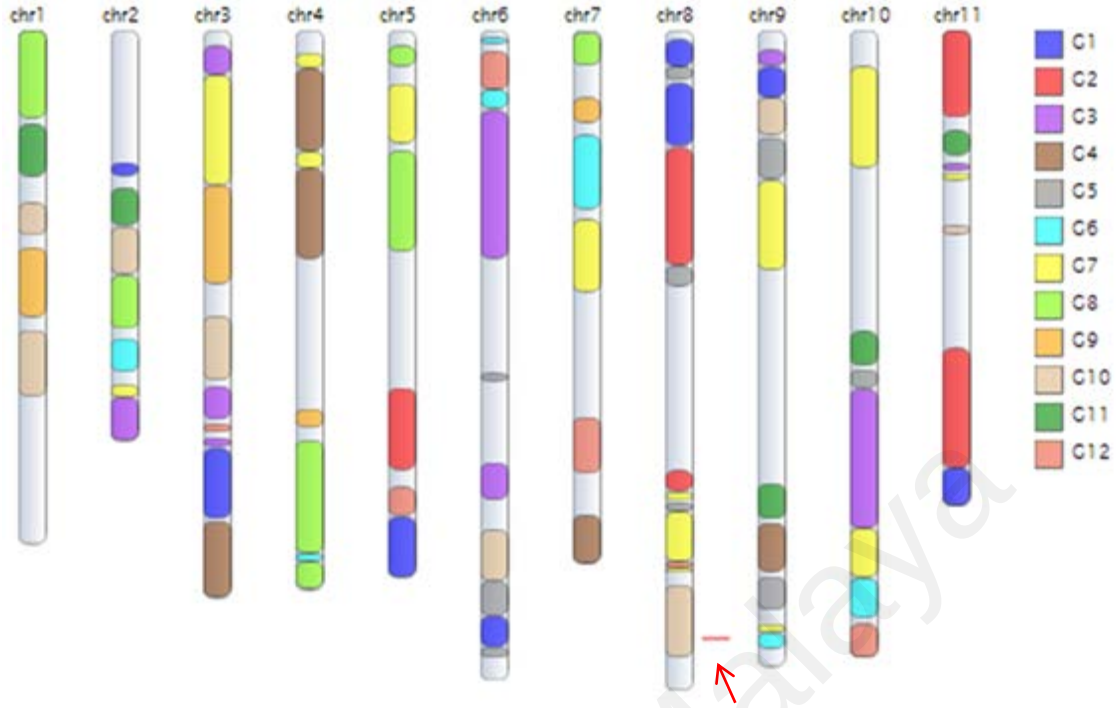



**Figure 4:5:** Banana genome chromosomes showed the location of *MAN-RF* at chromosome 8.

The similarity was 100% matched a putative disease resistance protein RPM1, a gene which confer resistance to *Pseudomonas syringae* in *Arabidopsis thaliana.*

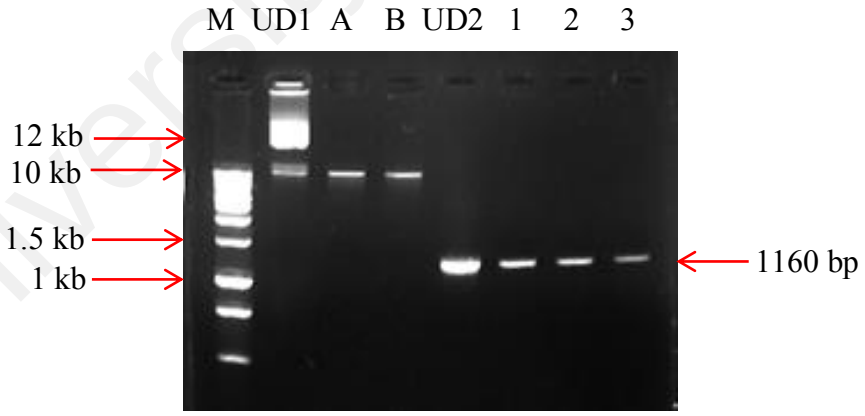



**Figure 4:6:** The purified product of digested pCAMBIA-1304 and MAN-RF fragments using same restriction enzyme, *Bgl*II and *Spe*I.

M : Marker- 1 kb DNA Ladder (Fermentas)
UD1 : Undigested pCAMBIA1304 plasmid
A & B : Linearized pCAMBIA1304 with *Bgl*II and *Spe*I
UD2 : Undigested MAN-RF
1-3 : Digested MAN-RF with *Bgl*II and *Spe*I

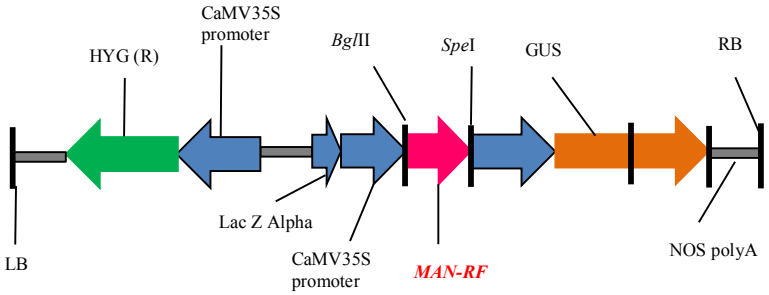



**Figure 4:7:** Schematic representation of the T-DNA region of binary vector pCAMBIA1304-MAN-RF.

The T-DNA region consist of LB (T-DNA left border); HYG(R) (Hygromycin selectable marker); CaMV35S (cauliflower mosaic virus promoter); *Bgl*II and *Spe*I: restriction sites; *MAN-RF* (*NBS-type RGC* gene); GUS (ß-glucuronidase); NOS (Nopaline synthase terminator); RB (T-DNA right border).

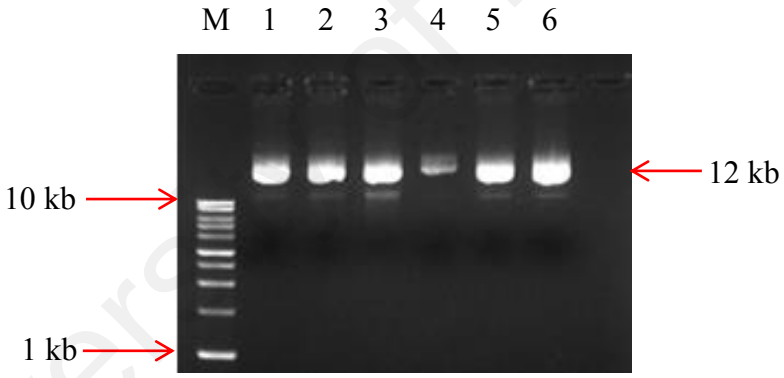



**Figure 4:8:** Plasmid of pCAMBIA1304-MAN-RF construct from different positive colonies of *E. coli*.

M : Marker- 1 kb DNA Ladder (Fermentas)
1-6 : Plasmid pCAMBIA1304-MAN-RF

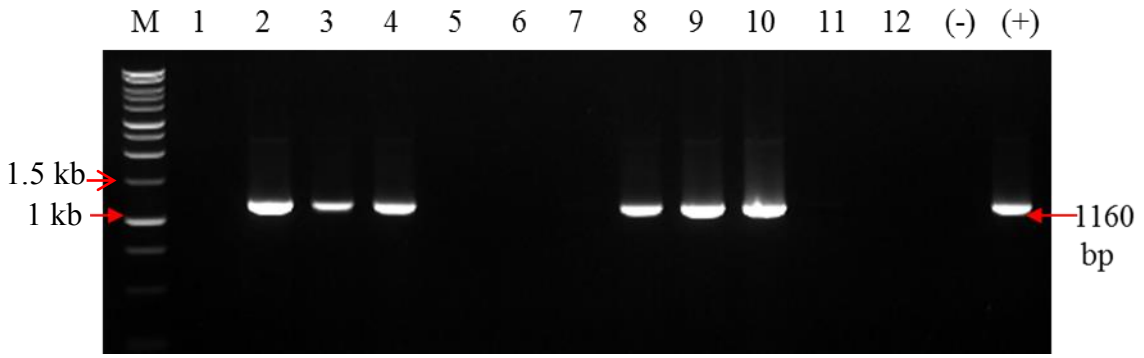



**Figure 4:9:** PCR amplification of *MAN-RF* from *E. coli* colonies with NF and NR primers.

| | |
|---|---|
| M | : Marker- 1 kb DNA Ladder (Fermentas) |
| 1-12 | : DNA fragments of *MAN-RF* from different *E. coli* colonies |
| (-) | : Negative control (PCR mixture without the DNA template) |
| (+) | : Positive control (plasmid extract of pCAMBIA1304 harbouring *MAN-RF* as DNA template) |

### 4.1.4 Transformation of the constructed plasmid into *A. tumefaciens*

The constructed plasmid from Section 4.1.3 was transformed into *A. tumefaciens* LBA4404 strain. The colonies survived on LB kanamycin and rifampicin resistant agar were selected for colony PCR using NF and NR primers. As shown in Figure 4.10, the expected fragment size of 1,160 bp was observed among 11 randomly selected colonies. The pCAMBIA1304 plasmid without *MAN-RF* insert was also transformed into *A. tumefaciens* LBA4404 as a positive control in the plant transformation.

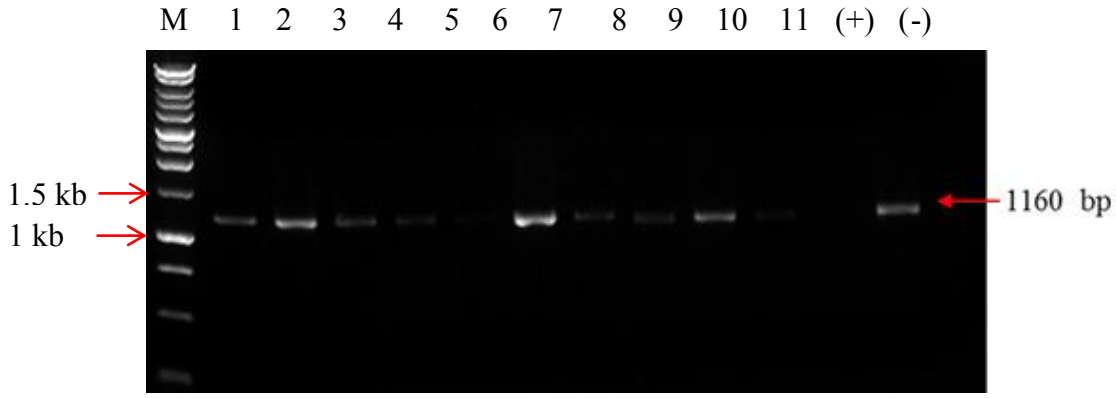



**Figure 4:10:** PCR amplification of *A. tumefaciens* colonies with NF and NR primer pairs.

M : Marker- 1 kb DNA Ladder (Fermentas)
1-11 : Colony PCR of pCAMBIA1304-MAN-RF
(+) : Positive control (plasmid extract of pCAMBIA1304 harbouring *MAN-RF* as DNA template)
(-) : Negative control (without DNA template)

### 4.1.5 Regeneration of transgenic tobacco *Nicotiana tabacum* L. cv. SR1

Leaf discs of 3 months old *Nicotiana tabacum* L. cv. SR1 transformed with *A. tumefaciens* harbouring pCAMBIA1304 and pCAMBIA1304-MAN-RF vectors were grown on hygromycin (25 mg/L) selection media. Leaf discs transformed with pCAMBIA1304 generated shoots when cultured on TSM media, whereas slow growth of new shoots was observed on leaf discs transformed with pCAMBIA1304-MAN-RF (Figure 4.11). Untransformed leaf discs showed necrosis on hygromycin selection media. Despite low transformation rate, few putative transgenic plants were regenerated during multiplication stage.


**Figure 4:11:** Regeneration of *Nicotiana tabacum* L. cv. SR1 plants.

The regenerated (a) Shoot proliferation from leaf disc infected with *A. tumefaciens* harbouring pCAMBIA1304-*MAN-RF*; (b) Shoot proliferation from leaf disc infected with *A. tumefaciens* harbouring pCAMBIA1304; (c) Plant regeneration from untransformed leaf disc; (d) Putative transgenic plantlets on height measurement; 1-3: Plantlets harbouring pCAMBIA1304; 4-6: Plantlets harbouring pCAMBIA1304-MAN-RF. *Bar* = 2 cm.

### 4.1.6 Regeneration of transgenic banana *Musa acuminata* cv. 'Berangan'

To establish transgenic bananas, the developed embryogenic cell suspension cultures of *Musa acuminata* cv. 'Berangan' were transformed with *A. tumefaciens* harbouring pCAMBIA-1304 and pCAMBIA1304-MAN-RF. The transformants were then grown in culture medium containing hygromycin (200 µg/L). Shoots generated from whitish embryos on M4 media containing BAP were cultured on rooting media for root initiation (Figure 4.12). Untransformed embryogenic cells were found to become necrosis on hygromycin selection media, whereas positive control showed shoot and root regeneration on media without hygromycin. However, the numbers of putative transgenic cells were decreased during maintenance and multiplication, mainly due to bacterial contamination.

### 4.1.7 Molecular characterization of transformed plants

The semi-quantitative PCR screening for the transformed bananas was successfully carried out using primers targeting *MAN-RF* and *hpt* genes. The PCR products from 4 randomly selected putative transgenic banana lines showed 1,160 bp (Figure 4.13) and 618 bp fragments (Figure 4.14) for the *MAN-RF* and *hpt* genes, respectively.

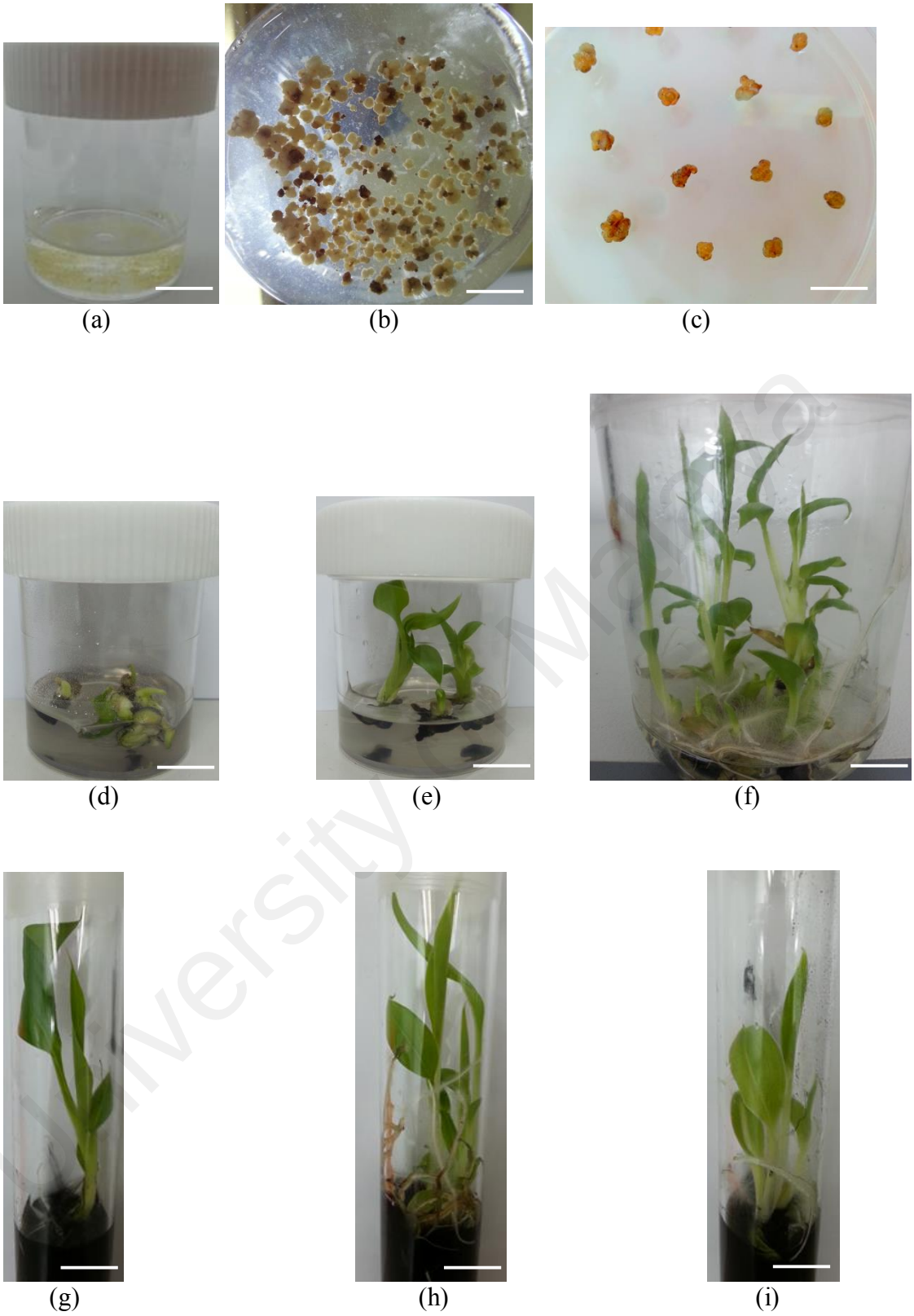



**Figure 4:12:** Regeneration of transgenic *Musa acuminata* cv. 'Berangan' plants overexpressing *MAN-RF*.

The regenerated (a) Transformed embryogenic cells in M2 liquid media; (b) Transformed embryogenic cells in M3 liquid media; (c) Transformed embryogenic cells in M4 solid media with 8 mg/L of BAP; (d) Shoot proliferation on M4 solid media with

4 mg/L of BAP; (e)-(f) Shoot proliferation on M4 solid media with 2 mg/L of BAP concentration; (g)-(i) Root proliferation of single plantlet on M5 media. *Bar* = 1.5 cm.

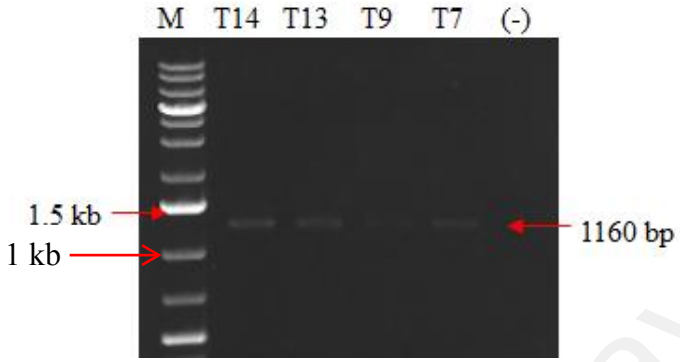



**Figure 4:13:** Genomic DNA PCR analysis of the putative transgenic banana lines (T7, T9, T13 and T14) showed *MAN-RF* fragment with 1,160 bp length.

| | |
|---|---|
| M | : Marker- 1 kb DNA Plus DNA Ladder (Fermentas) |
| T14, T13, T9, T7 | : Putative transgenic banana lines with *MAN-RF* insert |
| (-) | : Negative control (without DNA template) |

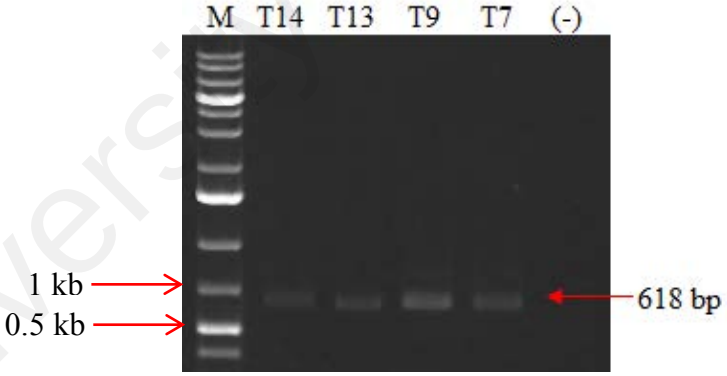
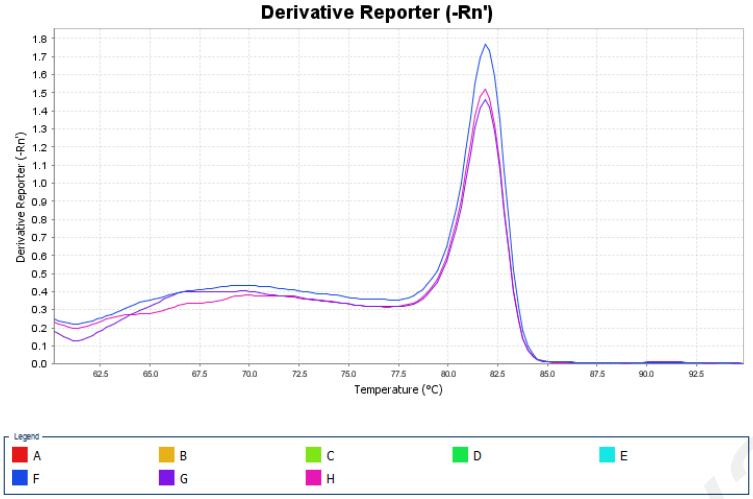



**Figure 4:14:** Genomic DNA PCR analysis of the putative transgenic banana lines (T7, T9, T13 and T14) showed *hpt* fragment at 618 bp length.

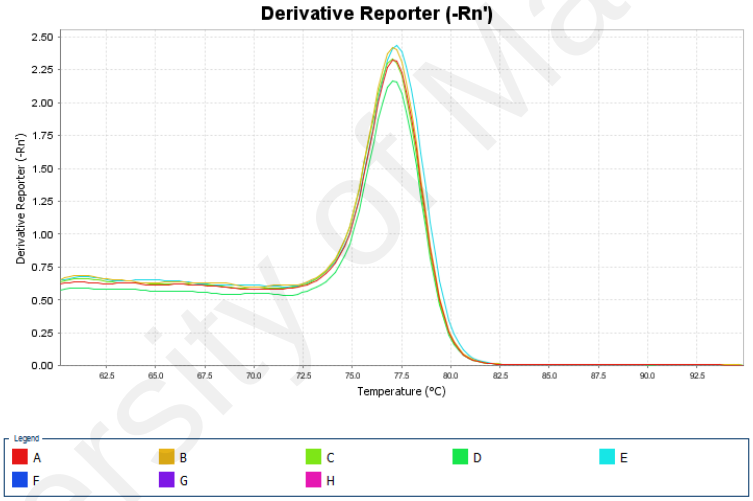
| | |
|---|---|
| M | : Marker- 1 kb DNA Plus DNA Ladder (Fermentas) |
| T14, T13, T9, T7 | : Putative transgenic banana lines with *MAN-RF* insert |
| (-) | : Negative control (without DNA template) |

### 4.1.8 Expression profiles of *MAN-RF* gene in transgenic *Musa acuminata* cv. 'Berangan'

qPCR analysis was carried out to analyze the expression of *MAN-RF* in the transgenic lines with two reference genes, *U6* and *RPS2*. The amplification of *U6* and *RPS2* using transgenic (Figure 4.15) and untransformed plants (Figure 4.16) produced a single peak in melting curve, confirming the specificity of the primers. The primer pair for *MAN-RF* also showed a single peak in melting curve for both transgenic and untransformed plants (Figure 4.17). The analysis of *MAN-RF* expression level using $2^{-\Delta\Delta Ct}$ method in T7, T13 and T14 transgenic lines resulted in 2.47, 3.29 and 4.69-fold changes, respectively, compared to the untransformed plant (Table 4.1 and Figure 4.18).

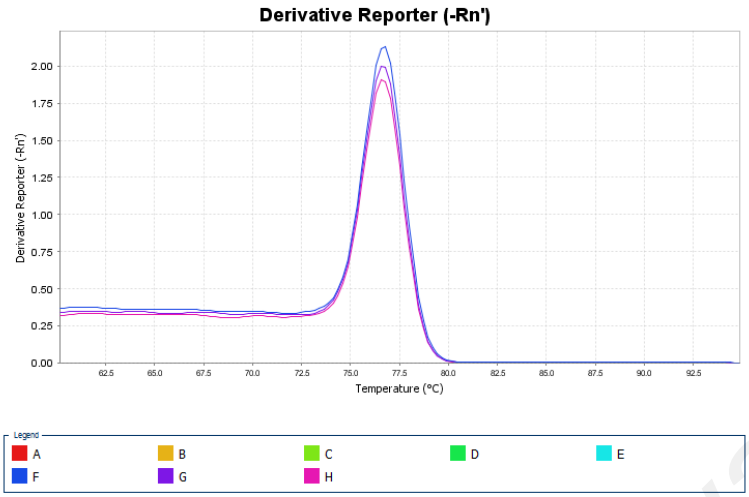



(a)

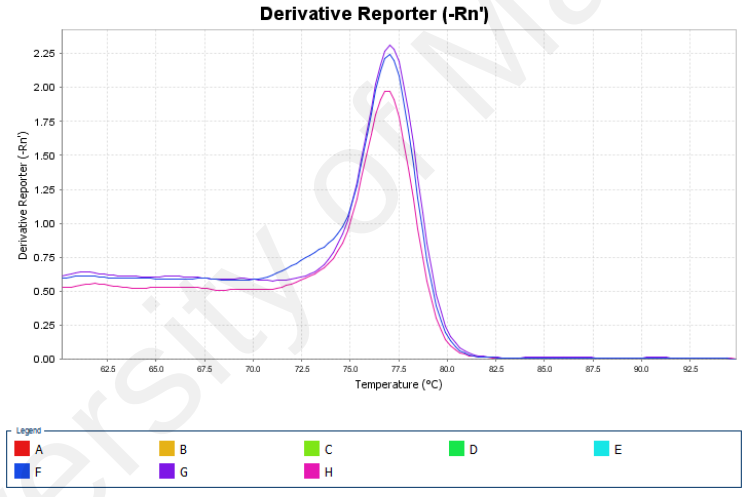



(b)

**Figure 4:15:** Melting curve of housekeeping gene primer in transgenic samples *Musa acuminata* cv. 'Berangan' (a) *RPS2*; (b) *U6*.

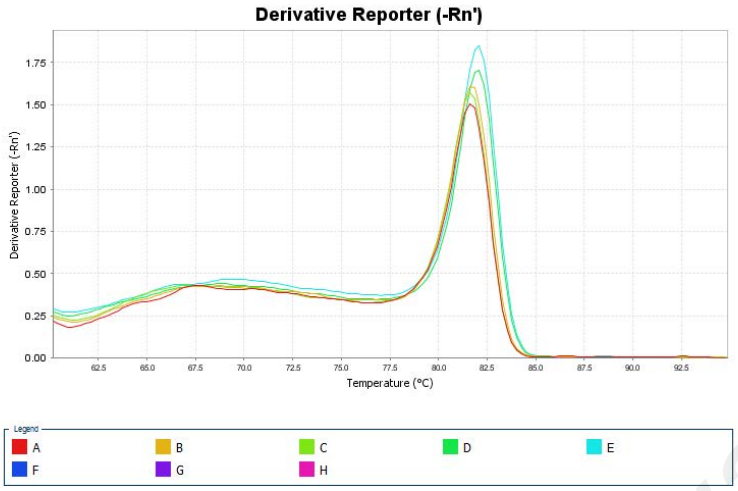



(a)

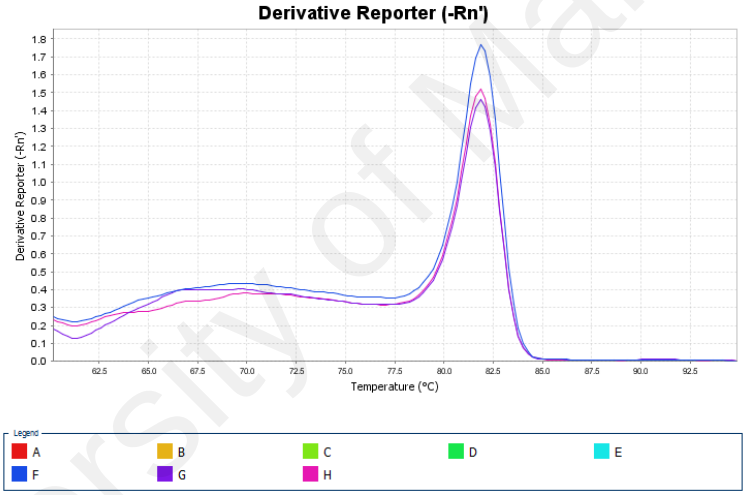



(b)

**Figure 4:16:** Melting curve of housekeeping gene primer in untransformed *Musa acuminata* cv. 'Berangan' (a) *RPS2*; (b) *U6*.




(a)




(b)

**Figure 4:17:** Melting curve of gene specific primer, 2F-NBS and 2R-NBS in (a) transgenic and (b) untransformed *Musa acuminata* cv. 'Berangan'.

**Table 4.1:** qPCR analysis of *MAN-RF* gene in transgenic *Musa acuminata* cv. 'Berangan'.

| | CT | | | | | | | Standard deviation (SD) | | | | SD DIFF | SE OF DIFF |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Samples** | ***MAN-RF*** | **Average *MAN-RF*** | ***RPS2*** | **Average *RPS2*** | ***U6*** | **Average *U6*** | **Average *RPS2* & *U6*** | **SD *MAN-RF*** | **SD *RPS2*** | **SD *U6*** | **Average SD *RPS2* & *U6*** | | |
| $T7_1$ | 31.358 | | 21.994 | | 20.512 | | | | | | | | |
| $T7_2$ | 31.945 | | 22.000 | | 22.130 | | | | | | | | |
| $T7_3$ | 30.691 | 31.331 | 21.093 | 21.696 | 21.296 | 21.313 | 21.505 | 0.627 | 0.522 | 0.809 | 0.666 | 0.915 | 0.528 |
| $T13_1$ | 30.772 | | 21.299 | | 21.702 | | | | | | | | |
| $T13_2$ | 30.527 | | 20.900 | | 21.117 | | | | | | | | |
| $T13_3$ | 30.172 | 30.490 | 20.369 | 20.856 | 21.064 | 21.294 | 21.075 | 0.302 | 0.467 | 0.354 | 0.410 | 0.509 | 0.294 |
| $T14_1$ | 30.498 | | 22.956 | | 20.756 | | | | | | | | |
| $T14_2$ | 29.778 | | 21.562 | | 20.170 | | | | | | | | |
| $T14_3$ | 30.206 | 30.161 | 21.874 | 22.131 | 20.223 | 20.383 | 20.257 | 0.362 | 0.732 | 0.324 | 0.528 | 0.640 | 0.370 |
| $UTC_1$ | 32.694 | | 21.210 | | 21.440 | | | | | | | | |
| $UTC_2$ | 31.844 | | 20.884 | | 21.209 | | | | | | | | |
| $UTC_3$ | 31.981 | 32.173 | 20.467 | 20.854 | 21.026 | 21.225 | 21.040 | 0.456 | 0.372 | 0.207 | 0.290 | 0.541 | 0.312 |

**Table 4.1,** continued.

| **Samples** | **ΔCT** | **ΔΔCT** | **$2^{\wedge}$-(ΔΔCT)** |
|---|---|---|---|
| $T7_1$ | | | |
| $T7_2$ | | | |
| $T7_3$ | 9.826 | -1.307 | 2.474 |
| $T13_1$ | | | |
| $T13_2$ | | | |
| $T13_3$ | 9.415 | -1.718 | 3.290 |
| $T14_1$ | | | |
| $T14_2$ | | | |
| $T14_3$ | 8.904 | -2.229 | 4.688 |
| $UTC_1$ | | | |
| $UTC_2$ | | | |
| $UTC_3$ | 11.133 | 0 | 1.000 |

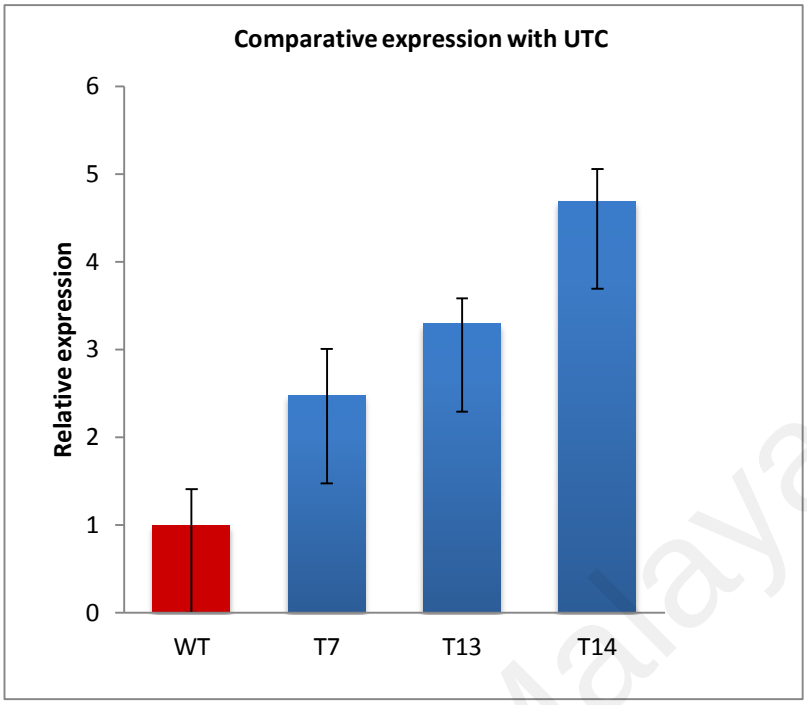



**Figure 4:18:** Gene expression level of *MAN-RF* in transgenic lines T7, T13 and T14 compared to untransformed control of *Musa acuminata* cv. 'Berangan' (UTC).

## 4.2 Analysis of *MamWRKY* (WRKY transcription factor from *Musa acuminata* ssp. *malaccensis*)

### 4.2.1 Partial gene isolation and sequence analysis

The partial cDNA sequence of *WRKY* obtained from cDNA library of *M. acuminata* cv. 'Mutiara' was 1.4 kb in size (Figure 4.19). The nucleotide sequence of partial cDNA *WRKY* is attached in Appendix C2a and C2b. The partial cDNA sequence was 80% identical with *Oryza sativa* (Japonica cultivar-group) WRKY13 mRNA, complete sequence (AY870602.1) with a coverage of 28%. This partial cDNA sequence was used as reference to isolate full length sequence of *MamWRKY*.

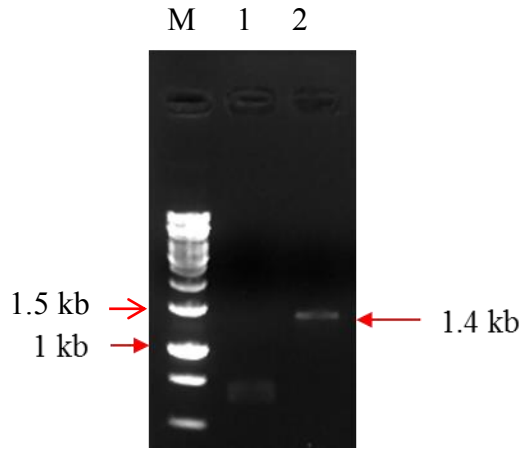



**Figure 4:19:** PCR amplification of partial cDNA sequence with T7 and T3 primers pair.

M : Marker- 1 kb DNA Ladder (Fermentas)
1 : Band from other gene (not in this study)
2 : Partial cDNA sequence of *WRKY*

### 4.2.2 Full length gene amplification

#### 4.2.2.1 RACE and sequence analysis

In order to amplify the 5' and 3' end sequence of *MamWRKY*, RNA was isolated from young leaf of *Musa acuminata* ssp. *malaccensis*. The RNA was further purified with DNase I kit to remove the genomic DNA contamination. Two rRNA fragments, 26S and 18S were observed (Figure 4.20). The RNA extract exhibited $OD_{260/280}$ with a range of 1.90 to 2.10.

The purified RNA was amplified using RACE. The 5'- and 3'- RACE produced two fragments of 298 bp and 324 bp, respectively (Figures 4.21 and 4.22). The sequencing result of 5' inner RACE was attached in Appendix C3a and C3b. The sequencing result of 3' inner RACE was attached in Appendix C4.

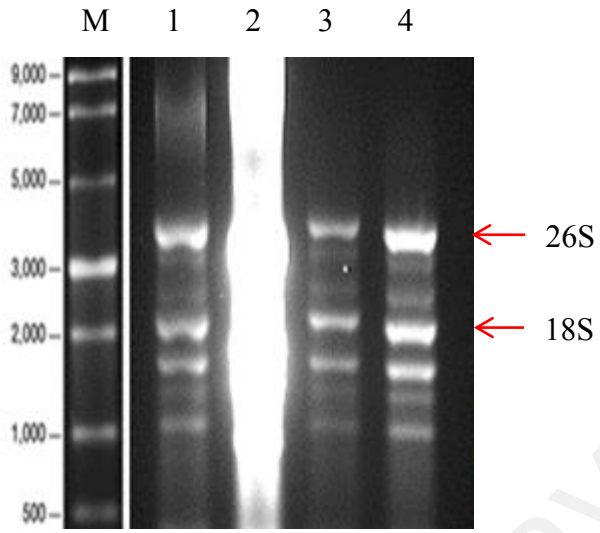



**Figure 4:20:** Total RNA extracted from leaf sample of *Musa acuminata* ssp. *malaccensis* for RACE experiment.

M : Marker; RNA ladder (NEB)
1-2 : Total RNA before DNase treatment
3-4 : Total RNA after DNase treatment

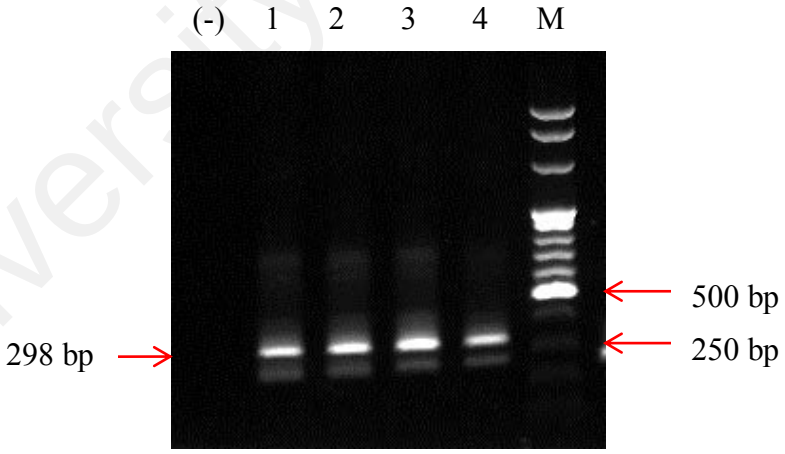



**Figure 4:21:** PCR amplification of 5' inner RACE of *MamWRKY* in cDNA of *Musa acuminata* ssp. *malaccensis.*

(-) : Negative control (PCR mixture without the DNA template)
1 - 4 : 5' inner RACE product (298bp)
M : Marker- 1 kb DNA Ladder (Fermentas)

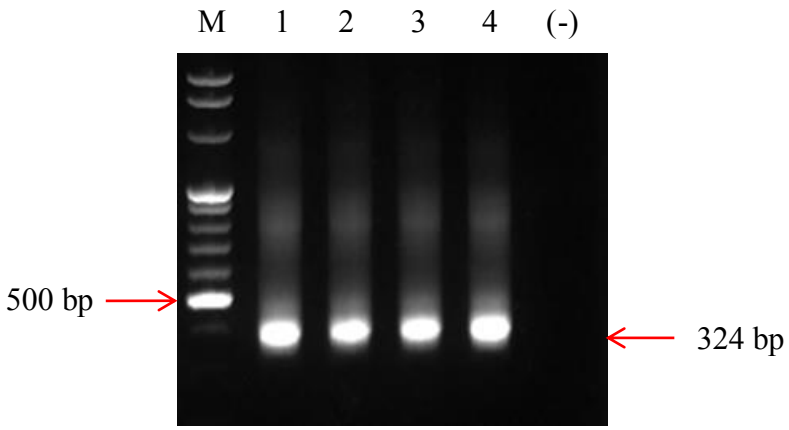



**Figure 4:22:** PCR amplificationof 3' inner RACE of MamWRKY in cDNA of *Musa acuminata* ssp. *malaccensis.*

M : Marker 1 kb DNA Ladder (Fermentas)
1 - 4 : 3' inner RACE product (324bp)
(-) : Negative control (PCR mixture without the DNA template)

#### 4.2.2.2 Full length gene amplification and sequence analysis

The full length cDNA was amplified from 3'- and 5'- end RACE template and generated 1,224 bp in length (Figure 4.23, Appendix C5a and Appendix 5b), whereas the amplification of genomic DNA (gDNA) generated 1,414 bp in length (Figure 4.23, Appendix C6a and Appendix C6b).

Comparative analysis of the genomic DNA and the isolated cDNA sequences indicated the presence of two introns, 98 bp and 92 bp in *MamWRKY* (Figure 4.24). The cDNA of *MamWRKY* consisted of 861 nucleotides, encoding a putative protein of 286 amino acids (Figure 4.25) with a predicted molecular mass of 30.16 kDa and a theoretical *pI* of 5.05. Domain in *MamWRKY* shared identity with probable *WRKY65* from *M. acuminata* ssp. *malaccensis* (99%) (Figure 4.26). The *MamWRKY* gene was found at different locus in chromosomes 3, 4, 5, 7, 8, 9, 10 and 11 but it showed the highest percentage of identity, 99%, with putative *WRKY69* (1,223 bp coverage) in chromosome 4, spanning from 11,741,594 to 11,742,644 bp (Figure 4.27).

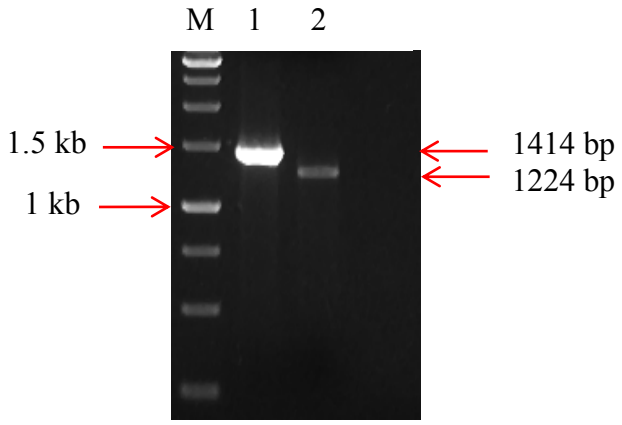



**Figure 4:23:** Full length *MamWRKY* from gDNA and cDNA of *Musa acuminata* ssp. *malaccensis*.

M : Marker 1 kb DNA Ladder (Fermentas)
1 : *MamWRKY* from gDNA
2 : *MamWRKY* from cDNA

MamWRKY protein is classified as Group IIe based on the single WRKY domain and the zinc finger motif (CSSSKGCPARKQVERSRVDPNVIVVTYAFDHNH). It has same conserved sequence of 60 amino acids, including WRKYGQK at the N-terminus, and a novel zinc-finger motif, either $Cx_{4-5}Cx_{22-23}HxH$ or $Cx_7Cx_{23}HxC$ with the AtWRKY65 (accession no. AT1G29280.1) and AtWRKY69 (AT3G58710.1) from *Arabidopsis thaliana*, TaRKY13 (ABO15543.1) from *Triticum aestivum*, StWRKY5 (NP_001274847.1) from *Solanum tuberosum*, GmWRKY52 (NP_001237726.1) from *Glycine max*, GhWRKY30 (AGV75938.1) from *Gossypium hirsutum*, PtWRKY65 (XP_002317397.1) from *Populus trichocarpa*, OsWRKY (BAB56055.1) from *Oryza sativa* Japonica Group, and ZmWRKY14 (ACG45823.1) from *Zea mays* (Figure 4.28). The phylogenetic analysis indicated that MamWRKY, TaWRKY13 (ABO15543.1) from *Triticum aestivum*, and OsWRKY (BAB56055.1) from *Oryza sativa* Japonica were classified in the same cluster (Figure 4.29).

```
gDNA    GAAAATGTGATTTTAATACGATGGATGGGTGCGTGCTATGAGTTTTAAAACCGCCTCCGT 60
cDNA    GAAAATGTGATTTTAATACGATGGATGGGTGCGTGCTATGAGTTTTAAAACCGCCTCCGT 60
        ************************************************************

gDNA    CTGTCCCCATTTGCACCCCCCACCAAACACCCTATGCCTCCCTCTCTCTCTTCTTCCTCT 120
cDNA    CTGTCCCCATTTGCACCCCCCACCAAACACCCTATGCCTCCCTCTCTCTCTTCTTCCTCT 120
        ************************************************************

gDNA    TCCTCTTCCTCTTCTAGTATTGTACAAAGCTTCTTCTTGTACGGTCGGAGGACGTGAGCG 180
cDNA    TCCTCTTCCTCTTCTAGTATTGTACAAAGCTTCTTCTTGTACGGTCGGAGGACGTGAGCG 180
        ************************************************************

gDNA    AGTGCGTGCTCTGGATCAAGGATCGAGAAGACAATTGGTAGAGAAAACTATATTGCTTCT 240
cDNA    AGTGCGTGCTCTGGATCAAGGATCGAGAAGACAATTGGTAGAGAAAACTATCTTGCTTCT 240
        ************************************************* ********

gDNA    TCTCGGATTGTGATGAACGGGAGCTGCAGCAACGAGCTCGACGCATGCGAGACCGAGGAG 300
cDNA    TCTCGGATTGTGATGAACGGGAGCTGCAGCAACGAGCTCGACGCATGCGAGACCGAGGAG 300
        ************************************************************

gDNA    GTCGAGATCGCATCCGAGATCAATGATGCGAGGCCGGGATCGCCTGGATCAGGCGATGAC 360
cDNA    GTCGAGATCGCATCCGAGATCAATGATGCGAGGCCGGGATCGCCTGGATCAGGCGATGAC 360
        ************************************************************

gDNA    TCGAAGCCCGTACACTCTTTGGGTGCCTCCACTTCATCTCCCTATCCCAAGAGAAGGTGG 420
cDNA    TCGAAGCCCGTACACTCTTTGGGTGCCTCCACTTCATCTCCCTATCCCAAGAGAAG---- 416
        ********************************************************

gDNA    ATCTTCATTTTCTCCAGCTACTCTTTGATCCTCGTTTCGTGCCCTTCTTAGTCCCTTTAG 480
cDNA    ------------------------------------------------------------


gDNA    TTGCTGGCATTAAATTCGGCTCATAAATGAACAGCCGGCGAGGAGTGCAGAAGCGGGTGG 540
cDNA    ---------------------------------CCGGCGAGGAGTGCAGAAGCGGGTGG 442
                                           **************************

gDNA    TGACGGTACCGATCAGCGACTCGAAGGGCGCCGGCGAGGGGGCTCCGCCACCTGATTCGT 600
cDNA    TGACGGTACCGATCAGCGACTCGAAGGGCGCCGGCGAGGGGGCTCCGCCACCTGATTCGT 502
        ************************************************************

gDNA    GGACCTGGAGGAAGTACGGCCAGAAGCCCATAAAAGGCTCGCCTTTTCCCAGGTATGAAC 660
cDNA    GGACCTGGAGGAAGTACGGCCAGAAGCCCATAAAAGGCTCGCCTTTTCCCAGG------- 555
        ************************************************

gDNA    GGGATTTGCAGTAAACTCCTCCGCCTCGGGGTGGAGGCGCACTGGAAGATGGATCCAAAG 720
cDNA    ------------------------------------------------------------


gDNA    TCGTGACTTATGTGGGACGTGCAGGGGCTACTACAGGTGCAGCAGCTCCAAGGGGTGCCC 780
cDNA    -------------------------GGCTACTACAGGTGCAGCAGCTCTAAGGGGTGCCC 590
                                  ********************** ***********

gDNA    GGCGAGGAAGCAGGTGGAACGAAGCCGCGTCGACCCAACCGTTATCGTGGTTACCTACGC 840
cDNA    GGCGAGGAAGCAGGTGGAGCGAAGCCGCGTCGACCCAAACGTTATCGTGGTTACCTACGC 650
        ****************** ****************** **********************

gDNA    CTTCGACCACAACCACACCTCGCCGCTCCCCAAAAACCACCACCACAAGCACGCGGCAGC 900
cDNA    CTTCGACCACAACCACACCTCGCCGCTCCCCAAAAACCACCACCACAAGCACGCGGCAGC 710
        ************************************************************

gDNA    CGCCGCGCAGCCCGTCGAGGAGCAGCCGCTCACGCCACAGCTGAACCAGTCCGGCACGCC 960
cDNA    CGCCGCGCAGCCCGTCGAGGAGCAGCCGCTCACGCCGCAGCTGAACCAGTCCGGCACGCC 770
        ********************************** ***********************

gDNA    GGACTCCGCGGAGCGCGACGAGAAGTTCTCCGACCTGATCACCGAGGAGGAGTCGGCGTT 1020
cDNA    GGACTCCGCGGAGCGCGACGAGAAGTTCTCCGACCTGATCACCGAGGAGGAGTCGGCGTT 830
        ************************************************************
```

**Figure 4:24:** Nucleotide sequence alignment of gDNA and cDNA of *MamWRKY*.

```
gDNA    CACGGTCCACGCCGGCGGCTGCTTCCCATGGTTCGCCGACGTCTGCTCGGTGCACCCGAC 1080
cDNA    CACGGTCCACGCCGGCGGCTGCTTCCCATGGTTCGCCGACGTCTGCTCGGTGCACCCGAC 890
        ************************************************************

gDNA    CTCCCCCTCTGCGGCGGACTCCGACGAGTTGCTCTACGGCTCGGTCCTCTTTGCCGGTGC 1140
cDNA    CTCCCCCTCTGCGGCGGACTCCGACGAGTTGCTCTACGGCTCGGTCCTCTTTGCCGGTGC 950
        ************************************************************

gDNA    CGCCACTGGCGCAGCGCTGCCCGAGGAACTGGAGGAGGCCGCAGGGGGCGGCGGCGGGGG 1200
cDNA    CGCCACTGGCGCAGCGCTGCCTGAGGAACTGGAGGAGGCCGCAGGGGGCGGCGGCGGGGG 1010
        ******************** ************************************

gDNA    TGACGACGACTCTCTGTTCGCGGGGCTCGGGGAGCTGCCGGAGTACACGGTGGTGCTCCG 1260
cDNA    TGACGACGACTCGCTGTTCGCGGGGCTCGGGGAGCTGCCGGAGTACACGGTGGTGCTCCG 1070
        ************ ********************************************

gDNA    CTGGGGGCTAGCATCGGCATCGTGGGTGGGGACCGCTGGGTGACCACTTTGCCGTCGATT 1320
cDNA    CTGGGGGCTAGCATCGGCATCGTGGGTGGGGACCGCTGGGTGACCACTTTGCCGTCGATT 1130
        ************************************************************

gDNA    CGATCGAGGGGAATAGCCGGCATTGACGTATATAAATTGTCTTCTTCCTGATTTCTTTTC 1380
cDNA    CGATCGAGGGGAATAGCCGGCATTGACGTATATAAATTGTCTTCTTCCTGATTTCTTTTC 1190
        ************************************************************

gDNA    CTTTTTTTCTTTAGGTTCTTTATTTTTACCTTCA 1414
cDNA    CTTTTTTTCTTTAGGTTCTTTATTTTTACCTTCA 1224
        ************************************
```

**Figure 4.24,** continued.

```
221     TCTCGGATTGTGATGAACGGGAGCTGCAGCAACGAGCTCGACGCATGCGAGACCGAGGAG
1                    M  N  G  S  C  S  N  E  L  D  A  C  E  T  E  E

281     GTCGAGATCGCATCCGAGATCAATGATGCGAGGCCGGGATCGCCTGGATCAGGCGATGAC
17      V  E  I  A  S  E  I  N  D  A  R  P  G  S  P  G  S  G  D  D

341     TCGAAGCCCGTACACTCTTTGGGTGCCTCCACTTCATCTCCCTATCCCAAGAGAAGCCGG
37      S  K  P  V  H  S  L  G  A  S  T  S  S  P  Y  P  K  R  S  R

401     CGAGGAGTGCAGAAGCGGGTGGTGACGGTACCGATCAGCGACTCGAAGGGCGCCGGCGAG
57      R  G  V  Q  K  R  V  V  T  V  P  I  S  D  S  K  G  A  G  E

461     GGGGCTCCGCCACCTGATTCGTGGACCTGGAGGAAGTACGGCCAGAAGCCCATAAAAGGC
77      G  A  P  P  P  D  S  W  T  W  R  K  Y  G  Q  K  P  I  K  G

541     TCGCCTTTTCCCAGGGGCTACTACAGGTGCAGCAGCTCTAAGGGGTGCCCGGCGAGGAAG
97      S  P  F  P  R  G  Y  Y  R  C  S  S  S  K  G  C  P  A  R  K

601     CAGGTGGAGCGAAGCCGCGTCGACCCAAACGTTATCGTGGTTACCTACGCCTTCGACCAC
117     Q  V  E  R  S  R  V  D  P  N  V  I  V  V  T  Y  A  F  D  H

661     AACCACACCTCGCCGCTCCCCAAAAACCACCACCACAAGCACGCGGCAGCCGCCGCGCAG
137     N  H  T  S  P  L  P  K  N  H  H  H  K  H  A  A  A  A  A  Q

721     CCCGTCGAGGAGCAGCCGCTCACGCCGCAGCTGAACCAGTCCGGCACGCCGGACTCCGCG
157     P  V  E  E  Q  P  L  T  P  Q  L  N  Q  S  G  T  P  D  S  A

781     GAGCGCGACGAGAAGTTCTCCGACCTGATCACCGAGGAGGAGTCGGCGTTCACGGTCCAC
177     E  R  D  E  K  F  S  D  L  I  T  E  E  E  S  A  F  T  V  H

841     GCCGGCGGCTGCTTCCCATGGTTCGCCGACGTCTGCTCGGTGCACCCGACCTCCCCCTCT
197     A  G  G  C  F  P  W  F  A  D  V  C  S  V  H  P  T  S  P  S

901     GCGGCGGACTCCGACGAGTTGCTCTACGGCTCGGTCCTCTTTGCCGGTGCCGCCACTGGC
217     A  A  D  S  D  E  L  L  Y  G  S  V  L  F  A  G  A  A  T  G

961     GCAGCGCTGCCTGAGGAACTGGAGGAGGCCGCAGGGGGCGGCGGCGGGGGTGACGACGAC
237     A  A  L  P  E  E  L  E  E  A  A  G  G  G  G  G  G  D  D  D

1021    TCGCTGTTCGCGGGGCTCGGGGAGCTGCCGGAGTACACGGTGGTGCTCCGCTGGGGGCTA
257     S  L  F  A  G  L  G  E  L  P  E  Y  T  V  V  L  R  W  G  L

1081    GCATCGGCATCGTGGGTGGGGACCGCTGGGTGA
277     A  S  A  S  W  V  G  T  A  G  -
                                      *
```



**Figure 4:25:** The complete cDNA and deduced amino acid sequences of *MamWRKY*.

In the WRKY domains (shaded in gray), the conserved amino acid residues W, R, K, Y, G, Q, and Y are underlined along with the two cysteines (C) and two histidines (H)(boxed).

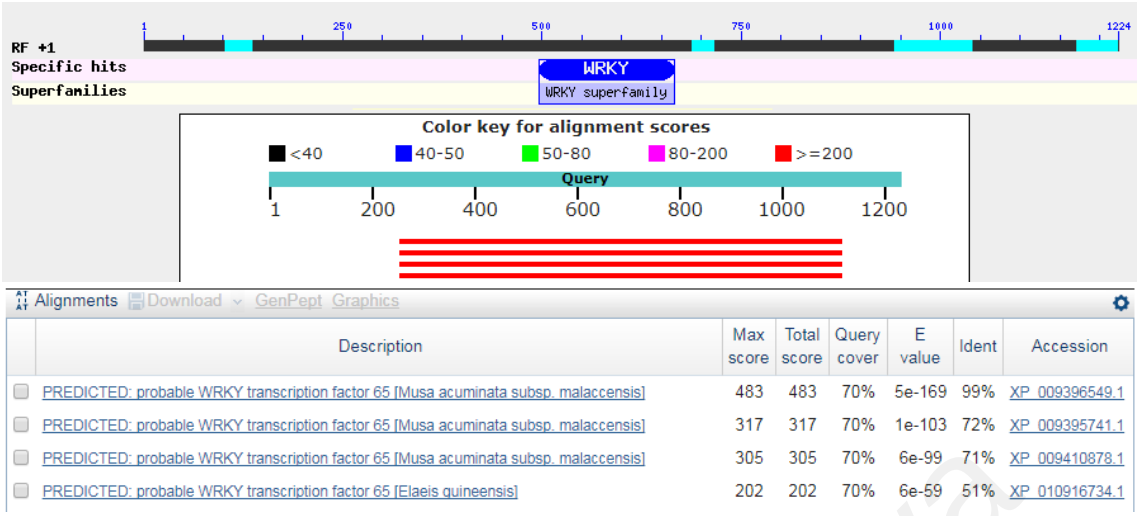



**Figure 4:26:** Graphical summary of BLAST results of the deduced amino acids of MamWRKY on the conserved domains.

The conserved WRKY superfamily domains were detected on the sequences.

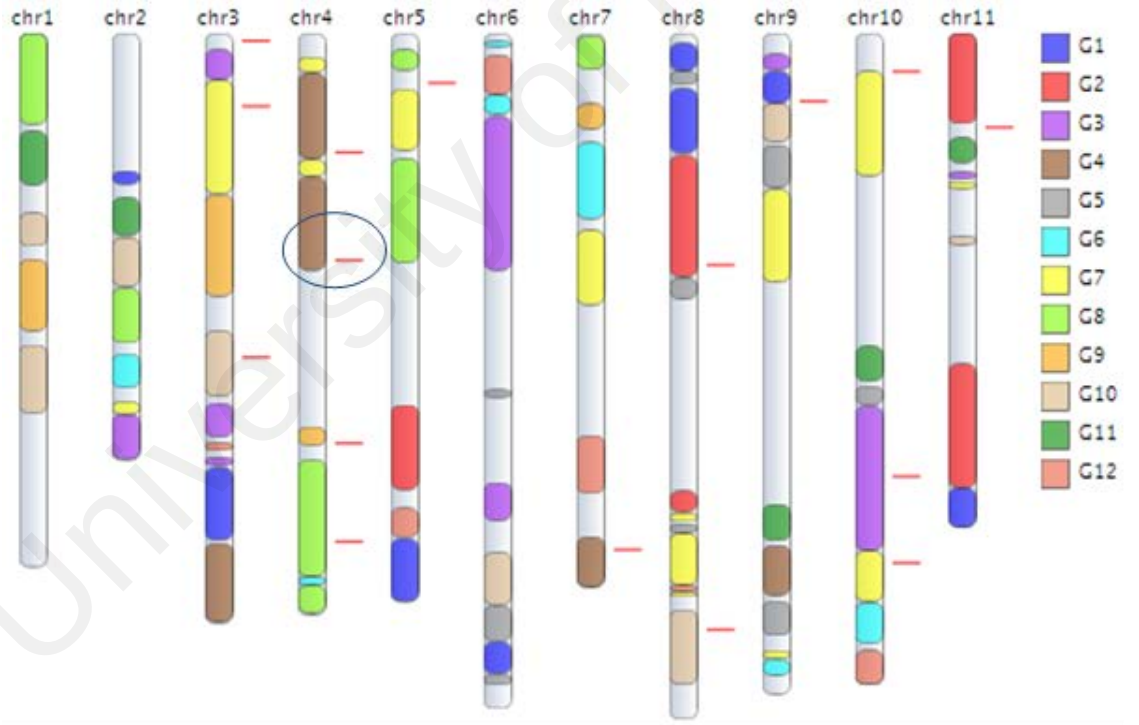



**Figure 4:27:** Banana genome chromosomes showed the localization of *MamWRKY* at chromosome 4.

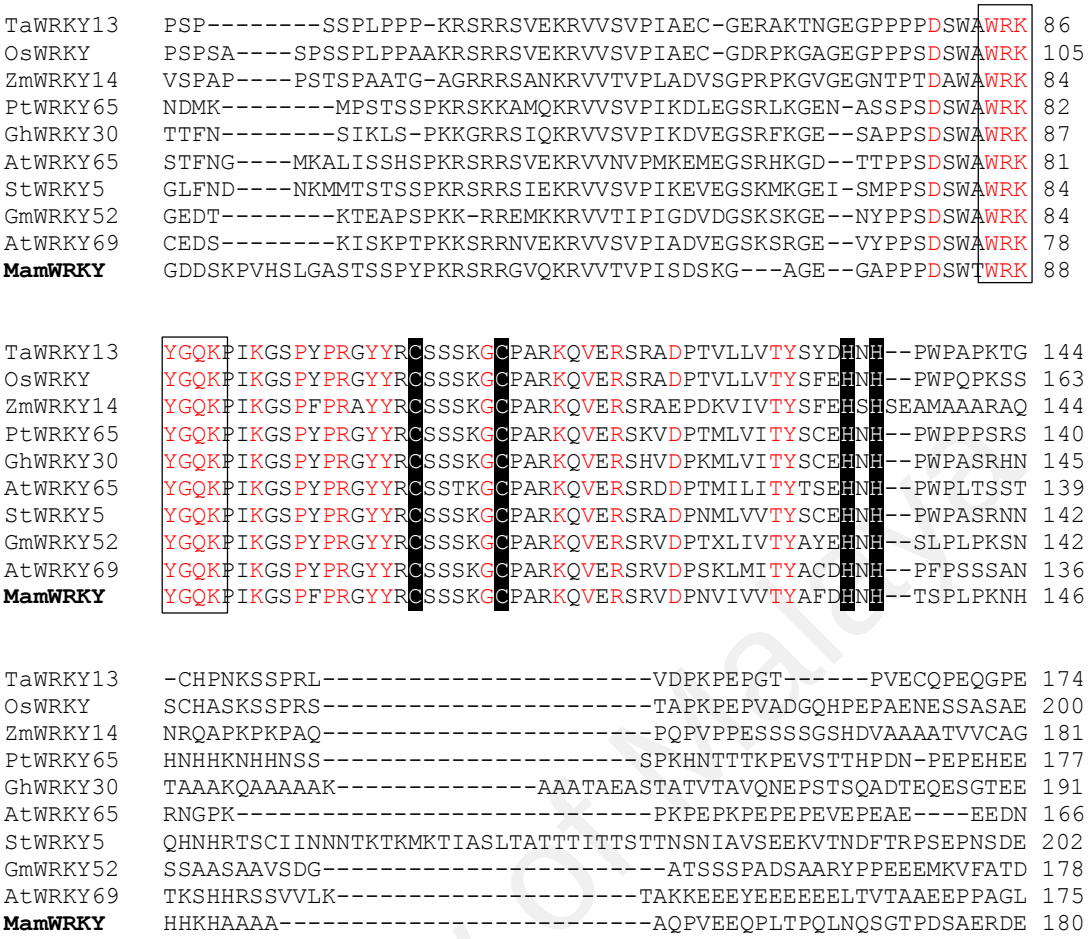



**Figure 4:28:** Multiple alignments of the deduced amino acids of the full-length sequence of *MamWRKY* with other WRKY sequences from other plants obtained from the GenBank database in the NCBI website.

They were AtWRKY65 (accession no. AT1G29280.1) and AtWRKY69 (AT3G58710.1) from *Arabidopsis thaliana*, TaRKY13 (ABO15543.1) from *Triticum aestivum*, StWRKY5 (NP_001274847.1) from *Solanum tuberosum*, GmWRKY52 (NP_001237726.1) from *Glycine max*, GhWRKY30 (AGV75938.1) from *Gossypium hirsutum*, PtWRKY65 (XP_002317397.1) from *Populus trichocarpa*, OsWRKY (BAB56055.1) from *Oryza sativa* Japonica Group and ZmWRKY14 (ACG45823.1) from *Zea mays*.

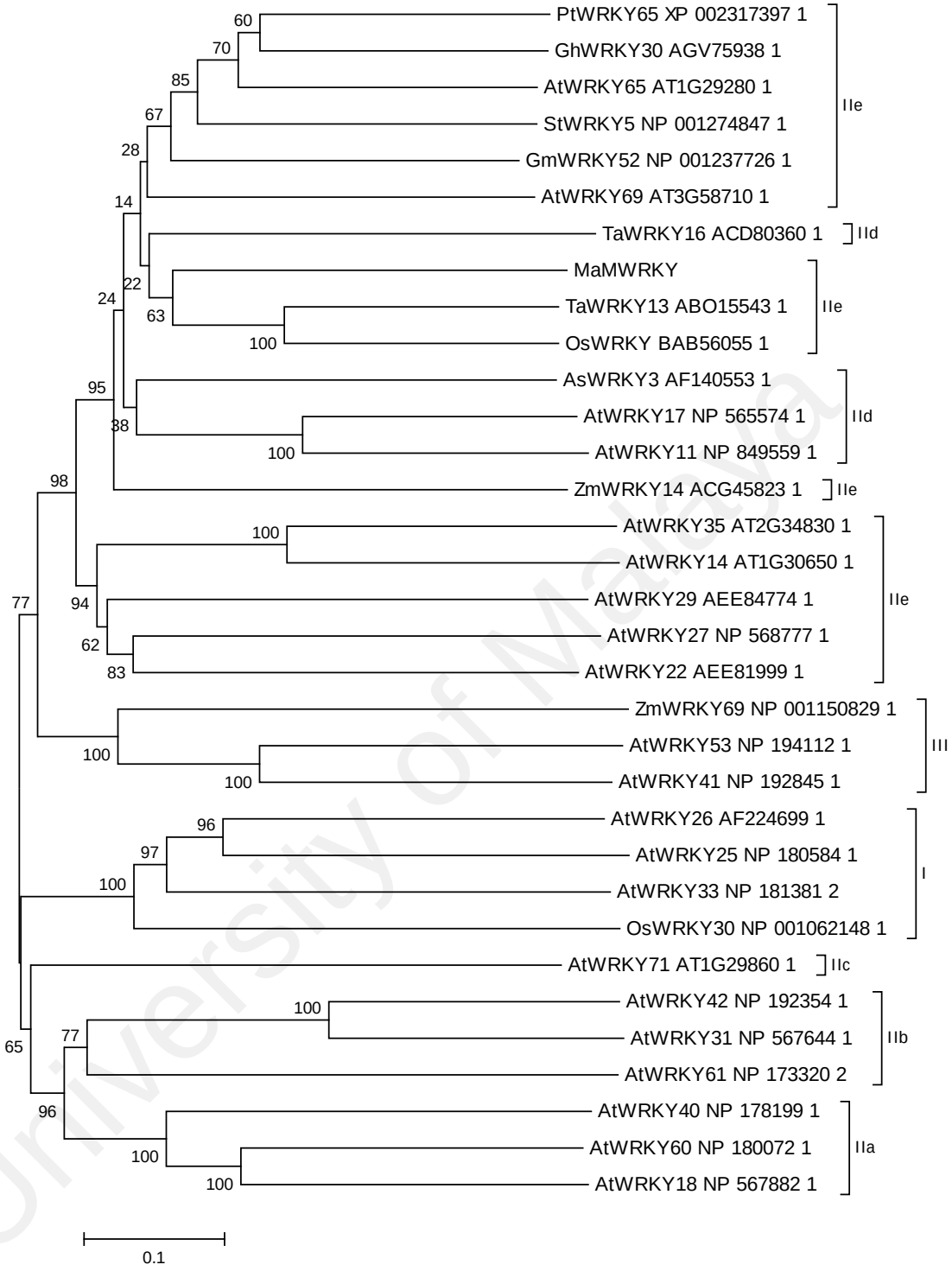



**Figure 4:29:** Phylogenetic tree generated with MamWRKY proteins and other WRKY proteins by using MEGA 6 of neighbor-joining method with 100 bootstrapping.

WRKY proteins that used to construct the phylogenetic are from monocots and dicots.

### 4.2.3 Digestion and ligation of pCAMBIA1304 and *MamWRKY* fragment

The digested products from pCAMBIA1304 and full length cDNA of *MamWRKY* using *Bgl*II and *Spe*I enzymes resulted in a single fragment size of 12 kb and 1.3 kb, respectively (Figure 4.30). In another digestion, both plasmid of pCAMBIA1304 and CDS of *MamWRKY* digested by *Bgl*II and *Spe*I enzymes produced a single fragment size of 12 kb and 861 bp, respectively (Figure 4.31).

All digested products were ligated using DNA ligase before cloned into *E. coli*. This ligation formed a construct pCAMBIA1304 with *MamWRKY* gene which is driven by CaMV35S (cauliflower mosaic virus) promoter (Figure 4.32). The verification of the ligation product was carried out after the *E. coli* transformation experiment.

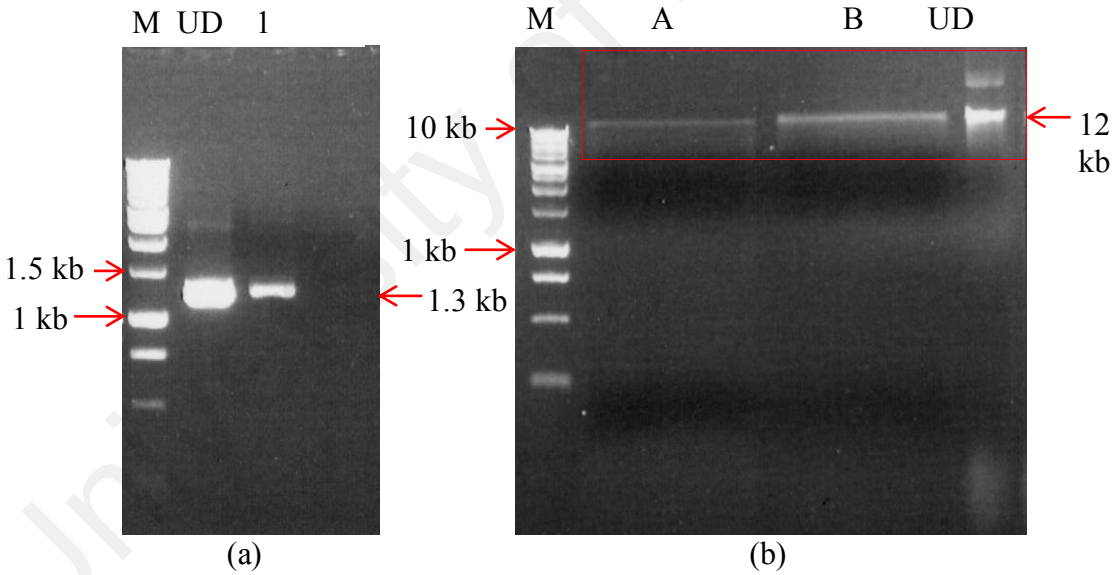



**Figure 4:30:** The purified product of digested (a) full cDNA of *MamWRKY* fragment; (b) pCAMBIA1304 plasmid using same restriction enzyme, *Bgl*II and *Spe*I.

M : Marker- 1 kb DNA Ladder (Fermentas)
UD : Undigested *MamWRKY* (a) and pCAMBIA1304 plasmid (b) fragment as control
1 : Digested *MamWRKY*
A-B : Linearized pCAMBIA1304

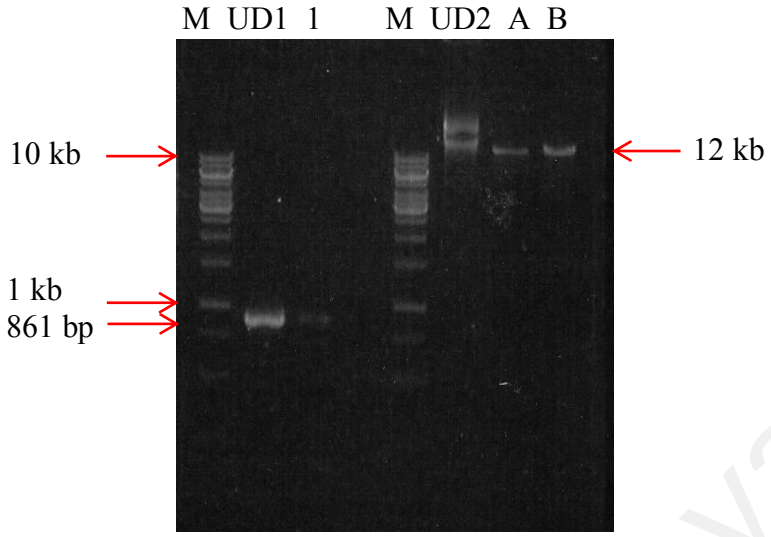



**Figure 4:31:** The purified product of digested *MamWRKY* CDS fragment and pCAMBIA1304 plasmid using same restriction enzyme, *Bgl*II and *Spe*I.

M : Marker- 1 kb DNA Ladder (Fermentas)
UD1 : Undigested *MamWRKY* CDS fragment
UD2 : Undigested pCAMBIA1304 plasmid
1 : Digested *MamWRKY* CDS fragment
A & B : Linearized pCAMBIA1304 plasmid

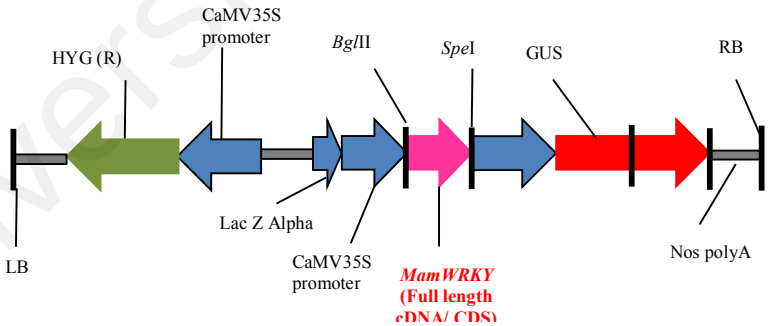



**Figure 4:32:** Schematic representation of the T-DNA region of binary vector pCAMBIA1304-MamWRKY.

The T-DNA region consist of LB (T-DNA left border); HYG(R) (Hygromycin selectable marker); CaMV35S (cauliflower mosaic virus promoter); *Bgl*II and *Spe*I: restriction sites; *MamWRKY* gene (Full length cDNA/ CDS); GUS (ß-glucuronidase); NOS (Nopaline synthase terminator); RB (T-DNA right border).

### 4.2.4 Transformation into *E. coli* competent cells

The ligation reaction of pCAMBIA1304 and full cDNA of *MamWRKY* fragments that formed a plasmid construct of pCAMBIA1304-MamWRKY was transformed into *E. coli* TOP 10 competent cells. The colonies appeared on LB kanamycin resistant agar were proceed with colony PCR with WFL_F and WFL_R WC_F and WC_R primer pair to confirm the positive colonies that harbored the plasmid construct of pCAMBIA1304-MamWRKY. As shown in Figure 4.33, the expected fragment size at ~1.3 kb was observed among 8 randomly selected colonies. The positive colonies were cultured and proceed with plasmid extraction procedure. The extracted plasmids (Figure 4.34) were sent for sequencing for further verification of whole sequences. The $OD_{260/280}$ reading of plasmid extract was in range of DNA purity 1.90-2.20.

The amplification of *MamWRKY* CDS was performed in this plasmid extract via PCR screening using WC_F and WC_R primer pair. The expected fragment size of 861 bp was observed (Figure 4.35). The ligation reaction with pCAMBIA1304 was carried out and proceeds with the transformation into *E. coli*. The colony PCR showed the expected fragment size of *MamWRKY* CDS in all 9 randomly selected colonies (Figure 4.36). The extracted plasmids (Figure 4.37) were sent for sequencing for further verification of whole sequences.

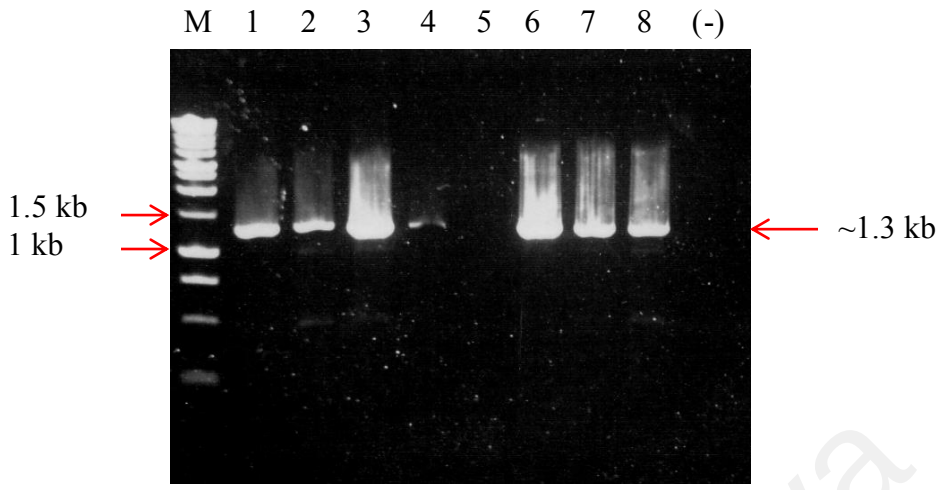



**Figure 4:33:** PCR screening of *MamWRKY* in *E. coli* colonies with WFL_F and WFL_R primer pairs.

| | |
|---|---|
| M | : Marker- 1 kb DNA Ladder (Fermentas) |
| 1-8 | : DNA fragment of *MamWRKY* from different *E. coli* colonies |
| (-) | : Negative control (PCR mixture without the DNA template) |

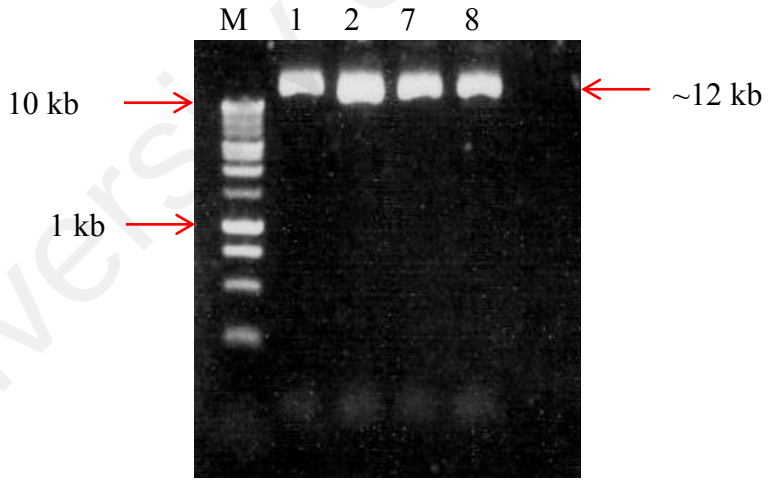



**Figure 4:34:** Plasmid of pCAMBIA1304-MamWRKY full length cDNA construct from different positive colonies of *E. coli*.

| | |
|---|---|
| M | : Marker- 1 kb DNA Ladder (Fermentas) |
| 1, 2, 7, 8 | : Plasmid of pCAMBIA1304-MamWRKY full length cDNA |

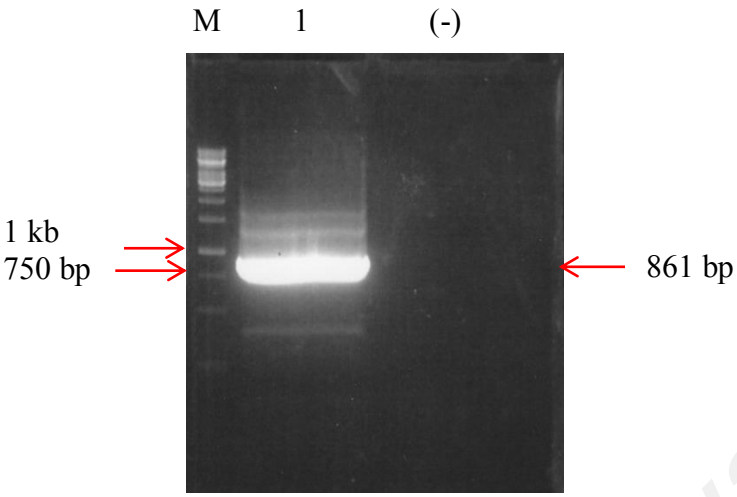



**Figure 4:35:** PCR amplification of *MamWRKY* CDS in pCAMBIA1304-MamWRKY plasmid.

M : Marker- 1 kb DNA Ladder (Fermentas)
1 : DNA fragment of *MamWRKY* CDS
(-) : Negative control (PCR mixture without the DNA template)

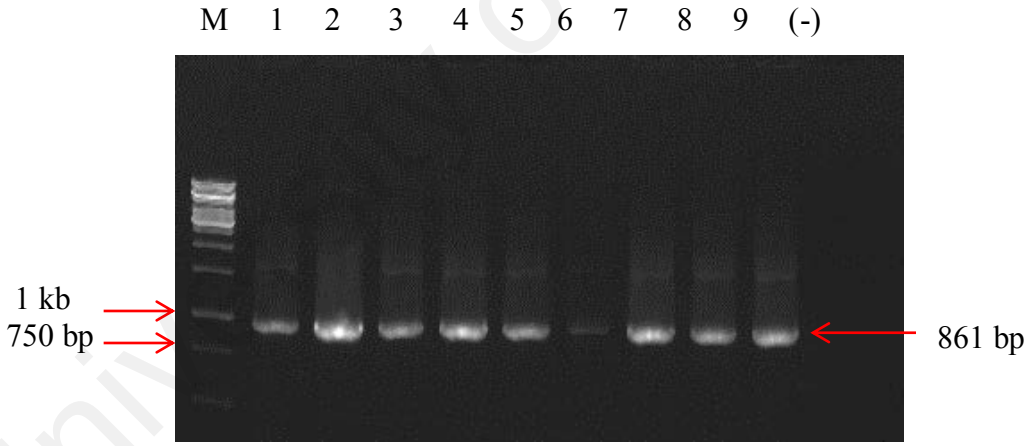



**Figure 4:36:** PCR screening of *MamWRKY* CDS in *E. coli* colonies with WC_F and WC_R.

M : Marker- 1 kb DNA Ladder (Fermentas)
1-9 : DNA fragment of *MamWRKY* CDS from different *E. coli* colonies
(-) : Negative control (PCR mixture without the DNA template)

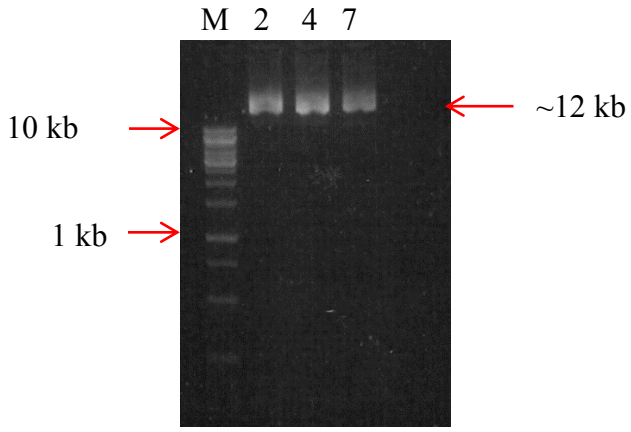



**Figure 4:37:** Plasmid of pCAMBIA1304-MamWRKY CDS construct from different positive colonies of *E. coli*.

M : Marker- 1 kb DNA Ladder (Fermentas)
2, 4, 7 : pCAMBIA1304-MamWRKY CDS

### 4.2.5 Transformation of constructed plasmid into *A. tumefaciens*

The pCAMBIA1304-MamWRKY CDS were transformed into *A. tumefaciens* LBA4404 strain. The colonies were amplified using WC_F and WC_R primers to confirm the presence of pCAMBIA1304-MamWRKY. As shown in Figure 4.38, the expected fragment size of 861 bp was observed among 10 randomly selected colonies.

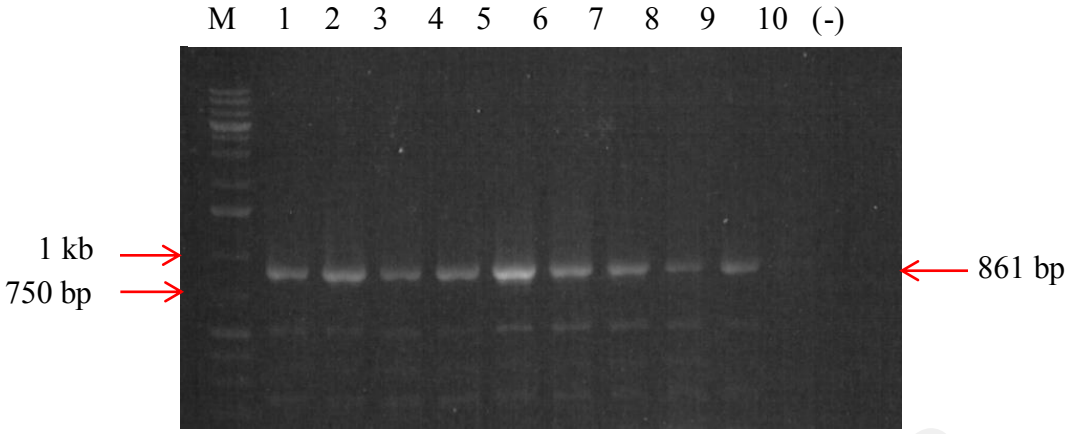



**Figure 4:38:** PCR screening of *MamWRKY* CDS in *A. tumefaciens* colonies with WC_F and WC_R primer pairs.

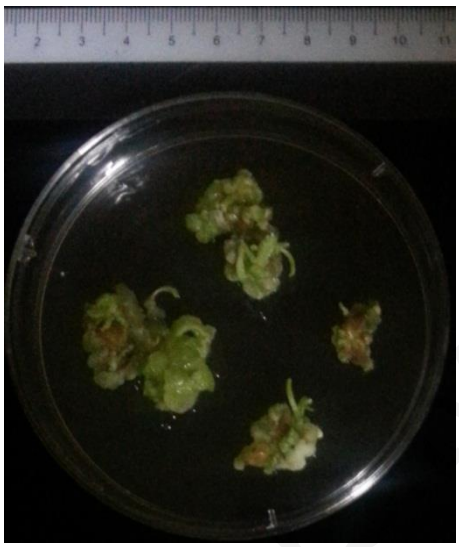
| | |
|---|---|
| M | : Marker- 1 kb DNA Ladder (Fermentas) |
| 1-10 | : DNA fragment of *MamWRKY* CDS in different *A. tumefaciens* colonies |
| (-) | : Negative control (PCR mixture without the DNA template) |

### 4.2.6 Regeneration of transgenic tobacco *Nicotiana tabacum* L. cv. SR1

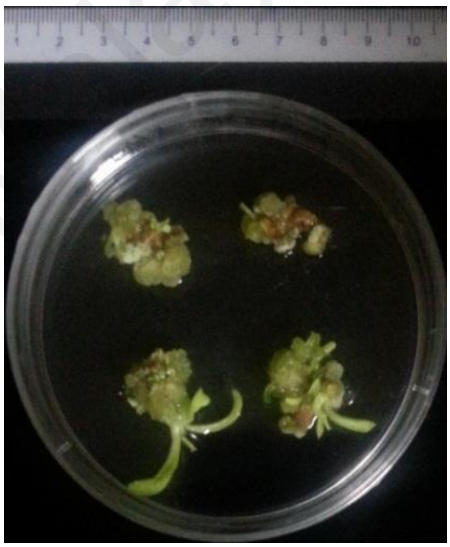
Tobacco leaf discs infected and co-cultivated with *A. tumefaciens* harbouring pCAMBIA1304-MamWRKY expression cassette were cultured on TSM media containing hygromycin. The hygromycin-resistant leaf explants produced shoots on TSM media (Figure 4.39), whereas the untransformed leaf discs without selection and grown on TSM selection media turned brownish and died (figure not shown). Leaves from four putative transgenic $T_0$ lines were harvested for GUS histochemical assay. The blue colorations were observed on the transformed tissues, whereas the untransformed tissues remained colorless (Figure 4.40). The rooted tobacco plantlets were transferred into potted soil and acclimatized in a control room environment (Figure 4.41). The transgenic tobacco did not show any flowering compared to the positive control and untransformed plants. Verification of the gene integration and expression were achieved through semi-quantitative PCR and qPCR, respectively, on 3 month-old $T_0$ tobacco plants.

(a)

(b)



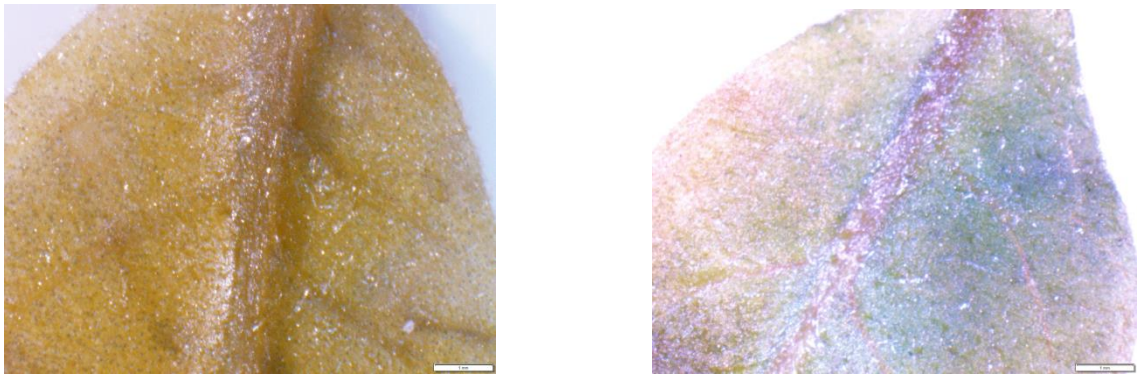
(c)



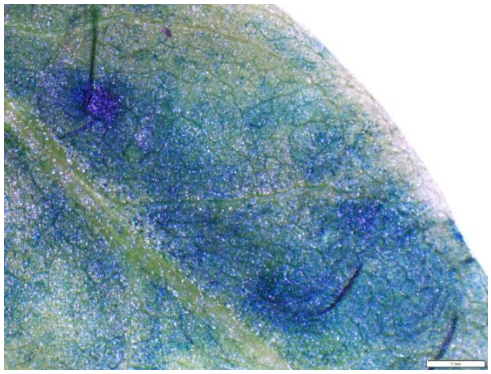
(d)

**Figure 4:39:** Regenerated leaf disc of untransformed and transformed *Nicotiana tabacum* L. cv. SR1 with pCAMBIA1304 and pCAMBIA1304-MamWRKY.

The regenerated leaf discs of a) Untransformed control; b) Transformed leaf discs with pCAMBIA1304; c-d) Transformed leaf discs with pCAMBIA1304-MamWRKY.



(a) (b)



(c)

**Figure 4:40:** Transient histochemical *gusA* gene expression in untransformed and transformed leaf of *Nicotiana tabacum* L. cv. SR1 with pCAMBIA1304 and pCAMBIA1304-MamWRKY.

The leaf of (a) Untransformed control; (b) pCAMBIA1304-MamWRKY insert and (c) pCAMBIA1304 insert. *Bar* = 1 cm.

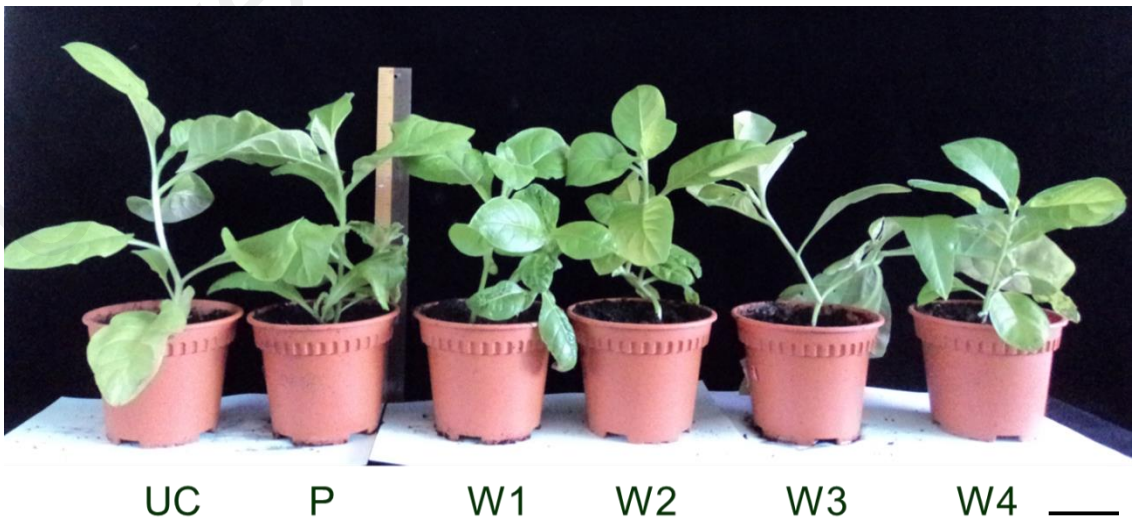



**Figure 4:41:** Untransformed and transformed *Nicotiana tabacum* L. cv. SR1 plants with pCAMBIA1304 and pCAMBIA1304-MamWRKY were hardened in potted soil (UC- untransformed control plant; P- pCAMBIA1304 insert; W1, W2, W3 and W4- transgenic lines). *Bar* = 8.25 cm.

### 4.2.7 Molecular characterization of transformed tobacco plants

Semi-quantitative PCR screening of genomic DNA showed that fragment size of 618 bp from the *hpt* gene was amplified in all four hygromycin-resistant lines, whereas no amplification was detected in the untransformed plants (Figure 4.42).

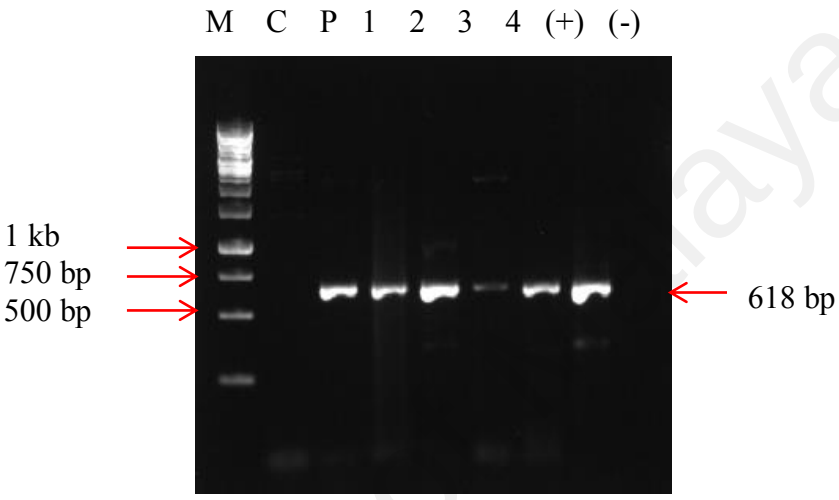


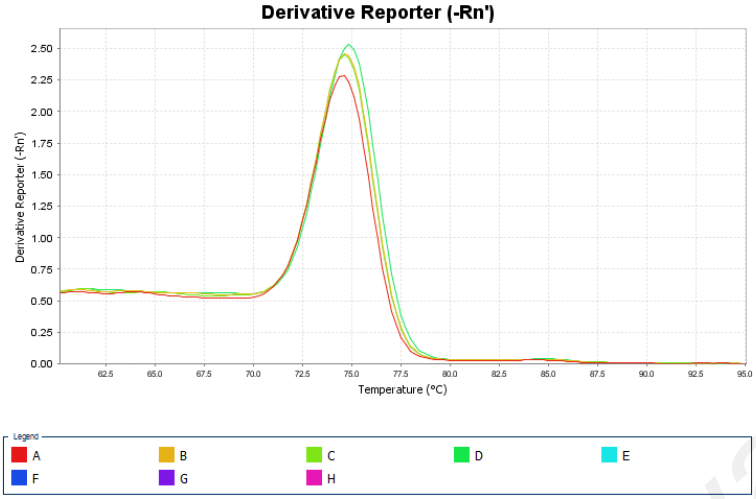

**Figure 4:42:** PCR screening of *hpt* gene in *Musa acuminata* cv. 'Berangan'.

M : Marker- 1 kb DNA Ladder (Fermentas)
C : Untransformed control plant
P : Transformed plant with pCAMBIA1304 insert
1-4 : Putative transgenic lines harbouring pCAMBIA1304-MamWRKY
(+) : Positive control (plasmid extract of pCAMBIA1304 harbouring *MamWRKY* as DNA template)
(-) : Negative control (PCR mixture without the DNA template)

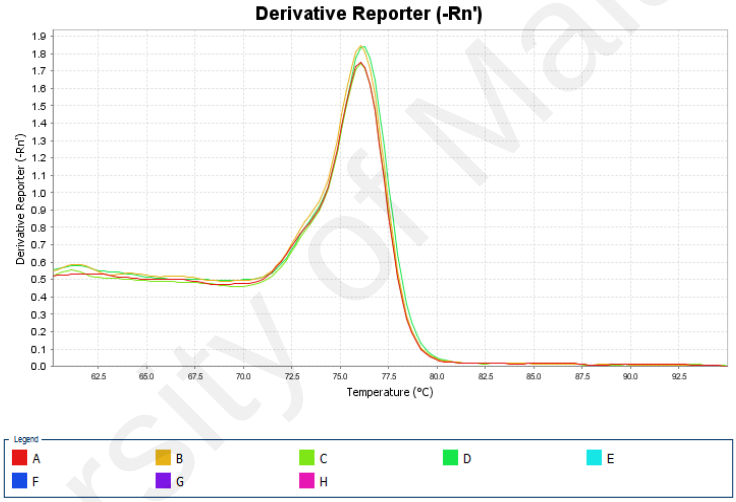
### 4.2.8 Expression profiles of *MamWRKY* in tobacco

qPCR analysis was carried out to analyze the expression of *MamWRKY* in the transgenic lines using two references genes, *Actin (Tac9)* (GenBank accession: X69885) and *EF-1α* (*elongation factor 1α*) (GenBank accession: AF120093). The primer pair to amplify the reference gene, *Actin (Tac9)* and *EF-1α* in transgenic (Figure 4.43) and untransformed plants (Figure 4.44) showed a single peak in the melting curve,

confirming the specificity of the primers. The primer pair targeting *MamWRKY* also showed a single peak in the melting curve in transgenic plants but not in untransformed plants (Figure 4.45). The analysis of *MamWRKY* expression level using $2^{-\Delta\Delta Ct}$ method in transgenic lines W1, W2 and W4 resulted in 809.002, 739.804 and 1153.659-fold changes, respectively, compared to the untransformed plants (Table 4.2 and Figure 4.46). While, the expression level of *PR1a* transcript in transgenic lines W1, W2 and W4 was at 968.763, 23.984 and 6812.648-fold changes, respectively, higher than untransformed plants (Figure 4.47).

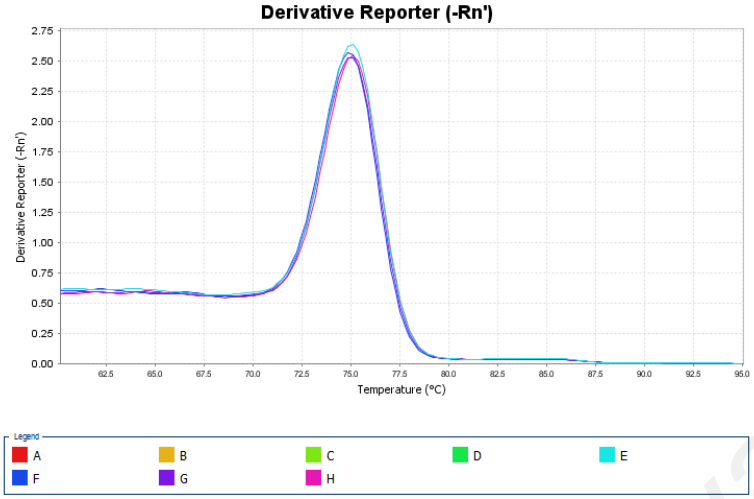



(a)

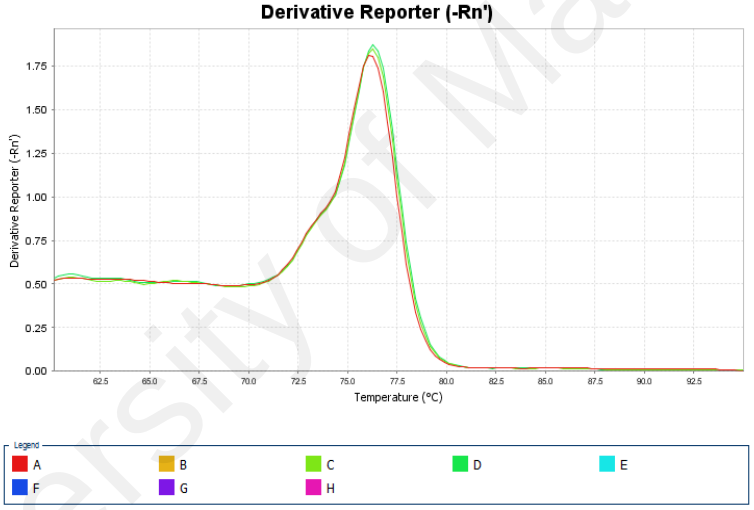



(b)

**Figure 4:43:** Melting curve of housekeeping gene primer in transgenic samples *Nicotiana tabacum* L. cv. SR1 (a) *Actin (Tac9)* and (b) *EF-1α.*

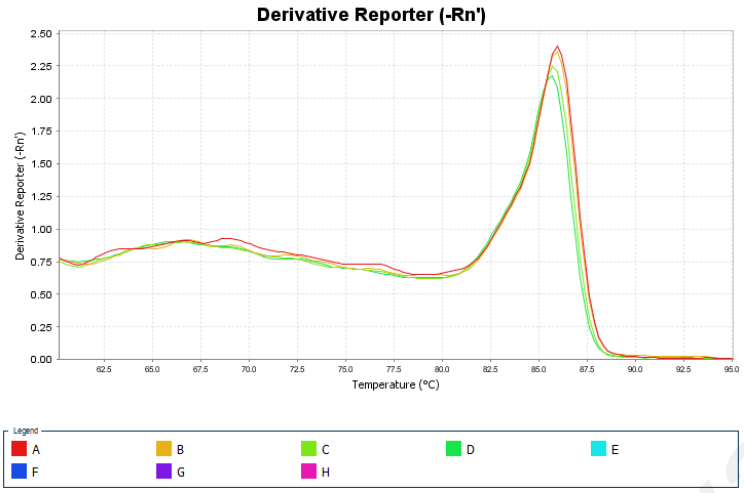



(a)

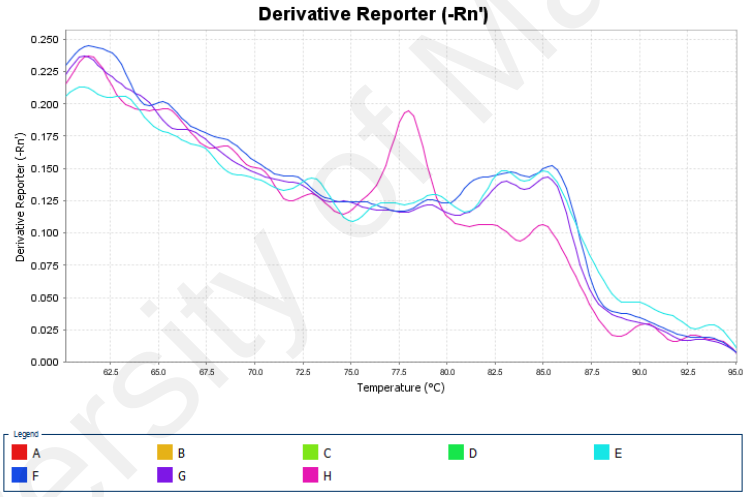



(b)

**Figure 4:44:** Melting curve of housekeeping gene primer in non-transgenic samples *Nicotiana tabacum* L. cv. SR1 (a) *Actin (Tac9)* and (b) *EF-1α.*




(a)




(b)

**Figure 4:45:** Melting curve of gene specific primer, W2_F and W2_R in (a) transgenic and (b) untransformed *Nicotiana tabacum* L. cv. SR1.

**Table 4.2:** qPCR analysis of *MamWRKY* gene expression in transgenic and non-transgenic compared to untransformed control (UTC) of *Nicotiana tabacum* L. cv. SR1.

| | CT | | | | | | | Standard deviation (SD) | | | | SD DIFF | SE OF DIFF |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Samples** | ***Mam WRKY*** | **Average *Mam WRKY*** | **Actin (*Tac9*)** | **Average Actin (*Tac9*)** | ***EF-1α*** | **Average *EF-1α*** | **Average Actin (*Tac9*) & *EF-1α*** | **SD *Mam WRKY*** | **SD Actin (*Tac9*)** | **SD *EF-1α*** | **Average SD Actin (*Tac9*) & *EF-1α*** | | |
| $W1_1$ | 25.254 | | 19.922 | | 17.786 | | | | | | | | |
| $W1_2$ | 25.242 | | 20.044 | | 17.804 | | | | | | | | |
| $W1_3$ | 25.369 | 25.288 | 19.964 | 19.977 | 17.677 | 17.756 | 18.867 | 0.070 | 0.062 | 0.069 | 0.065 | 0.096 | 0.055 |
| $W2_1$ | 25.800 | | 20.565 | | 18.480 | | | | | | | | |
| $W2_2$ | 26.184 | | 20.220 | | 18.525 | | | | | | | | |
| $W2_3$ | 25.857 | 25.947 | 20.128 | 20.304 | 18.463 | 18.489 | 19.397 | 0.207 | 0.230 | 0.032 | 0.131 | 0.245 | 0.142 |
| $W4_1$ | 23.630 | | 19.490 | | 16.034 | | | | | | | | |
| $W4_2$ | 23.659 | | 19.438 | | 16.039 | | | | | | | | |
| $W4_3$ | 23.553 | 23.614 | 19.346 | 19.425 | 15.883 | 15.985 | 17.705 | 0.055 | 0.073 | 0.089 | 0.081 | 0.098 | 0.056 |
| $UTC_1$ | 34.793 | | 20.027 | | 17.200 | | | | | | | | |
| $UTC_2$ | 34.734 | | 20.031 | | 17.171 | | | | | | | | |
| $UTC_3$ | 34.671 | 34.733 | 20.154 | 20.071 | 17.325 | 17.232 | 18.652 | 0.061 | 0.072 | 0.082 | 0.077 | 0.098 | 0.057 |

**Table 4.2,** continued.

| Samples | ΔCT | ΔΔCT | $2^{\wedge-(\Delta\Delta CT)}$ |
|---|---|---|---|
| $W1_1$ | | | |
| $W1_2$ | | | |
| $W1_3$ | 6.421 | -9.660 | 809.002 |
| $W2_1$ | | | |
| $W2_2$ | | | |
| $W2_3$ | 6.550 | -9.531 | 739.804 |
| $W4_1$ | | | |
| $W4_2$ | | | |
| $W4_3$ | 5.909 | -10.172 | 1153.659 |
| $UTC_1$ | | | |
| $UTC_2$ | | | |
| $UTC_3$ | 16.081 | 0 | 1.000 |

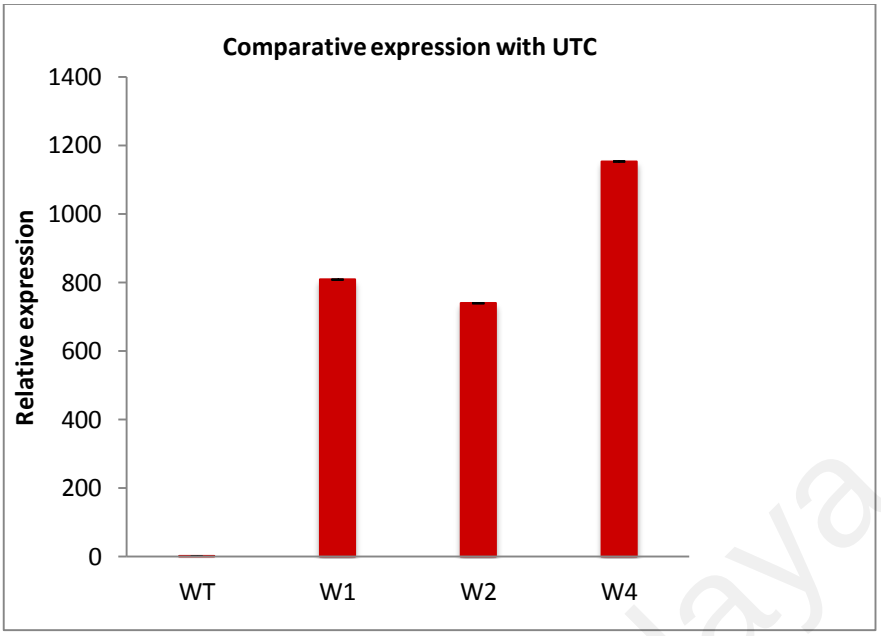



**Figure 4:46:** Gene expression level of *MamWRKY* in transgenic lines W1, W2 and W4 compared to untransformed control of *Nicotiana tabacum* L. cv. SR1 (UTC).

**Table 4.3:** qPCR analysis of *PR1a* gene expression in transgenic and non-transgenic compared to untransformed control (UTC) of *Nicotiana tabacum* L. cv. SR1.

| | CT | | | | | | | Standard deviation (SD) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Samples** | ***PR1a*** | **Average *PR1a*** | **Actin (*Tac9*)** | **Average Actin (*Tac9*)** | ***EF-1α*** | **Average *EF-1α*** | **Average Actin (*Tac9*) & *EF-1α*** | **SD *PR1a*** | **SD Actin (*Tac9*)** | **SD *EF-1α*** | **Average SD Actin (*Tac9*) & *EF-1α*** | **SD DIFF** | **SE OF DIFF** |
| $W1_1$ | 21.161 | | 20.310 | | 16.947 | | | | | | | | |
| $W1_2$ | 21.052 | | 20.593 | | 16.711 | | | | | | | | |
| $W1_3$ | 21.305 | 21.173 | 20.385 | 20.429 | 16.999 | 16.886 | 18.658 | 0.127 | 0.147 | 0.153 | 0.15 | 0.197 | 0.113 |
| $W2_1$ | 26.873 | | 20.824 | | 17.168 | | | | | | | | |
| $W2_2$ | 26.766 | | 20.700 | | 17.257 | | | | | | | | |
| $W2_3$ | 26.935 | 26.858 | 20.820 | 20.781 | 17.276 | 17.234 | 19.008 | 0.085 | 0.07 | 0.058 | 0.064 | 0.107 | 0.062 |
| $W4_1$ | 17.837 | | 20.276 | | 16.689 | | | | | | | | |
| $W4_2$ | 17.855 | | 19.998 | | 15.539 | | | | | | | | |
| $W4_3$ | 17.730 | 17.807 | 20.533 | 20.269 | 15.600 | 15.943 | 18.106 | 0.068 | 0.268 | 0.647 | 0.457 | 0.462 | 0.267 |
| $UTC_1$ | 30.675 | | 20.242 | | 16.887 | | | | | | | | |
| $UTC_2$ | 31.018 | | 20.840 | | 17.126 | | | | | | | | |
| $UTC_3$ | 31.658 | 31.117 | 19.970 | 20.351 | 17.026 | 17.013 | 18.682 | 0.499 | 0.445 | 0.12 | 0.283 | 0.573 | 0.331 |

**Table 4.3,** continued.

| **Samples** | **ΔCT** | **ΔΔCT** | **2^-(ΔΔCT)** |
|---|---|---|---|
| $W1_1$ | | | |
| $W1_2$ | | | |
| $W1_3$ | 2.515 | -9.920 | 968.763 |
| $W2_1$ | | | |
| $W2_2$ | | | |
| $W2_3$ | 7.851 | -4.584 | 23.984 |
| $W4_1$ | | | |
| $W4_2$ | | | |
| $W4_3$ | -0.299 | -12.734 | 6812.648 |
| $UTC_1$ | | | |
| $UTC_2$ | | | |
| $UTC_3$ | 12.435 | 0 | 1.000 |

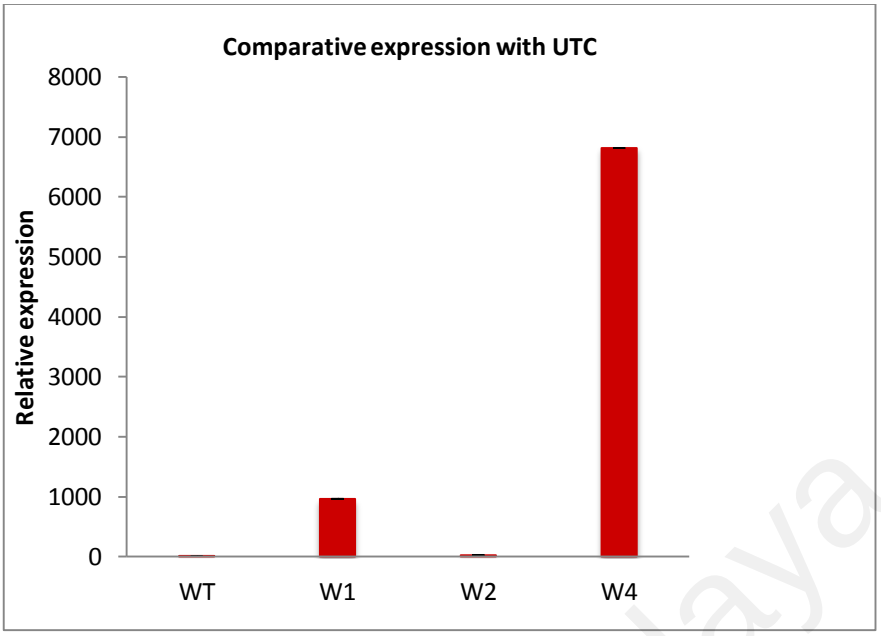



**Figure 4:47:** Gene expression level of *PR1a* in transgenic lines W1, W2 and W4 compared to untransformed control of *Nicotiana tabacum* L. cv. SR (UTC).

# CHAPTER 5: DISCUSSION

## 5.1 *MAN-RF*

It has been demonstrated that resistance gene (*R*) from wild type banana, *Musa acuminata* ssp. *malaccensis* exhibited resistance to Foc race 4 (Ploetz & Pegg, 2000). Based on that, Dumin (2007) has isolated and characterized the highly conserved motif of *R* gene, the NBS domain sequences from this wild type banana. In this current work, *NBS-type RGC* sequence from *Musa acuminata* ssp. *malaccensis*, designated as *MAN-RF* was cloned into *A. tumefaciens* and transformed into leaf disc of *Nicotiana tabacum* L. cv. SR1 and ECS of *Musa acuminata* cv. 'Berangan' for the expression analysis.

### 5.1.1 Analysis of *MAN-RF* cDNA and protein structure

Genes that encode an NBS-containing sequence are common in plant genomes. In the previous study, degenerate primers have been designed and used to isolate NBS-type domain sequence of resistance gene candidate (*RGC*) from *Musa acuminata* ssp. *malaccensis* using PCR method. In this present study, PCR amplification has successfully isolated the NBS- type sequence from *Musa acuminata* ssp. *malaccensis*, namely, *MAN-RF* with cDNA fragment size of 1,160 bp. In the nucleotide BLAST results, *MAN-RF* showed 99% identity to the partial CDS of resistance gene and NBS-type protein of *RGC1* gene from *Musa acuminata* ssp. *malaccensis*, suggesting that *MAN-RF* has the function of resistance gene. *RGC1* expression has been identified to be associated with Foc race 4 resistance lines of *Musa acuminata* ssp. *malaccensis* (Peraza-Echeverria et al., 2008). Meanwhile, referring to Banana Genome Hub database, *MAN-RF* shared 100% identity matches with putative disease resistance protein RPM1 conferring resistance to *Pseudomonas syringae* in *Arabidopsis thaliana*. This indicated that *MAN-RF* might putatively function to confer resistance to *Fusarium*

wilt of banana and bacterial infection. The most conserved motifs for NBS domain in *Musa* species were P-loop (GMGGVGKT), kinase-2 (VLDDVW) and kinase-3 (GLPL) (Azhar & Heslop-Harrison, 2008). The NBS protein domain with these motifs is essential for catalytic activities (Saraste et al., 1990). These conserved motifs were also found in deduced amino acid of MAN-RF and other highly conserved motifs described by Meyers et al. (1999) in *Oryza sativa* and *Arabidopsis*. This suggests that *MAN-RF* may be involved in the catalytic activities. The p-loop was involved in ATP and GTP binding protein by interacting directly with the phosphate of bound NTPs and kinase-2 motif consisting of aspartate residues important for coordinating $Mg^{2+}$ in phospho-transfer reactions in NBS domains (Saraste et al., 1990; Traut, 1994). The NBS sequence was divided into two distinct groups of diverse sequences based on the conserved motifs found within the domain (Traut, 1994). One group comprised of sequences encoding an N-terminal domain with TIR domain including known resistance genes, *N*, *M*, *L6*, *RPP1* and *RPP5* from dicots and totally absent in monocots species. The other group did not encode a TIR domain or non-TIR encoding sequences including the known resistance genes, *RPS2*, *RPM1*, *I2*, *Mi*, *Dm3*, *Pi-B*, *Xa1*, *RPP8*, *RPS5* and *Prf* from monocots and dicots. Two domains within the NBS clearly distinguished TIR and non-TIR sequences which were novel motifs of RNBS-A and RNBS-D where RNBS-D was well-conserved among non-TIR sequences (Meyers et al., 1999). Meanwhile, the kinase-2 domain motif was LVLDDVD in the TIR-NBS-LRR type of *R* gene found in eudicotyledons and LVLDDVW in the CC-NBS-LRR gene found in monocotyledons including *Musa* (Azhar & Heslop-Harrison, 2008). TIR or non-TIR sequence was not identified in the MAN-RF protein sequence. However, kinase-2 motif in protein sequence of MAN-RF was LLILDDVW which was similarly found in CC-NBS-LRR gene in *Musa* species (Azhar & Heslop-Harrison, 2008). This indicated that *MAN-RF* was grouped in non-TIR type sequences.

### 5.1.2 Construction of recombinant plasmid

In this study, plant expression vector, pCAMBIA1304 as a constitutive expression cassette to clone the *MAN-RF* was used. Restriction enzyme of *Bgl*II and *Spe*I were used in the cloning strategy of *MAN-RF* cDNA sequences as these enzymes recognition sites were absent in gene sequences. *MAN-RF* was successfully inserted in the T-DNA region of pCAMBIA1304 between *Bgl*II and *Spe*I to produce recombinant plasmid construct of pCAMBIA1304-MAN-RF. In Figure 4.6, UD1 is the undigested fragment of plasmid pCAMBIA1304 with the top band is the nicked DNA and two bottom band which not separated well is the linear and supercoiled DNA, run faster on gel. Meanwhile, UD2 is the undigested fragment of MAN-RF and as an indicator for the digested MAN-RF with *Bgl*II and *Spe*I to view on gel. Somehow, the size cannot be differentiated by the gel view. The recombinant plasmid was transformed into plant cell culture. The regenerated plantlets were maintained and harvested for gene expression analysis. To date, a single domain of *NBS-type RGC* has not been reported for any plant transformation except using the NBS-LRR region of TIR or non-TIR resistance gene.

### 5.1.3 Development of transgenic tobacco *Nicotiana tabacum* L. cv. SR1

The procedure for the *Agrobacterium*-mediated transformation of tobacco leaf discs has been established (Burow et al., 1990). Tobacco is a model species for genetic transformation as it is a fast-growing plant, ameanable to transform and could be regenerated into transgenic plants. The recombinant plasmid construct of pCAMBIA1304-MAN-RF was successfully transformed into *A. tumefaciens* LBA4404 using freeze-thaw method (Go, 2013). In this present study, for the first time an NBS-type of RGC, *MAN-RF* gene from *Musa acuminata* ssp. *malaccensis* was transferred into tobacco cells using *A. tumefaciens* transformation system. The transferring of

*MAN-RF* resulted in slow regeneration of the transformed leaf discs compared to the positive control transformants (Figure 4.11). As a result, few putative transgenic plantlets were produced with stunted growth compared to the positive control and untransformed control plant. The introduction of *MAN-RF* gene might promote enhances defense response in tobacco plant but inhibited the plant growth as response towards the plant immunity. Plant growth is often inhibited with increased defense response resulting in dwarf phenotypes in occurrence of autoimmune in plant (Zou et al., 2014). Primarily, expression of *R* genes is important for enhancement of defense response and balancing of plant immunity, growth and development (Dangl & Jones, 2001). The mutation of *BON1*, a negative regulator of an NB-LRR-encoding *R* gene *SNC1* (*bon1*) has showed enhanced disease resistance in *Arabidopsis* towards pathogen infection but plant growth showed stunted (Hua et al., 2001). This indicated that the expression of *MAN-RF* in tobacco had effected the growth of tobacco plant.

### 5.1.4 Development of transgenic banana *Musa acuminata* cv. 'Berangan'

Almost all banana and plantain cultivars are highly susceptible to race 4 Foc including *Musa acuminata* cv. 'Berangan' found in tropical peninsular Malaysia (Pérez-Vicente, 2004). Therefore, this banana cultivar was chosen in this study to develop resistant banana to Foc. Most of the previous transgenic work on banana involved transformation of ECS via *Agrobacterium*-mediated transformation (Khanna et al., 2004; Kosky et al., 2010; Mahdavi et al., 2012; Tripathi et al., 2012; Sreedharan, et al., 2013; Mohandas et al., 2013; Ghag et al., 2014). ECS has become an important tool for plant genetic transformation due to its high proliferation potential, minimal genetic instability and single-cell origin which in turn reduces the formation of chimera (Sowmya et al., 2016).

Putative transgenic *Musa acuminata* cv. 'Berangan' plantlets were successfully rooted. *A. tumefaciens* was eliminated by cefotaxime throughout the regeneration of ECS but bacteria contamination occurred in putative transgenic lines during maintenance and multiplication of plantlets in rooting media. As the absence of antibiotic in rooting media, the contamination might be due to the introduction of microbial contaminants from frequent subculture handling. The standard sterilization of tissue culture utensils, preparation of media and aseptic technique application during handling tissue culture could help to reduce or eliminate environmental bacterial and fungal contaminants. Besides that, the co-cultivation of *Musa acuminata* cv. 'Berangan' ECS with *A. tumefaciens* harbouring *MAN-RF* gene was performed in liquid media. Liquid co-cultivation was preferred because it was more efficient than semi-solid co-cultivation in banana as it could reduce the browning of the ECS (Hu et al., 2010). However, over the next regeneration procedure, liquid co-cultivation led to bacterial overgrowth in later regeneration phases (Ghosh et al., 2009). The young leaves from surviving plantlets were harvested for molecular analysis.

### 5.1.5 Expression analysis of *MAN-RF* in putative transgenic *Musa acuminata* cv. 'Berangan'

The presence of *MAN-RF* gene in the genome of transformed plants was analysed based on the genomic DNA of four transformed plants which used as samples in the PCR amplification. The *MAN-RF* and *hpt* gene were observed at the expected size which suggested the presence of *MAN-RF* in banana genome. The confirmation of presence of the *MAN-RF* was also performed in the cDNA of the same plants. PCR results also showed the same expected bands as in banana genome PCR. The plants that gave positive results at PCR from both DNA and cDNA were also analysed using qPCR to determine the *MAN-RF* gene expression level. qPCR results indicated that although

all of these four plants have *MAN-RF* expression, they exhibit different levels of gene expression. As the expression of *MAN-RF* gene was higher than untransformed control plants in qPCR analysis, this indicated that *MAN-RF* gene was successfully expressed in banana plants with normal growth. Different level of *MAN-RF* gene expression was obtained because the transgenic banana is derived from the transformation of banana suspension cells which might have different plant transformation efficiency. But all transgenic banana showed high gene expression compared to the untransformed control plant with small different of expression level. However, due to shortage of transgenic plants sample, further analysis on the Foc infection was not conducted.

## 5.2 *MamWRKY*

In a separate experiment, full length cDNA of WRKY transcription factor has been successfully isolated from *Musa acuminata* ssp. *malaccensis*, named as *MamWRKY*. The *MamWRKY* CDS was transformed into *Nicotiana tabacum* L. cv. SR1 and the gene expression was analysed. This experiment was carried out following previous study by Lim (2006), where the cDNA microarray analysis has identified the WRKY transcription factor as one of transcriptionally responsive gene after Foc infection in tolerant *Musa* ssp. 'Mutiara'.

### 5.2.1 Analysis of *MamWRKY* cDNA and protein sequence

WRKY family contains a large number of members which made it complex to be well understood especially from the non-model plants. *Musa* contains the second largest WRKY family (153 members) next to *Glycine max* (176 members) (D'hont et al., 2012). In the current study, the full-length cDNA of *MamWRKY* has been generated

from partial gene of *MamWRKY* in *Musa acuminata* ssp. *malaccensis*. A full-length cDNA comprises of 5' and 3' end of untranslated regions (UTRs). However, obtaining full-length cDNAs with accurate sequences from the 5' end through to the 3' end can be challenging. In order to obtain the full-length cDNA of *MamWRKY* gene, 3' and 5' RACE PCR method was used. RACE is the most effective method as it is simple and quick to obtain the ends of cDNAs when only partial sequences are available (Zhang, 2003). The full-length *MamWRKY* cDNA resulted in 1,224 nucleotides from the extended 3' and 5' RACE PCR of partial sequence. The completion of full-length *MamWRKY* cDNA was identified when poly-A tail and adapter sequences were obtain at 3' and 5' ends, respectively. The full length sequence of MamWRKY gDNA comprises of two introns, 98 bp and 92 bp lengths which were determined by comparing the sequence alignment of genomic DNA and cDNA of *MamWRKY*. Although *MamWRKY* cDNA sequence showed localization matches on 3, 4, 5, 7, 8, 9, 10 and 11 chromosomes, the highest percentage of identity was showed with putative *WRKY69* (1,223 bp coverage) at chromosome 4, spanning from 11,741,594 to 11,742,644 bp. The genome-wide analysis of the WRKY gene in *Musa acuminata* by Goel et al. (2016) also reported that *WRKY* genes abundantly present on chromosome 4 and 7.

While, the first *WRKY* gene isolated from banana was *MusaWRKY71* by Shekhawat at al. (2011). This gene obtained via RACE has resulted in 843 nucleotides of CDS that encodes a protein with 280 amino acids with predicted molecular weight of 30.69 kDa and a theoretical p*I* of 8.80 (Shekhawat et al., 2011). Similarly, *MamWRKY* CDS obtained in this present study have few nucleotides differences which consisted of 861 nucleotides that encode a putative protein of 286 amino acids with predicted molecular weight of 30.16 kDa and a theoretical p*I* of 5.05. Within this protein sequences, a single WRKY domain and zinc finger motif (CSSSKGCPARKQVERSRVDPNVIVVTYAFDHNH) were observed which

comprised of 60 amino acids. These characteristics of WRKY are consistent with description by Rushton et al. (2010) which stated that WRKY gene consist of conserved 60 amino acids, including WRKYGQK at the N-terminus and a novel zinc-finger motif, either $Cx_{4-5}Cx_{22-23}HxH$ (C2-H2) or $Cx_7Cx_{23}HxC$ (C2-HC). WRKY gene family members were classified into three groups according to WRKY and zinc finger domains. Group I consists of two WRKY domains while Group II contains only one WRKY domain with both of these groups have same zinc finger motif pattern of C2-H2 but was further classified into five (a–e) subgroups (Eulgem et al., 2000). Group III consists of one WRKY domain with a different zinc finger motif pattern of C2-HC. In *Musa acuminata*, 24 genes were categorized in Group I whereas 106 and 16 identified as members of Group II and Group III, respectively. Meanwhile, the *MamWRKY* genes present in Group IIa, Group IIb, Group IIc, Group IId, and Group IIe contains of 11, 21, 32, 25, and 17 members respectively (Goel et al., 2016). MamWRKY protein in this study is classified as Group IIe based on a single WRKY domain and zinc finger motif pattern of C2-H2 which might be also one of 17 members of WRKY genes in *Musa acuminata*.

### 5.2.2 Construction of recombinant plasmid

In this study, plant expression vector, pCAMBIA1304 was used as a constitutive expression cassette to clone *MamWRKY*. Restriction enzyme of *Bgl*II and *Spe*I were used to digest the *MamWRKY* cDNA sequences as these enzymes recognition sites were absent in gene sequences. *MamWRKY* was successfully inserted in the T-DNA region of pCAMBIA1304 between *Bgl*II and *Spe*I to produce recombinant plasmid construct of pCAMBIA1304-MamWRKY.

### 5.2.3 Development of transgenic tobacco *Nicotiana tabacum* L. cv. SR1

The recombinant plasmid construct of pCAMBIA1304-MamWRKY was successfully transformed into *A. tumefaciens* LBA4404 using freeze-thaw method (Go, 2013). In this work, the regulation of defense-related gene associated with constitutive expression of *MamWRKY* gene was analysed. Interestingly, the transgenic tobacco overexpressing *MamWRKY* did not showed any flowering compared to the positive control and untransformed control plants. The WRKY proteins function by binding to the W-box (TTGACC/T) present in the target promoters of genes involved in a variety of developmental and physiological processes (Rushton et al., 2010). It has been demonstrated that *AtWRKY6*, *AtWRKY53*, *GsWRKY20*, *MlWRKY12* and *OsWRKY11* were all involved in the determination of flowering time (Robatzek & Somssich, 2002; Miao et al., 2004; Luo et al., 2013; Yu et al., 2012; Cai et al., 2014). This result suggested that the *MamWRKY* transcription factor was inhibited the flowering in tobacco (refer 5.2.4) and might be negative regulator of flowering gene.

### 5.2.4 Expression analysis of *MamWRKY* CDS in putative transgenic *Nicotiana tabacum* L. cv. SR1

The transformed tobacco plants that were tested with GUS histochemical assay showed blue colored on the leaves surfaces while the untransformed leaves plants remained colorless indicated the transferred of *MamWRKY* in the tobacco leaves. The presence of *MamWRKY* gene in the genome of transformed plants was analysed based on the genomic DNA of four transformed plants that gave positive results in GUS histochemical assay as samples in the PCR amplification. The *MamWRKY* and *hpt* gene were observed at the expected size which confirmed the integration of *MamWRKY* in banana genome. The confirmation of *MamWRKY* integration in plants was also performed in the cDNA of the same plants. PCR results also showed the same expected

bands as in banana genome PCR. The plants that showed positive results in the semi-quantitative PCR from both DNA and cDNA were proceed with qPCR analysis to determine the *MamWRKY* gene expression level. qPCR results indicated that all of these three plants exhibited high level of *MamWRKY* expression of about 809, 740 and 1154-fold changes compared to the untransformed control plants. This result demonstrated that *MamWRKY* was successfully overexpressed in tobacco plants.

All of the transgenic tobacco lines showed no visible phenotypic changes except for non-flowering physiological change compared to positive control and untransformed control plants (Figure 5.1) which were correlated with lines exhibited constitutive high elevated levels of *MamWRKY* transcript (Table 4.2). This overexpression of *MamWRKY* probably has downregulated the genes involved in the flowering pathway. Alternatively, this could be due to the absence of W-boxes in the promoter region of flowering genes and overexpression also causes deleterious effects on plant growth and development. It was shown in *Arabidopsis* that *AtWRKY70*-overexpressing lines produced smaller plant size than control plants and exhibited changes in morphology with delayed flowering (Li et al., 2004). Correspondingly, *Dlf1* gene in rice was found to encode a WRKY transcription factor, *OsWRKY11* that acted as a trans-activator to downregulate *Ehd2*/*RID1*/*OsId1* and caused late flowering (Cai et al., 2014). On the contrary, *WRKY71* in *Arabidopsis* was shown to bind to the W-boxes in the FLOWERING LOCUS T and LEAFY promoters in vitro and in vivo that accelerated flowering (Yu et al., 2016).

(a) (b)

**Figure 5:1:** (a) Transgenic tobacco overexpressing *MamWRKY* without flower; (b) Untransformed control tobacco with flower.

### 5.2.5 Expression of putative *MamWRKY* target gene, *PR1a* in *MamWRKY* overexpressing lines

Although WRKY transcription factor had shown up-regulated expression during the infection of Foc, the role of *MamWRKY* to regulate defense-related gene in banana plant have not been investigated. The transgenic banana cannot be developed due to low transformation efficiency from two years old ECS culture. Thus, to understand the role of *MamWRKY* in regulating defense-related gene, the identification of defense-related gene which expression is modulated by *MamWRKY* overexpression was conducted. Many WRKY proteins have been found to bind selectively to the W-box motif (Eulgem et al., 2000) that is present in the promoter regions of a large number of defense genes, including the tobacco class I chitinase gene (*CHN50*) (Fukuda & Shinshi, 1994), parsley *PR1* gene (Rushton et al., 1996) and the *Arabidopsis NPR1* gene (Yu et al., 2001). It has thus been suggested that WRKY proteins might generally regulate defense-related genes in the plant kingdom. In this study, it was hypothesized that *MamWRKY* played an

important role in response to biotic stress. To support the hypothesis, the function of *MamWRKY* was analyzed by overexpressing it in tobacco plants and analyzing the expression of a pathogenesis-related gene, *PR1a*, an inducible defense gene which usually accumulates after *Tobacco mosaic virus* (TMV) infection. It has been reported that in tobacco, expression of *tWRKY3* and *tWRKY4* which encode one WRKY domain were rapidly induced when infected with TMV (Chen & Chen, 2000). The same effect was shown by the partial *MamWRKY* in *Musa* ssp. 'Mutiara' where its expression was up-regulated upon infection of Foc in the roots (Lim, 2006). *MamWRKY* was expected to give the same effect with banana infected by Foc. The finding in this study showed that *PR1a* transcript was higher in transgenic lines than in the untransformed plants, indicating *MamWRKY* might be a positive regulator for *PR1a* gene expression. In contrast, the overexpression of *MamWRKY* in W2 transgenic line was not seen to regulate *PR1a* gene expression compared to other transgenic lines. The results showed that the regulation of *PR1a* gene expression varied among the transgenic lines. The gene expression was related to the specific binding of *MamWRKY* to the W-box of *PR1a* gene promoter. Previously, van Verk et al. (2008) successfully identified the specific binding site W-box, which was induced by salicylic acid and bacterial elicitors, for *NtWRKY12* in the promoter of *PR1a* gene. In addition, two W-boxes were identified in the region between -125 and -69 in tobacco class 1 chitinase gene *CHN48* (Yamamoto et al., 2004). These W-boxes were found to be mediated by *NtWRKY1* and *NtWRKY4*. Hence, it was possible that *MamWRKY* was able to bind to the W-box element even without the induction by exogenous factor such as salicylic acid (van Verk et al., 2008).

## 5.3 Challenges and future recommendations

### 5.3.1 Improved cell culture quality for plant transformation

Throughout the research, the tobacco transformation for both genes, *MAN-RF* and *MamWRKY* resulted in low regeneration rate and produce low quantity of transgenic plants. This problem has led to insufficient samples for further analysis. In order to overcome this problem, a report has suggested that the use of six and seven weeks old plantlets are the best explant to induce callus for tobacco transformation because the leaf disc could produce good callus formation of the transgenic plants using *Agrobacterium* method (Kutty et al., 2011). It also reported that 5 weeks-old plantlets resulted in very low transformation compared to 7 weeks-old plantlets which showed high frequency of transformation. This suggested that the used of leaf disc from 8 weeks-old plantlets in this study has contributed to the low regeneration of the transformants.

The established ECS culture can result in somaclonal variation, microbial contamination and total loss of morphogenic capacity in a prolonged culture period (Strosse et al., 2006). The efficiency of *Agrobacterium* mediated transformation of ECS was subjected to several parameters including *Agrobacterium* strain, age of the ECS, time of infection, time of co-culture and bacterial cell density, the use of semi-solid or liquid medium for co-cultivation and *Agrobacterium vir* gene inducing compounds like acetosyringone (Khanna et al., 2004; Arinaitwe et al., 2004; Ghosh et al., 2009). The present study has utilized ECS from two years old cultures for the transformation which produced low regeneration rate of callus and transgenic plants. Cryopreservation therefore is an essential tool for the safe storage of established ECS. Cryopreservation is a long-term storage in liquid nitrogen at -196 ºC which could retained morphogenetic potential of ECS, eliminate the requirement of frequent subculturing and also the possibility of contamination by microbes (Panis et al., 1990; Sipen et al., 2011). It has

been applied by Strosse et al. (2006) where all the successfully established ECS were safely stored in liquid nitrogen using the method of Panis et al. (1990) for future used in genetic engineering of banana and plantains.

### 5.3.2 Candidate gene insert

For the development of transgenic plants with *MAN-RF* gene, there is a need to isolate the complete genes sequence for functional analysis study. The majority of R genes cloned encode proteins with a NBS and a LRR region (Bouktila et al., 2015). Furthermore, very little is known about regulation of their expression since most findings are focusing on the identification of new *R* gene sequences and the evolution of this large family. With access to the full-genome sequence of banana, NBS-LRR resistance genes can be identified and annotated through genome-wide analysis. Then the development of transgenic plant expressing resistance gene could be significant for functional analysis study.

Further evidences are needed to verify whether *PR* genes are direct targets of *MamWRKY* in conferring disease resistance in banana plant. The involvement of WRKY in response to biotic and abiotic stresses was linked to various regulatory processes (Eulgem et al., 2000). A large number of *WRKY* genes are induced by pathogens or plant defense signal molecules. Previous report has shown that, MusaWRKY71 was upregulated when treated with elicitor molecules of biotic stress response pathways like ethylene, salicylic acid and methyl jasmonic (Shekhawat & Ganapathi, 2013). This experiment can be applied to look for differential expression of genes which are known to be involved in Foc response pathways in banana.

# CHAPTER 6: CONCLUSION

*Fusarium* wilt caused by soil-borne fungus *Fusarium oxysporum* f. sp. *cubense* (Foc) is one of the most important threats to global banana production. The present study aimed to overexpress either *NBS-type RGC* or *WRKY* transcription factor genes in *Musa acuminata* cv. 'Berangan' to produce Foc-resistant transgenic bananas. Prior to transformation, the *NBS-type RGC* (*MAN-RF*) and *WRKY* transcription factor (*MamWRKY*) genes isolated from *Musa acuminata* ssp. *malaccensis* were molecularly characterized.

cDNA of *MAN-RF* was found to consist of 1,160 bp nucleotide with the presence of highly conserved motifs of resistance gene described by Meyer et al. (1999). These motifs are kinase-1/p-loop (GMGGVGKTT), kinase-2 (LLILDDVW), kinase-3a (GSRIVITTRL), kinase-3 (GLPLAI), RNBS-A and RNBS-D, which have been found to be important in catalytic activities. On the other hand, full-length gDNA and cDNA of *MamWRKY* isolated from *Musa acuminata* ssp. *malaccensis* using RACE were 1,224 bp and 1,414 bp in length, respectively. The *MamWRKY* CDS encoding 286 amino acids was predicted to have a molecular mass of 30.16 kDa and a theoretical p*I* of 5.05. The *MamWRKY* consisted of WRKY domain sequence and the structural features matched with group IIe in the WRKY proteins family.

Both *MAN-RF* and *MamWRKY* were ligated and cloned in pCAMBIA1304 before introduced into *Agrobacterium tumefaciens* LBA4404. *Agrobacterium* harbouring pCAMBIA1304-MAN-RF was used to infect and co-cultivate with tobacco and *Musa acuminata* cv. 'Berangan'. Since most of the *MAN-RF* transformed tobacco tissues showed stunted growth, therefore there are insufficient samples to proceed with molecular analysis. Putative transgenic *Musa acuminata* cv. 'Berangan', however, was verified by PCR for the transgene integration, whereas the expression of *MAN-RF* was performed using qPCR. The results showed an elevated expression of *MAN-RF* in

transgenic *Musa acuminata* cv. 'Berangan' compared to untransformed plants. However, functional analysis of *MAN-RF* was unable to carry out due to the shortage of samples after bacterial contamination. The transformed banana plants did not show any abnormality compared to untransformed plants.

For *MamWRKY*, CDS of this gene was introduced to leaf discs of *Nicotiana tabacum* L. cv. SR1 via *Agrobacterium*-mediated transformation. GUS histochemical assay and PCR analysis confirmed the stable integration for putative transgenic tobaccos. The transcript level of *MamWRKY* was highly expressed in three transgenic lines compared to untransformed plants. Since transgenic banana cannot be established due to low regeneration efficiency of two year-old embryogenic cell suspension culture, an attempt to identify downstream target genes of *MamWRKY* was performed. To study the differential regulation of downstream target of *MamWRKY*, specific primers targeting *PR1a* was used in quantitative qPCR. *PR1a* gene, a pathogen-responsive gene, showed higher expression in transgenic lines than the untransformed plants. This finding suggests that gene expression of *PR1a* is related to the specific binding of *MamWRKY* to the W-box of *PR1a* gene promoter and positively modulate *PR1a* expression. Interestingly, *MamWRKY*-overexpressing transgenic tobacco lines did not produce flowers compared to positive control and untransformed plants. It was suggested that promoter region of the flowering genes might not be targeted by *MamWRKY* and their overexpression also causes deleterious effects on plant growth and development.

In conclusion, the successful establishment of transgenic plants expressing *MAN-RF* and *MamWRKY* could be useful for gene functional study. The data generated from this study could serve as a reference to further identify full-length gene of *MAN-RF* and to understand the group IIe of other *WRKY* genes in more details. In addition, further study for the regulation of *MamWRKY* on *PR1a* and other defense-related genes might be important to understand the pathogen infection mechanism.

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# APPENDIX

## Appendix A: Sterilization

1) All glassware, micropipette tips, microcentrifuge tubes, PCR tubes, stock solutions, buffers and medium were autoclaved at 121 ºC with 15 psi for various of time according to different purpose of works:

   i. RNA works : 45 minutes

   ii. DNA works : 20 minutes

   iii. Bacteria & tissue culture works : 15 minutes

2) All glassware, micropipette tips, microcentrifuge tubes, PCR tubes, stock solutions, buffers and medium were autoclaved at 121 ºC with 15 psi for various of time according to different purpose of works:

   iv. RNA works : 45 minutes

   v. DNA works : 20 minutes

   vi. Bacteria & tissue culture works : 15 minutes

3) The bench and all materials for DNA, bacteria and plant tissue culture works was swab with 70% (v/v) ethanol before start works.

4) The bench and all materials for RNA works was swab with RNase Away for once and followed with 70% (v/v) ethanol before start works.

5) All tissue culture works were carried out in a laminar flow.

## Appendix B: Stock solutions, buffers and medium

### Appendix B1: Stock solutions buffers and medium for DNA and RNA works

1) Diethyl pycarbonate (DEPC)-treated water

   i. In a fume hood,1 mL DEPC was added to 1 L distilled water. (the bottle must be autoclave for 45 minutes before using).

ii. The bottle was capped nicely and shakes for a minute. The solution was left overnight in the fume hood.

iii. It was autoclaved in the next day. There should be no smell of DEPC in the treated water after autoclaving and kept in an RNase-free area.

2) Cetyltrimethylammonium bromide (CTAB) buffer

i. All the components below were add and mix accordingly:

- CTAB 2.0 g
- PVP 2.0 g
- NaCl 8.0 g
- $dH_2O$ 50.0 mL
- EDTA (0.5 M, pH 8) 5.0 mL
- DEPC-treated water 55 µL

ii. Stir and leave it overnight. Another day, shake and autoclave it.

iii. After autoclaved, 10 mL Tris 1 M pH 8.0 was added.

iv. Then, the solution was added up to 100 mL with DEPC-treated $H_2O$.

3) 70% (v/v) ethanol

i. The solution below was added and mixed well before kept in 4 ºC:

- Absolute ethanol 70 mL
- Sterile distilled $H_2O$ 30 mL

4) Other solutions preparation are as follows:

| **Solution** | **Materials and Methods** |
|---|---|
| i) 0.5M Tris pH 8.0 (50 mL) | 1) 7.882 g of Tris base was added up to 30 mL $dH_2O$<br>2) Then, Tris base was titrated with HCl to pH 8.0 that made up Tris-Cl.<br>3) The volume was adjusted to 50 mL with $dH_2O$.<br>4) Autoclave.<br>**Tris cannot be dissolved in DEPC-treated water because Tris will react with DEPC.*<br>*(Source: Wikipedia)* |
| ii) 3M NaOAc pH 5.2 (50 mL) | 1) 81.648 g NaOAc powder was dissolved in 180 mL DEPC-T $dH_2O$. It was stirred vigorously on a magnetic stirrer.<br>2) The pH was adjusted to 5.2 with glacial acetic acid.<br>3) Then, the solution was added with DEPC-T $dH_2O$ up to 200 mL<br>4) Autoclave. |
| iii) 0.5M EDTA pH 8.0 (100 mL) | 1) 11.462 g EDTA powder was diluted into 80 mL DEPC-T $dH_2O$. It was stirred vigorously on a magnetic stirrer.<br>2) The pH was adjusted to 8.0 with NaOH.<br>3)Then, the solution was added with DEPC-T $dH_2O$ up to 100 mL.<br>4) Autoclave. |
| iv)Chloroform : Isoamylalcohol (24:1) | 1) 240 mL chloroform was added with 10 mL isoamylalcohol in an autoclaved bottle. |

| | |
|---|---|
| (250 mL) | 2)The bottle was wrapped with aluminium foil. |

**Appendix B2: Stock solutions and medium preparation for bacterial culture**

1) Luria-Bertani (LB) broth

About 2 g of LB broth powder was dissolved in 100 mL of $dH_2O$. After autoclaved, the medium was kept at room temperature. Appropriate concentration of antibiotic was added for corresponding experiment.

2) Luria-Bertani (LB) agar

About 35 g of LB agar powder was dissolved in 1 L of $dH_2O$. After autoclaved, the medium was cooled down at about 50 ºC and added with appropriate concentration of antibiotic for corresponding experiment. The agar was allowed to solidify in sterile Petri dish. All solidified agar plates were kept at 4 ºC prior to use.

3) Calcium chloride ($CaCl_2$) 2.5 M

i. $CaCl_2$ powder, 5.55 g was dissolved in 20 mL $dH_2O$

ii. It was then filter sterilized through 0.22 μm filter and aliquot in microcentrifuge tubes. Then kept at 4 °C.

**Appendix B3: Stock solutions for plasmid preparation**

1) Alkaline Lysis Solution I

i. The solutions below was added and mixed well before autoclave and kept at 4 ºC.:

- 50 mM glucose

- 25 mM Tris-Cl (pH 8.0)
- 10 mM EDTA (pH 8.0)

2) Alkaline Lysis Solution II

i. The solutions below were added and mixed well:
   - 0.2 N NaOH
   - 1% (w/v) SDS
   - Sterile distilled water

ii. This solution was prepared freshly from the stock solutions during experiment

3) Alkaline Lysis Solution III

i. The solutions below were added and mixed well:
   - 5 M potassium acetate 60 mL
   - Glacial acetic acid 11.5 mL

ii. The mixture was adjusted to 100 mL with sterile distilled $H_2O$, autoclaved and stored at 4 ºC.

4) Glucose

i. Glucose powder, 19.82 g was dissolved in 100 mL $dH_2O$. It was stirred vigorously on a magnetic stirrer.

ii. It was then filter sterilized through 0.22 µm filter and stored at room temperature.

5) NaOH (10 M)

i. NaOH pellets, 40 g were dissolved in 80 mL $dH_2O$. It was stirred vigorously on a magnetic stirrer.

ii. When the pellets have dissolved completely, the solution was added with $dH_2O$ up to 100 mL.

iii. Sterilization is not necessary and the solution was kept at room temperature.

6) SDS (10%)

i. SDS powder, 25 g was dissolved in 200 mL $dH_2O$.

ii. The mixture was heated at 68 °C and stirred vigorously on a magnetic stirrer.

iii. The pH was adjusted to 7.2 with HCl.

iv. Then, the solution was added with $dH_2O$ up to 250 mL

v. Sterilization is not necessary and the solution was kept at room temperature.

**Appendix B4: Stock solutions and buffers for gel electrophoreses**

1) 5X TBE buffer for DNA use

| | |
|---|---|
| Tris base | 54g |
| Boric acid | 27.5g |
| 0.5 M EDTA (pH 8.0) | 20 mL |
| Distilled $H_2O$ to | 1000 mL |

i. Tris base, boric acid and EDTA were dissolved in distilled $H_2O$ and adjusted to the final volume of l000ml before sterilization. Then, the sterile solution was kept at room temperature.

ii. The solution was diluted to 1X working concentration for agarose gel electrophoresis.

2) 5X TBE buffer for RNA use

i. The solution was prepared as mentioned above and adjusted to the final volume of l000 mL before sterilization. Then, the sterile solution was kept at room temperature.

ii. The solution was diluted to 1X working concentration for agarose gel electrophoresis.

3) 1% agarose gel (v/v)

About 1 g of the agarose powder was dissolved in added into 100 mL of 1X TBE buffer by heating in a microwave at medium high setting for about 2 minutes. Approximately 5 µL of ethidium bromide were added into the gel and allowed to solidify at room temperature.

**Appendix B5: Stock solutions buffers and medium for plant tissue culture**

1) All the stock solutions to preapare tissue culture medium were prepared as in the table below:

| **Stock Solution** | **Ingredients** |
|---|---|
| Macroelements (10X)<br>1000 mL | 16.50 g of $NH_4NO_3$<br>19.00 g of $KNO_3$<br>4.40 g of $CaCl_2.2H_2O$<br>3.70 g of $MgS0_4.7H_2O$<br>1.70 g of $KH_2PO_4$<br>The powder was dissolved in $dH_2O$. Later, $dH_2O$ was added up to 1000 mL. |
| Microelements (100X)<br>500 mL | 0.0415 g of KI<br>0.310 g of $H_3BO_3$<br>0.845 g of $MnSO_4.H2O$<br>0.430 g of $ZnSO_4.7H2O$<br>0.0125 g of $Na_2MoO_4.2H2O$<br>0.0013 g of $CuSO_4.5H_2O$<br>0.0013 g of $CoCl_2.6H_2O$<br>The powder was dissolved in $dH_2O$. Later, $dH_2O$ was added up to 500 mL. |
| Iron source (100X)<br>500 mL (light sensitive) | 1.39 g of $FeSO_4.7H_2O$<br>1.865 g of $Na_2EDTA.2H_2O$<br>The powder was dissolved in $dH_2O$. Later, $dH_2O$ was added up to 500 mL. The solution was covered with aluminium foil. |
| Vitamins (100X)<br>500 mL (light sensitive) | 0.025 g of Nicotinic acid<br>0.025 g of Pyridoxine<br>0.025 g of Thiamine<br>0.100 g of Glycine<br>5.000 g of Myoinositol (add later)<br>The powder was dissolved in $dH_2O$. Later, $dH_2O$ was added up to 500 mL. The solution was covered with aluminium foil. |

| | |
|---|---|
| NAA (0.1 mg/mL) 250 mL | 0.025 g of NAA powder was dissolved in 1 M NaOH. Later, $dH_2O$ was added up to 250 mL. |
| BAP (0.1 mg/mL) 250 mL | 0.025 g of BAP powder was dissolved in 1 M NaoH. Later, $dH_2O$ was added up to 250 mL. |
| 2, 4-D (0.5 mg/mL) 250 mL | 0.125 g of 2, 4-D was dissolved in alcohol drops. Later, $dH_2O$ was added up to 250 mL. |
| Biotin (0.1 mg/mL) 250 mL | 0.025 g of biotin powder was dissolved in warm $dH_2O$ and later $dH_2O$ was added up to 250 mL. |

2) Co-culture medium

i. The components below were added accordingly:

| **Ingredients** | **Amount in 100 mL** |
|---|---|
| MS medium powder | 0.44 g |
| Sucrose | 3 g |
| NAA (1 mg/mL) | 10 µL |
| 6-BA (1mg/mL) | 100 µL |

ii. The ingredients were dissolved in 80 mL distilled $H_2O$ and stirred continuously. The pH was adjusted to 5.7 and $dH_2O$ was added up to 100 mL before autoclaved for sterilization.

iii. The sterile medium was stored at 4ºC.

3) Tobacco shoot multiplication medium (TSM)

i. The components below were added accordingly:

| **Ingredients** | **Amount in 1 L** |
|---|---|
| MS medium powder | 4.4 g |
| Sucrose | 30 g |
| NAA (1 mg/mL) | 100 µL |
| 6-BA (1mg/mL) | 1000 µL |
| Gel rite | 2.2 g |

ii. The ingredients were dissolved in 800 mL distilled $H_2O$ and stirred

continuously. The pH was adjusted to 5.7 and $dH_2O$ was added up to 1 L before adding gel rite and autoclaved.

iii. The sterile medium was stored at 4 ºC.

4) Tobacco root multiplication medium (TRM)

The medium is prepared as stated in TSM medium without 6-BA and aliquoted into sterile tissue culture jars before sealed with parafilm and kept at 4 ºC.

5) M2 medium

iv. The components below were added accordingly:

| **Ingredients** | **Amount in 1 L** |
|---|---|
| MS- Macroelements (10X) | 100.00 mL |
| MS- Microelements (100X) | 10.00 mL |
| MS- FeEDTA (100x) | 10.00 mL |
| MS- Dhed'a Vitamins (100X) | 10.00 mL |
| Ascorbic acid | 10.00 mL |
| 2, 4-D (1.0 mg/mL) | 2.2 mL |
| Sucrose | 20.00 g |
| Myoinositol | 0.10 g |
| Zeatin (added after autoclave-heat sensitive) | 0.25 mL |

v. The pH was adjusted to 5.7 and autoclaved.

vi. After autoclave, the medium was allowed to be cooled down at room temperature before zeatin was added in sterile condition. The medium was kept at 4 ºC.

6) M3 medium

i. The components below were added accordingly:

| **Ingredients** | **Amount in 1 L** |
|---|---|
| MS- Macroelements (10X) | 100.00 mL |
| MS- Microelements (100X) | 10.00 mL |
| MS- FeEDTA (100xX | 10.00 mL |

| | |
|---|---|
| Vitamins (100X) | 10.00 mL |
| Sucrose | 20.00 g |
| Myoinositol | 0.10 g |

ii. All the ingredients above were added together and diluted with $dH_2O$ up to 800 mL.

iii. The pH of the mixture was measured and adjusted to pH 5.7 and $dH_2O$ was added up to 1 L before autoclaved for sterilization.

iv. The autoclaved medium was cooled before L-glutamine (400 mg/L), hygromycin (0.2 $mgL^{-1}$) and cefotaxime (50 µg/mL) were added into the medium during the experiment in sterile condition. Before the experiment, the medium was kept at 4 ºC.

7) M4 medium supplemented with 8 mg/L BAP (M8B)

| **Ingredients** | **Amount in 1 L** |
|---|---|
| MS- Macroelements (10X) | 100.00 mL |
| MS- Microelements (100X) | 10.00 mL |
| MS- FeEDTA (100X) | 10.00 mL |
| Vitamins (100X) | 10.00 mL |
| BAP (1 mg/mL) | 8 mL |
| Sucrose | 30.00 g |
| Myoinositol | 0.10 g |
| Gel rite | 2.20 g |

i. All the ingredients above were added together and diluted with $dH_2O$ up to 800 mL.

ii. The pH of the mixture was measured and adjusted to pH 5.7. Distilled water was added up to 1 L before adding gel rite and autoclaved for sterilization.

iii. After autoclaved, the medium was cooled down at about 50 ºC and added with appropriate concentration of antibiotic for corresponding

experiment. The agar was allowed to solidify in sterile Petri dish. All solidified agar plates were kept at 4 ºC prior to use.

8) M4 medium supplemented with 4 mg/L BAP (M4B)

The medium was prepared as above (M8B) but with the addition of 4 mg/mL BAP. About 10 mL medium was aliquot into sterile white capped bottle and kept at room temperature.

9) M4 medium supplemented with 2 mg/L BAP (M2B)

The medium was prepared as above (M8B) but with the addition of 2 mg/mL BAP. About 50 mL medium was aliquot into sterile jar and kept at room temperature.

10) M5 (rooting medium)

| **Ingredients** | **Amount in 1 L** |
|---|---|
| MS- Macroelements (10X) | 100.00 mL |
| MS- Microelements (100X) | 10.00 mL |
| MS- FeEDTA (100X) | 10.00 mL |
| Vitamins (100X) | 10.00 mL |
| Sucrose | 30.00 g |
| Charcoal | 8.0 g |
| Gel rite | 2.20 g |

i. All the ingredients above were added together and diluted with $dH_2O$ up to 800 mL.

ii. The pH of the mixture was measured and adjusted to pH 5.7. Distilled water was added up to 1 L before adding gel rite and autoclaved for sterilization.

iii. After autoclaved, the medium was cooled down at about 50 ºC and added with appropriate concentration of antibiotic for corresponding

experiment. The agar was allowed to solidify in sterile jar. All solidified agar plates were kept at room temperature prior to use.

11) GUS assay buffer

i. 0.2 M $NaPO_4$ buffer pH 7.0

0.2 M $NaH_2PO_4$ (A) : 3.12 g of $NaH_2PO_4$ powder was diluted in 100 mL $dH_2O$

0.2 M $Na_2HPO_4$ (B) : 2.84 g of $Na_2HPO_4$ powder was diluted in 100 mL $dH_2O$

39 mL Solution (A) was added with 61 Solution (B) to make up 100 mL 0.2 M Then, the pH was adjusted to 7.0.

ii. 0.1M $K_3[Fe(CN)_6]$

| | |
|---|---|
| $K_3[Fe(CN)_6]$ | 3.293 g |
| Sterile distilled $H_2O$ to | 100 mL |

Potassium ferricyanide was dissolved in 80 mL sterile distilled $H_2O$ before adjusted to final volume of 100 mL. The solution was kept at 4ºC.

iii. 0.1M $K_4[Fe(CN)_6].3H_2O$

| | |
|---|---|
| $K_4[Fe(CN)_6].3H_2O$ | 3.293 g |
| Sterile distilled $H_2O$ to | 100 mL |

Potassium ferrocyanide was dissolved in 80 mL sterile distilled $H_2O$ before adjusted to final volume of 100ml. The solution was kept at 4 ºC.

iv. 20 M X-gluc

| | |
|---|---|
| X-gluc | 20mg |
| DMSO | 1 mL |

X-gluc was dissolved in DMSO and stored at -20 ºC before use. It is light sensitive.

v. 0.5% Triton X-100

Triton X-100 50 µL

Sterile distilled $H_2O$ to 10 mL

Triton X-100 was mixed with sterile distilled $H_2O$ and kept at 4 ºC.

vi. GUS histochemical buffer

| Components | Working concentration | |
|---|---|---|
| | Concentration | Volume, µL |
| 0.2 M $NaPO_4$ buffer (pH 7.0) | 0.1 M | 5000 |
| 0.1M $K_3[Fe(CN)_6]$ | 0.5 mM | 50 |
| 0.1M $K_4[Fe(CN)_6].3H_2O$ | 0.5 mM | 50 |
| 0.5M EDTA (pH8.0) | 10 mM | 200 |
| 20M X-gluc | 1 M | 500 |
| 0.5% Triton X-100 | 0.1% | 2000 |
| Methanol | 20% (v/v) | 2000 |
| Sterile distilled $H_2O$ | - | 200 |

All the components were added accordingly and kept at 4 ºC.

vii. FAA solution (fixing purpose solution after GUS histochemical assay was done:

| **Components** | **Volume** |
|---|---|
| Absolute ethanol | 45 mL |
| Glacial acetic acid | 5 mL |
| Formaldehyde | 5 mL |
| $dH_2O$ | 45 mL |

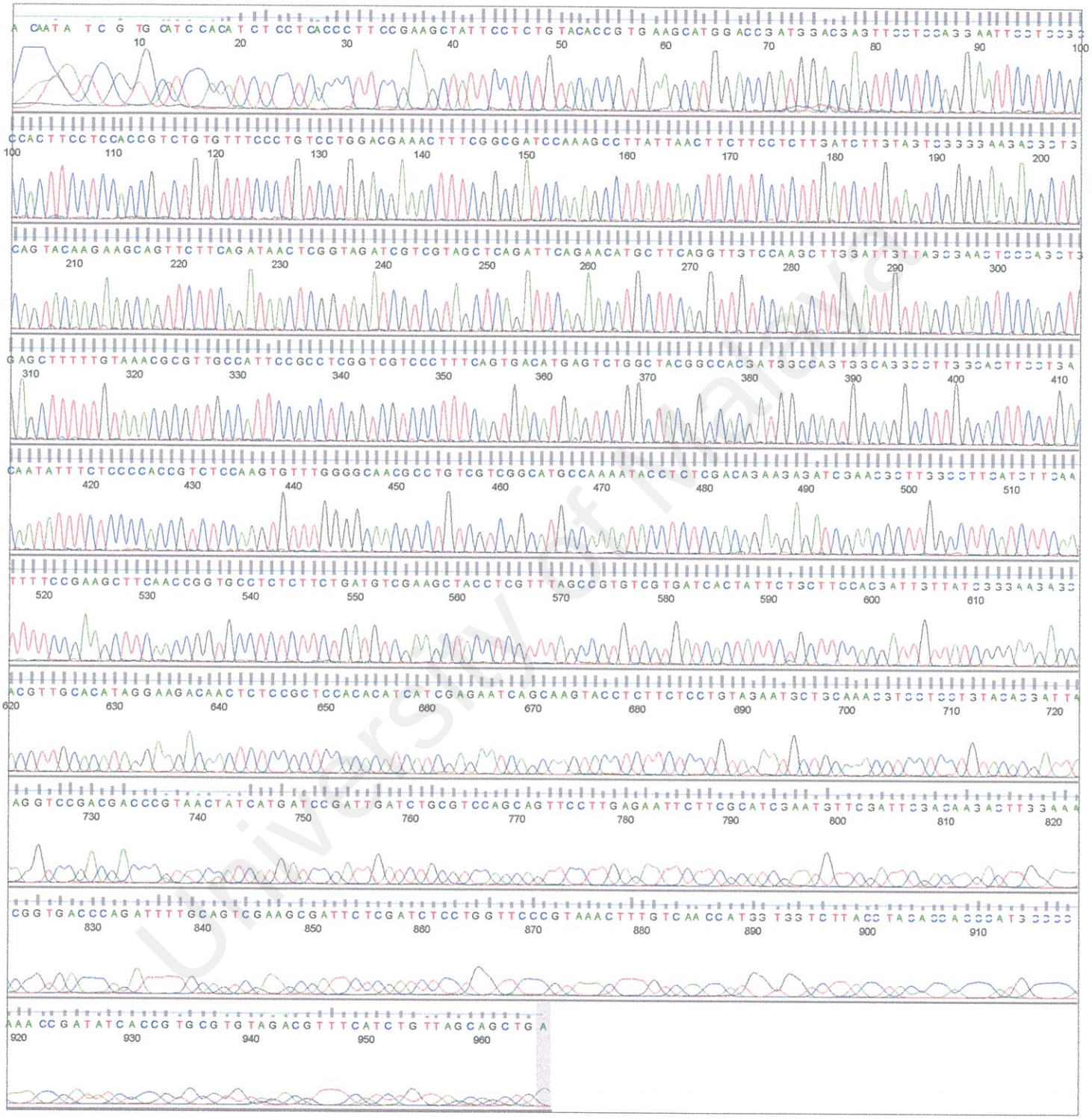
All the components were added accordingly and mix well. Then, it was autoclaved and kept at room temperature.

**File: Appendix C1- Partial MAN-RF cDNA sequences.ab1**

Geospiza
www.geospiza.com

Sample Name: Rb_rev
Mobility: KB_3730_POP7_BDTv3.mob
Spacing: 14.6724
Comment: n/a

Signal Strengths: A = 1643, C = 2246, G = 1648, T = 1823
Lane/Cap#: 25
Matrix: n/a
Direction: Native

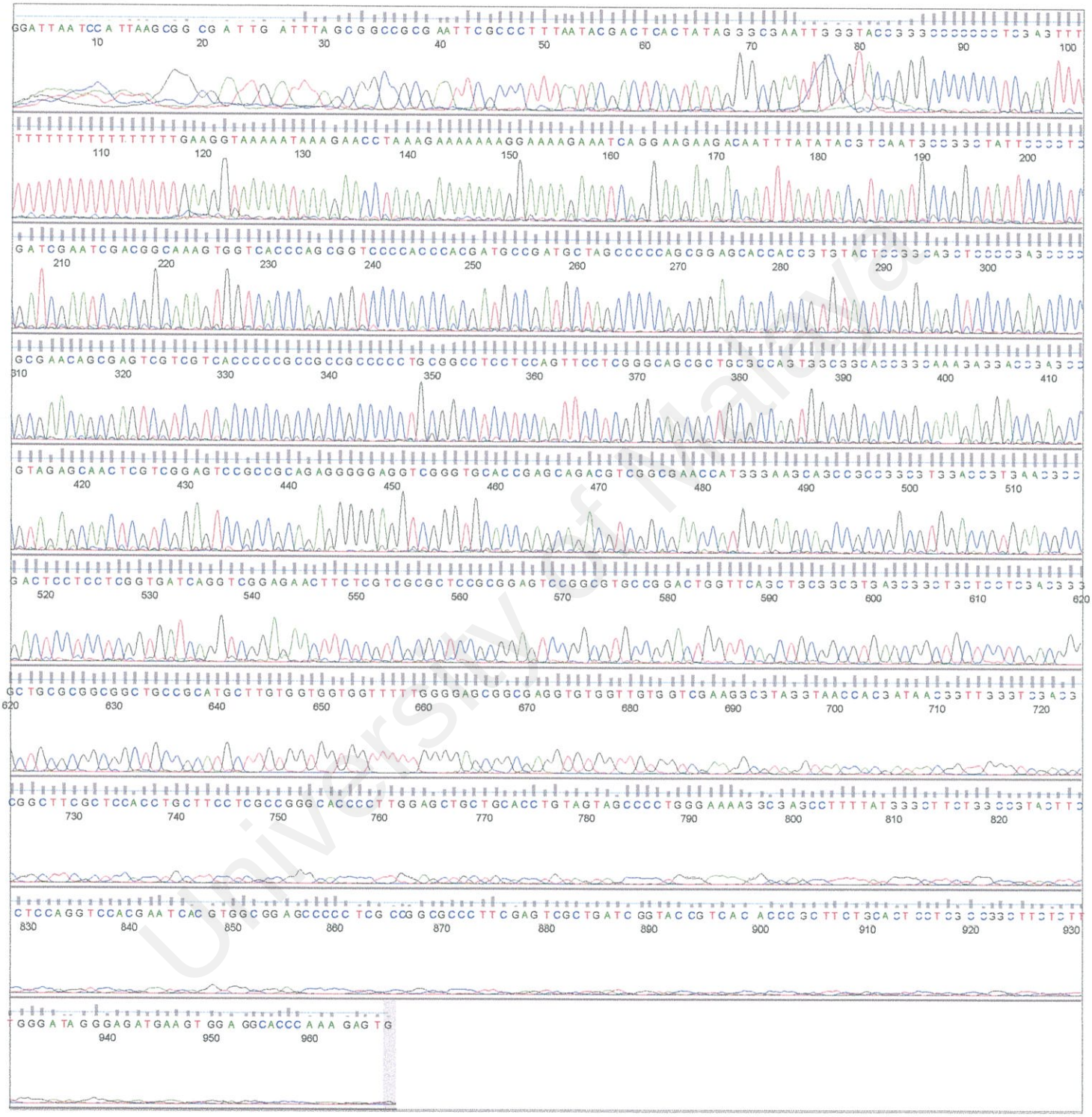


FinchTV v.1.4.0

**File: Appendix C2a- WRKY partial cDNA forward sequences (Musa acuminata cv. 'Mutiara').ab1**

Geospiza
www.geospiza.com

| | | | |
|---|---|---|---|
| Sample Name: | pW8_M13-forward | Signal Strengths: | A = 204, C = 287, G = 303, T = 200 |
| Mobility: | KB_3730_POP7_BDTv3.mob | Lane/Cap#: | 80 |
| Spacing: | 14.9288 | Matrix: | n/a |
| Comment: | n/a | Direction: | Native |





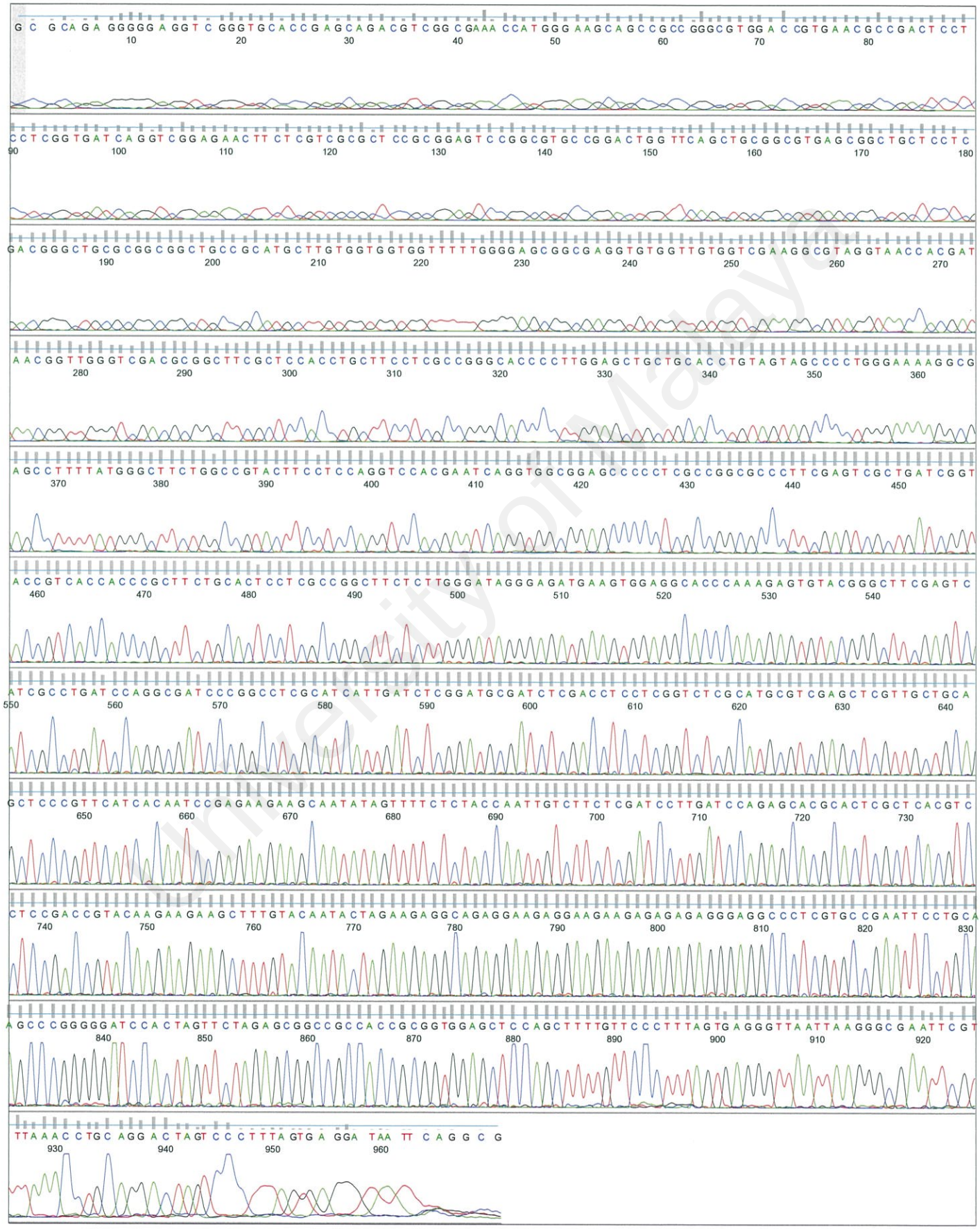
FinchTV v.1.4.0

**File: Appendix C2b- WRKY partial cDNA reverse complement sequencins (Musa acuminata cv. 'Mudia' ).ab1**



Sample Name: pW8_M13-reverse
Mobility: KB_3730_POP7_BDTv3.mob
Spacing: 15.009
Comment: n/a

Signal Strengths: A = 384, C = 452, G = 493, T = 346
Lane/Cap#: 78
Matrix: n/a
Direction: Reverse Complement



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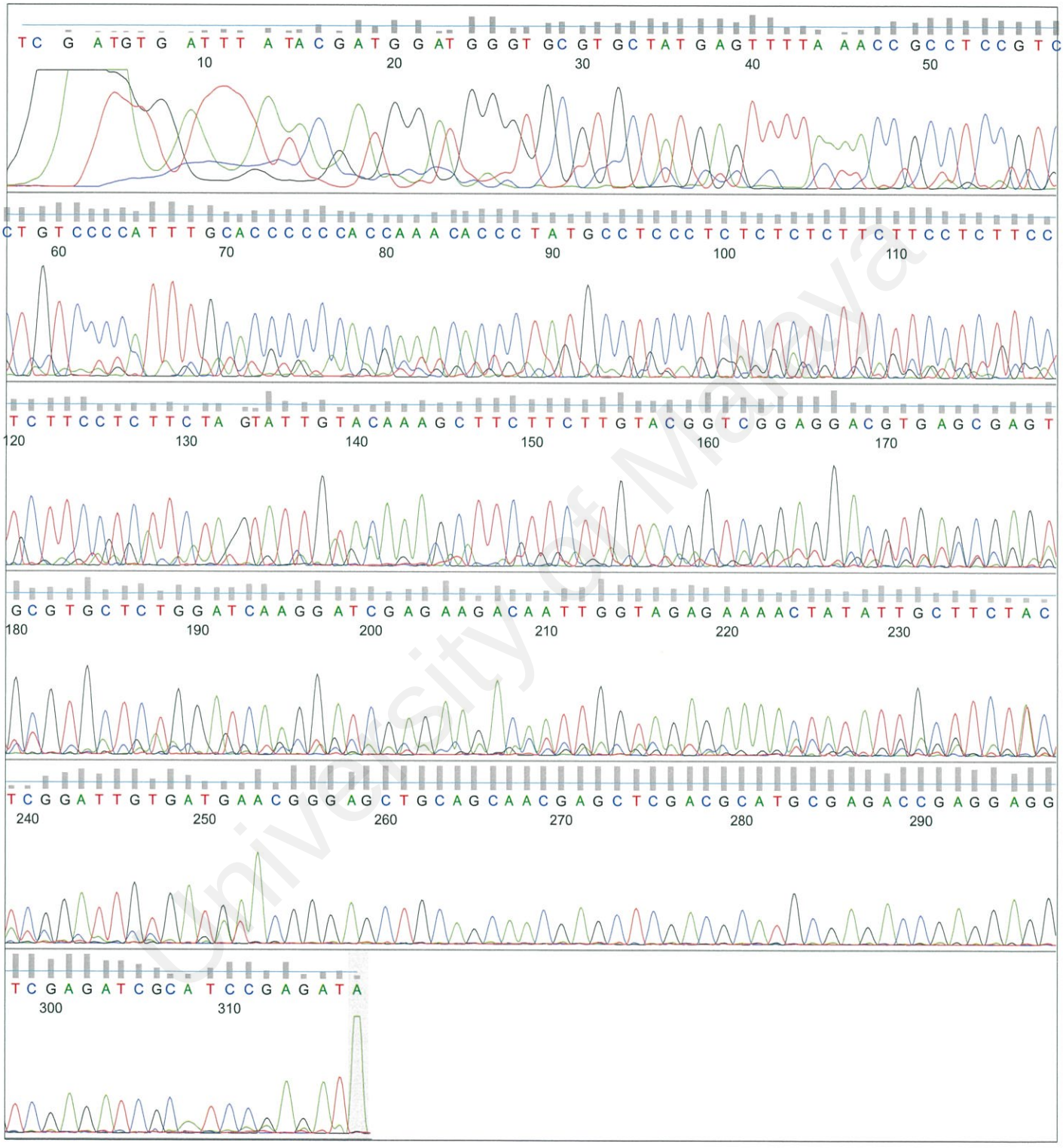
**File: Appendix C3a- 5' inner RACE MamWRKY forward sequences (Musa acuminata ssp. malaccensis).ab1**

Geospiza
www.geospiza.com

Sample Name: 56W5_5innRACE-forward
Mobility: KB_3730_POP7_BDTv3.mob
Spacing: 14.6806
Comment: n/a

Signal Strengths: A = 1666, C = 1771, G = 2110, T = 1631
Lane/Cap#: 31
Matrix: n/a
Direction: Native

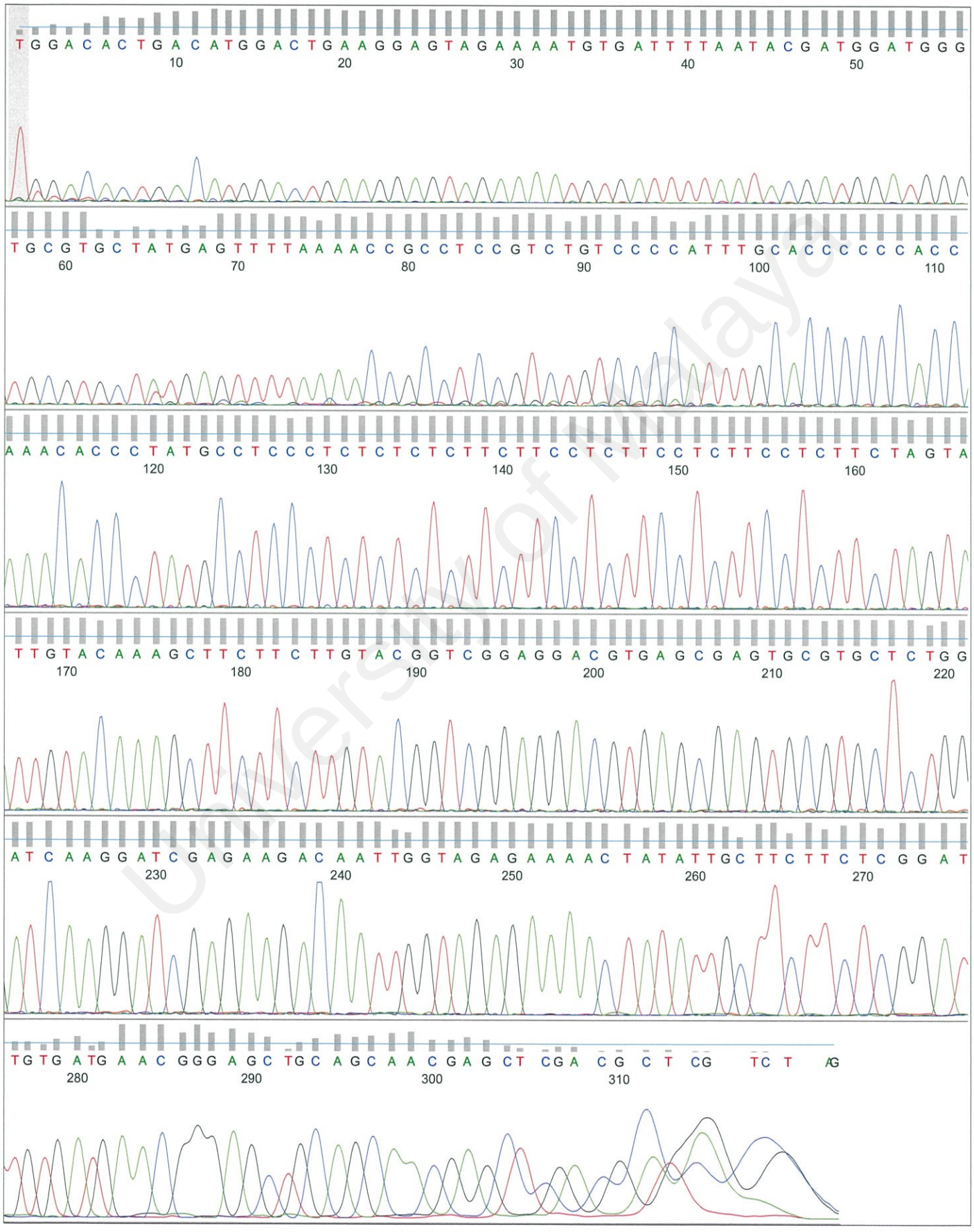





FinchTV v.1.4.0

Sample Name: 56W5_3Bingsp-reverse
Mobility: KB_3730_POP7_BDTv3.mob
Spacing: 16.1631
Comment: n/a

Signal Strengths: A = 2453, C = 2365, G = 2704, T = 2081
Lane/Cap#: 29
Matrix: n/a
Direction: Reverse Complement

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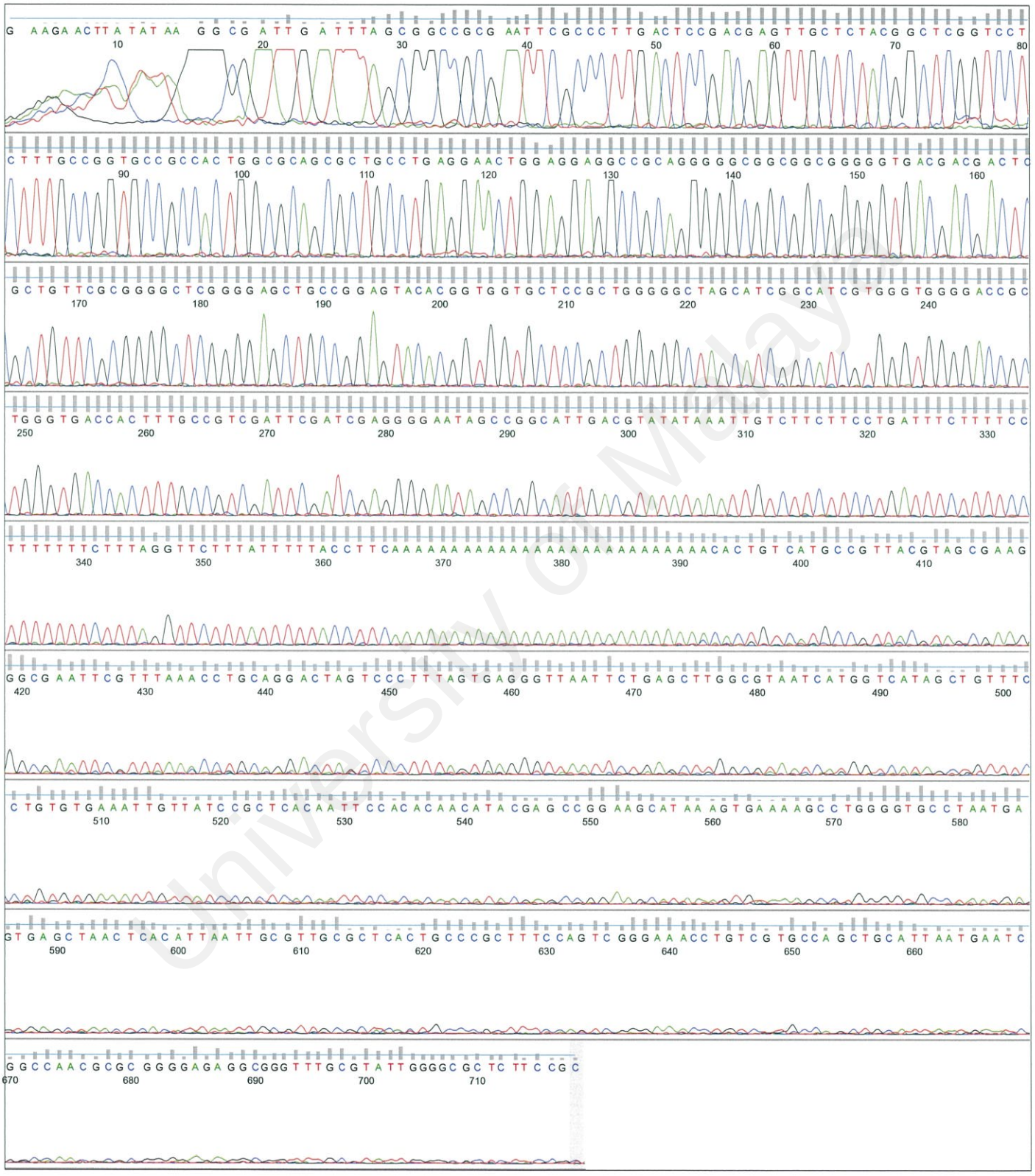
**File: Appendix C4- 3' inner RACE MamWRKY forward sequences (Musa acuminata ssp. malaccensis).ab1**

Geospiza
www.geospiza.com

Sample Name: 3innW6_M13-forward
Mobility: KB_3730_POP7_BDTv3.mob
Spacing: 14.683
Comment: n/a

Signal Strengths: A = 101, C = 132, G = 166, T = 120
Lane/Cap#: 72
Matrix: n/a
Direction: Native



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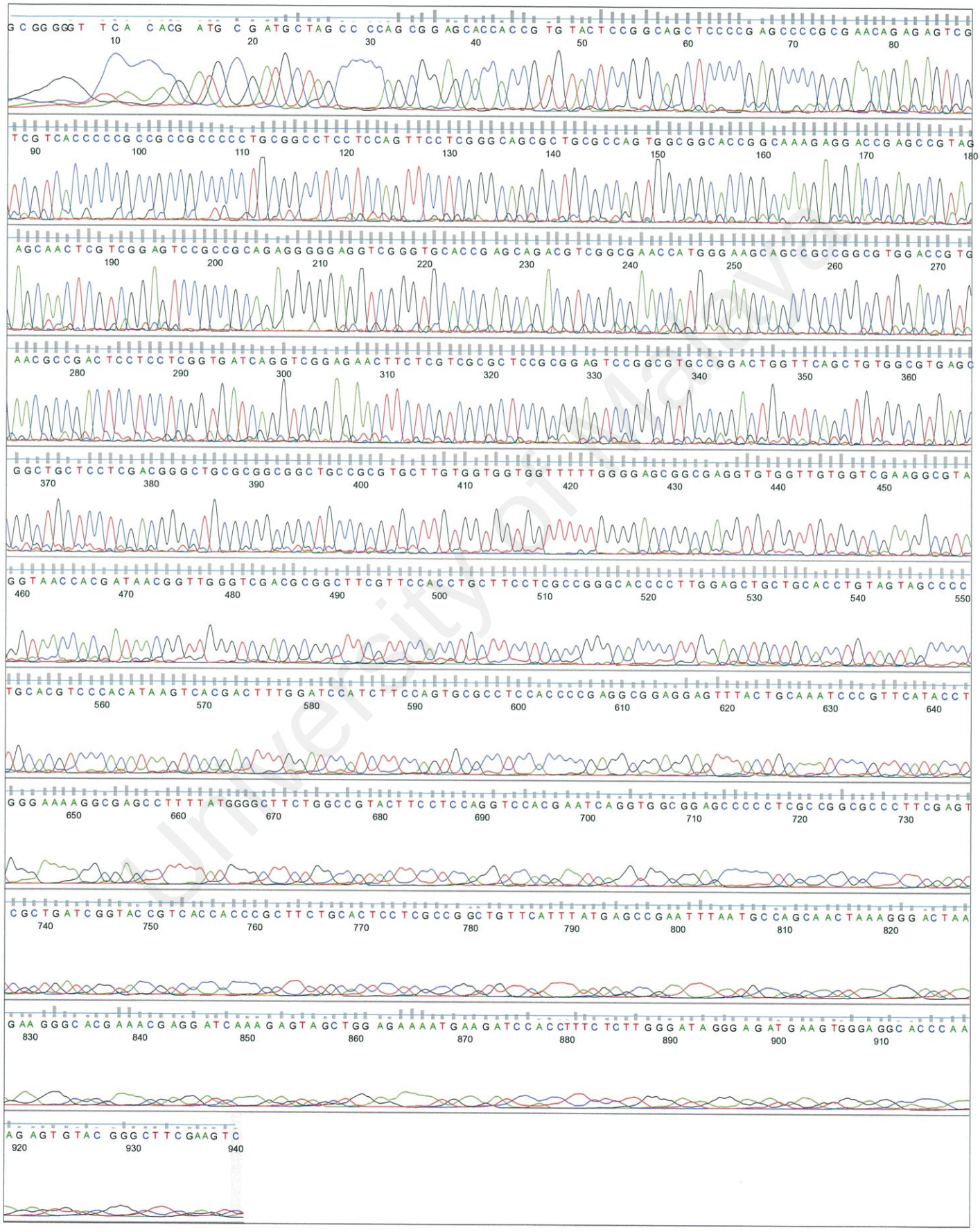
**File: Appendix C5a- MamWRKY genomic DNA forward sequences.ab1**

Geospiza
www.geospiza.com

Sample Name: gW_Wf(forward)
Mobility: KB_3730_POP7_BDTv3.mob
Spacing: 15.081
Comment: n/a

Signal Strengths: A = 1815, C = 3154, G = 2312, T = 1761
Lane/Cap#: 92
Matrix: n/a
Direction: Native

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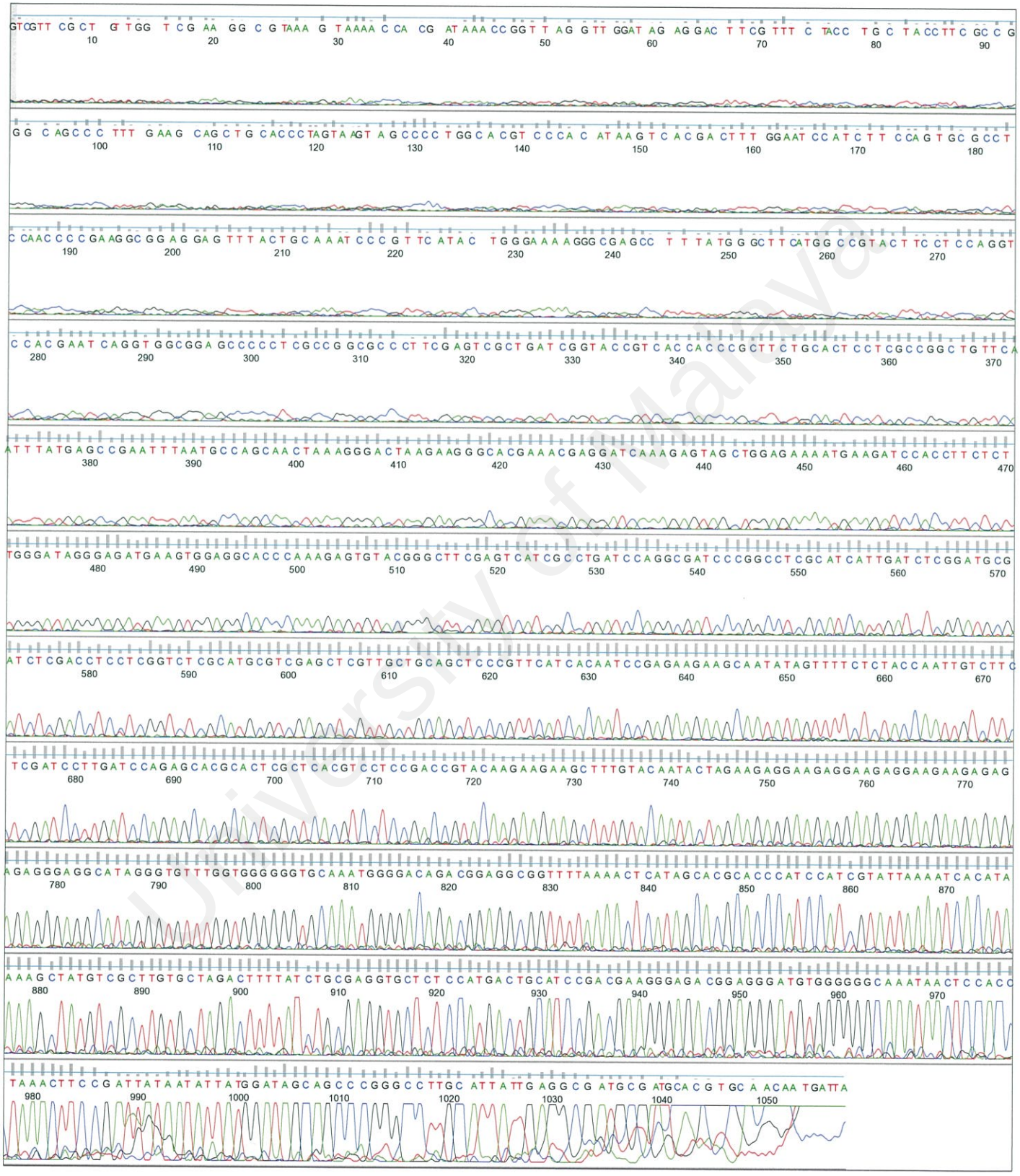
**File: Appendix C5b- MamWRKY genomic DNA reverse complement sequences.ab1**

Geospiza
www.geospiza.com

| | | | |
|---|---|---|---|
| Sample Name: | gW_Wr(reverse) | Signal Strengths: | A = 120, C = 138, G = 134, T = 127 |
| Mobility: | KB_3730_POP7_BDTv3.mob | Lane/Cap#: | 70 |
| Spacing: | 14.2652 | Matrix: | n/a |
| Comment: | n/a | Direction: | Reverse Complement |





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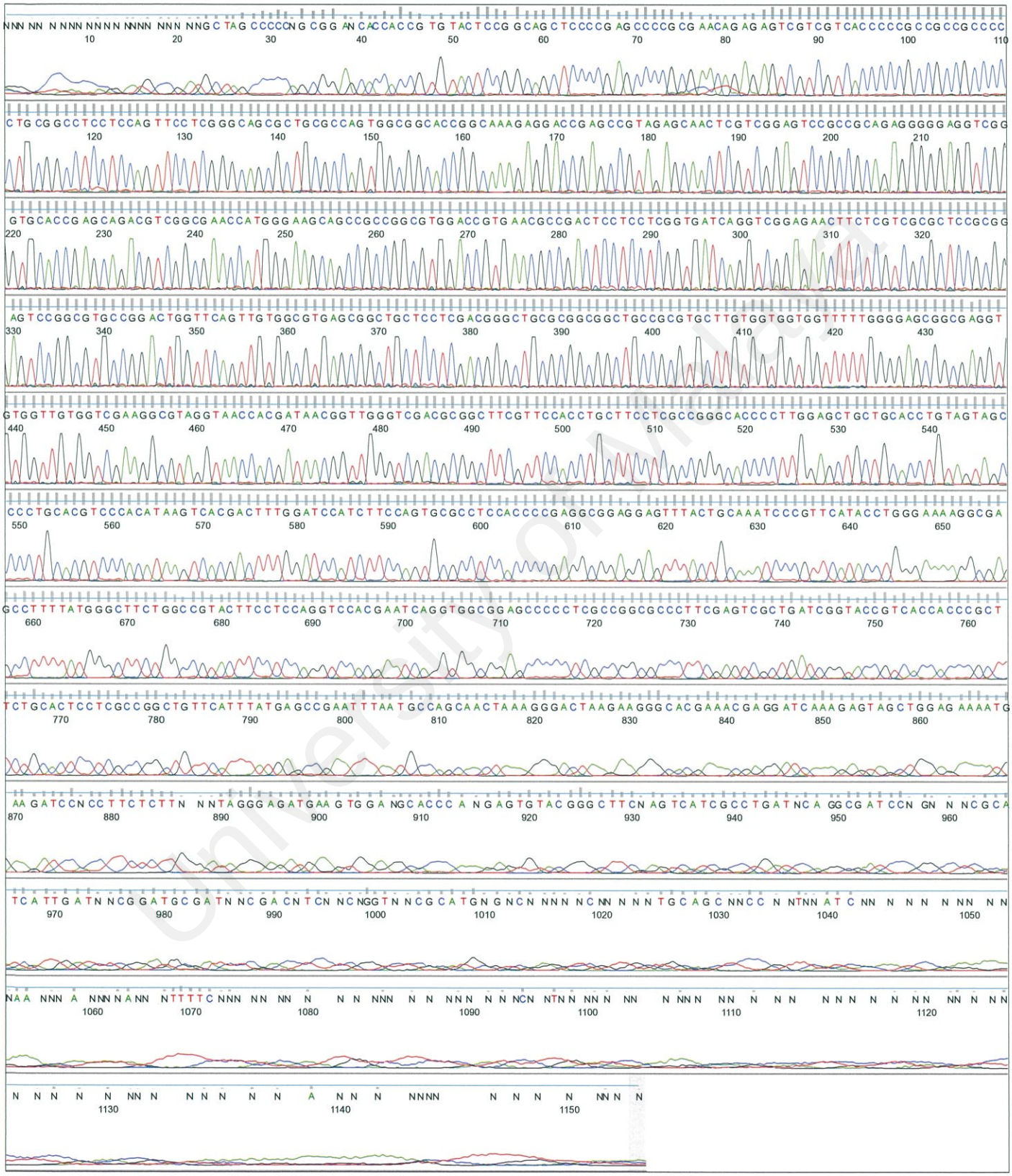
**File: Appendix C6a- MamWRKY cDNA forward sequences.ab1**

Geospiza
www.geospiza.com

Sample Name: B6W_Akmar_wf
Mobility: KB_3130_POP7_BDTv3.mob
Spacing: 12.44
Comment: n/a

Signal Strengths: A = 68, C = 91, G = 143, T = 67
Lane/Cap#: 1
Matrix: n/a
Direction: Native





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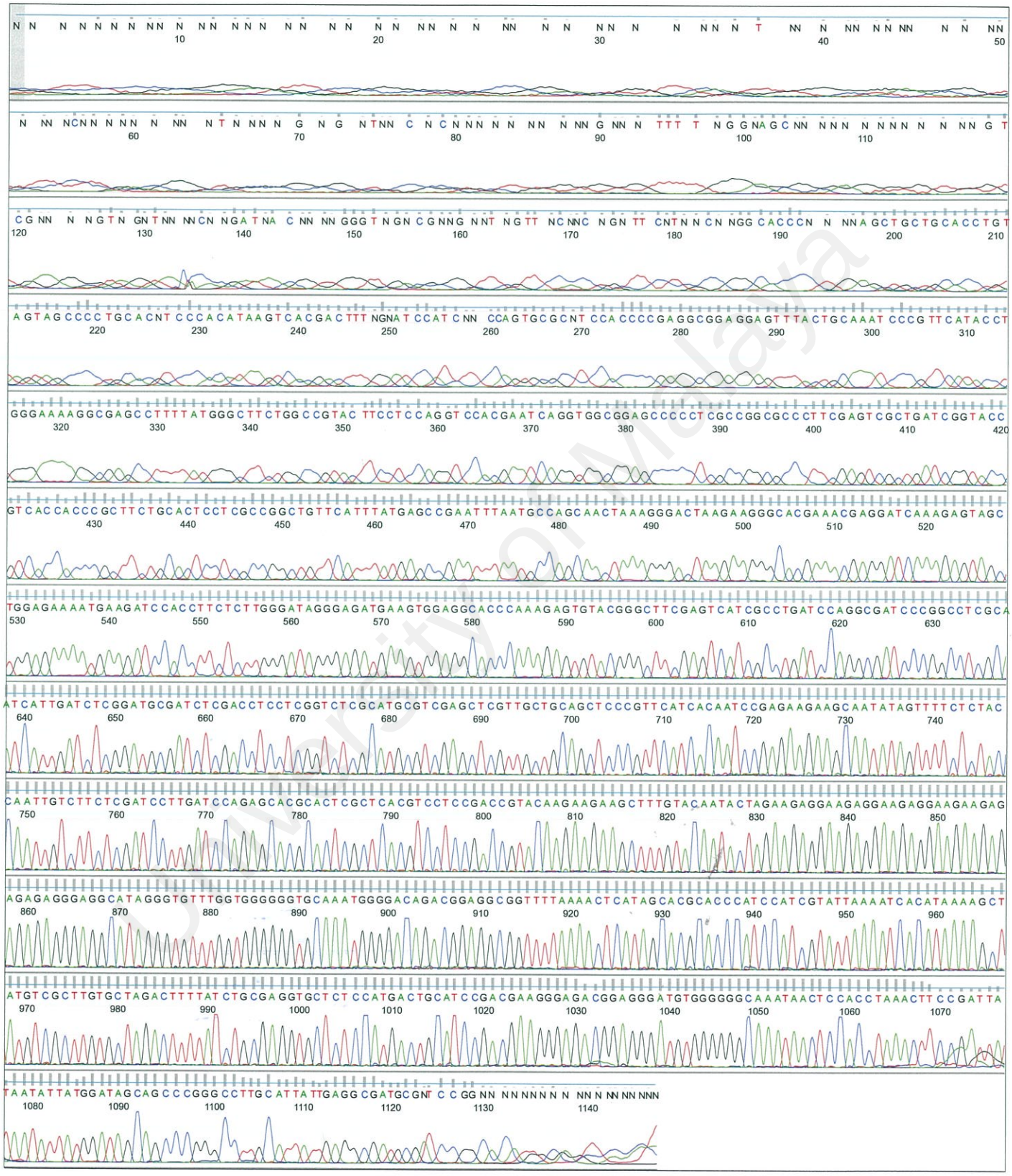
**File: Appendix C6b- MamWRKY cDNA reverse complement sequences.ab1**

Geospiza
www.geospiza.com

Sample Name: B6W_Akmar_wr
Mobility: KB_3130_POP7_BDTv3.mob
Spacing: 12.5963
Comment: n/a

Signal Strengths: A = 130, C = 139, G = 167, T = 142
Lane/Cap#: 2
Matrix: n/a
Direction: Reverse Complement





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