GENE EXPRESSION ANALYSIS OF EFFECTORS INVOLVED IN EARLY INTERACTION OF BANANA AND *Fusarium oxysporum* f. sp. *cubense*

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GENE EXPRESSION ANALYSIS OF EFFECTORS INVOLVED IN EARLY INTERACTION OF BANANA AND *Fusarium oxysporum* f. sp. *cubense*

ABSTRACT

Banana is a popular but disease-laden fruit crop. It is very susceptible to various phytopathogens such as viruses, bacteria, fungi and nematodes. A particularly infectious and economically important fungal disease in banana is Fusarium wilt caused by *Fusarium oxysporum* f. sp. *cubense* (*Foc*). The disease has been plaguing the banana industry since late 19th century. Despite the early discovery of this phytopathogen of banana, little is known on its the mechanism of pathogenicity. In other plants, fungal effectors have been found to function in manipulating plant mechanisms during infection and many have been identified and characterized. In this study, the presence of a well-studied group of effectors known as Secreted-in-xylem (SIX) were verified in the *Foc* Tropical Race 4 (*Foc*TR4) genome. Additionally, a computational pipeline was used to predict a pool of *Foc* effector candidates known as Small secreted cysteine-rich protein (SSCRP). The gene expression of selected effectors were characterized during the early interaction of the banana host and *Foc*. The *in silico* prediction pipeline successfully predicted 101 putative SSCRP and five of them were selected for gene expression analysis. The expression profiles of eight *FocSIX* genes identified in the genome were characterized with quantitative reverse transcriptase PCR (qRT-PCR). Four out of five *SSCRP* were found to be expressed *in planta* and all eight *FocSIX* genes were upregulated during early pathogenic interaction. Genomic features of the effector genes were also examined in this study. The results suggest *FocSIX* genes were actively involved in the early interaction of banana and *Foc*TR4. Future work should focus on the functional characterization of the SSCRP and FocSIX encoding genes.

Keywords: *Fusarium oxysporum* f. sp. *cubense*, Banana, Effector, Gene expression.

ANALISA PENGEKPRESAN GEN EFEKTOR YANG TERLIBAT DALAM INTERAKSI AWAL ANTARA PISANG DAN *Fusarium oxysporum* f. sp. *cubense*

ABSTRAK

Pisang adalah tanaman buah-buahan yang popular tetapi kerap diserangi penyakit. Ia sangat mudah diserang oleh pelbagai patogen tumbuhan seperti virus, bakteria, kulat dan nematod. Sejenis penyakit kulat yang sering menjangkiti pisang dan mempunyai kepentingan dari segi ekonomi adalah penyakit layu Fusarium yang disebabkan oleh *Fusarium oxysporum* f. sp. *cubense* (*Foc*). Penyakit ini melanda industri pisang sejak akhir abad ke-19. Walaupun patogen ini yang menjangkiti pisang telah lama diketahui, maklumat tentang mekanisme penjangkitannya amat kurang. Efektor kulat didapati berfungsi dalam memanipulasi mekanisme tumbuhan semasa jangkitan. Mereka telah dikenalpasti dan dicirikan di banyak patogen tumbuhan lain. Dalam kajian ini, satu kumpulan efektor yang dikenali sebagai *Secreted-in-xylem* (SIX) disahkan hadir dalam genom *Foc* Tropical Race 4 (*Foc*TR4). Selain itu, pengkomputeran digunakan untuk meramalkan sekumpulan calon efektor *Foc* yang dikenali sebagai *Small secreted cysteine-rich protein* (SSCRP). Pengekspresan gen efektor yang terpilih telah dicirikan pada peringkat awal interaksi antara pisang dengan *Foc*. Ramalan komputer telah mengenal pasti 101 calon *SSCRP* dan lima daripadanya dipilih untuk analisa pengekspresan gen. Lapan gen *FocSIX* yang dikenal pasti dalam genom telah dicirikan secara kuantitatif dengan tindak balas polimeras berantai transkripsi berbalik (qRT-PCR). Empat daripada lima *SSCRP* didapati diekspres secara *in planta* dan semua gen *FocSIX* diekspres dengan tahap tinggi sempena interaksi awal. Ciri-ciri genom gen efektor juga diperiksa dalam kajian ini. Hasilnya mencadangkan set gen ini terlibat secara aktif dalam peringkat interaksi awal antara pisang dan *Foc*TR4. Kerja-kerja masa depan harus menumpukan pada pencirian fungsi gen yang mengekodkan SSCRP dan FocSIX.

Kata kunci: *Fusarium oxysporum* f. sp. *cubense*, Pisang, Efektor, Pengekpresan gen.

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LIST OF SYMBOLS AND ABBREVIATIONS

°C	:	Degree Celsius
M	:	Molar
mg	:	Milligram
mL	:	Millilitre
ng	:	Nanogram
µg	:	Microgram
µL	:	Microlitre
µM	:	Micromolar
x g	:	Relative centrifugal force
Avr gene	:	Avirulence gene
BLAST	:	Basic local alignment search tool
cDNA	:	Complementary deoxyribonucleic acid
CTAB	:	Hexadecyltrimethylammonium bromide
DNA	:	Deoxyribonucleic acid
EDTA	:	Ethylenediaminetetraacetic acid
ETI	:	Effector-triggered immunity
Foc	:	*Fusarium oxysporum* f. sp. *cubense*
Fol	:	*Fusarium oxysporum* f. sp. *lycopersici*
f. sp.	:	forma speciales
GFP	:	Green fluorescence protein
hpi	:	Hour-post-inoculation
INDEL	:	Insertion and deletion
MITE	:	Miniature inverted-repeat transposable element
NaCl	:	Sodium chloride

NaOAc	:	Sodium acetate
no RT	:	No reverse transcriptase
NBS-LRR	:	Nucleotide-binding site leucine-rich repeat
PAMP	:	Pathogen-associated molecular pattern
PCR	:	Polymerase chain reaction
PDA	:	Potato dextrose agar
PDB	:	Potato dextrose broth
PTI	:	PAMP-triggered immunity
PVP	:	Polyvinylpyrrolidone
qRT-PCR	:	Quantitative reverse transcriptase polymerase chain reaction
R gene	:	Resistance gene
RNA	:	Ribonucleic acid
RT-PCR	:	Reverse transcriptase polymerase chain reaction
SSCRP	:	Small secreted cysteine-rich protein
SIX	:	Secreted-in-xylem
VCG	:	Vegetative compatibility group

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CHAPTER 1: INTRODUCTION

1.1 Research background

Banana (*Musa* spp.) is the second most cultivated fruit crop in Malaysia, but ranked top in terms of local fruit production in year 2017. The annual yield of 331 thousand metric tons banana was estimated to be worth RM 553 million (Department of Agriculture, 2018). Edible bananas are either a cultivar from the species *Musa acuminata* (A genome) or are hybrids of *M. acuminata* with *M. balbisiana* (B genome) (De Langhe et al., 2009; Simmonds & Shepherd, 1955). Half of the bananas growing in the country are of the commercial cultivars 'Berangan' or 'Cavendish', which are both triploid cultivars with an AAA genome (Department of Agriculture, 2018). Unfortunately, banana despite its popularity, is a disease-laden fruit crop, and it is susceptible to various phytopathogens such as viruses, bacteria, fungi and nematodes.

A particularly infectious fungal disease of banana, known as Fusarium wilt or Panama disease, is caused by *Fusarium oxysporum* f. sp. *cubense* (*Foc*) and has been plaguing the banana industry since the late 19th century (Ploetz, 2006a). As banana is an export commodity, Fusarium wilt has been a serious threat to the banana trade. In the 1950s, wide spread infection with *Foc* Race 1 had wiped out a then popular triploid banana cultivar, 'Gros Michel' also of AAA genome, from commercial plantations and international trade (Ploetz, 2005). A more resistant cultivar 'Cavendish' was subsequently introduced to the world market as a replacement. Nevertheless, Cavendish was found susceptible to Fusarium wilt too, by a more virulent race of *Foc* which was later named as *Foc* Tropical Race 4 (*Foc*TR4) (Ploetz, 2006b).

Two decades ago, *Foc*TR4 was first reported and was restricted to the Asian banana producing countries such as Malaysia and Indonesia, and subsequently also recorded in Northern Australia. But since the late 2013, *Foc*TR4 was reportedly found outside of South East Asia and the Pacific Areas for the first time. Incidences caused by *Foc*TR4 were reported from the Middle East (Garcia et al., 2014; Ordonez et al., 2016) and then appeared to spread into Africa (Zheng et al., 2018). More recently, it has been reported to spread to previously non-prevalent regions such as India and Latin America (Stokstad, 2019; Thangavelu et al., 2019) in addition to reoccurrence of Fusarium wilt in Queensland in 2015. In the light of *Foc*TR4 wide spreading, research community is actively seeking for effective solutions, such as the effort made by Dale et al. (2017) in creating the first *Foc*TR4-resistant transgenic banana.

Despite the discovery of the disease as early as the 1900s, and the various programs introduced to date, there is still no single effective control measure. *Foc* infection starts in the feeder root of the banana plant, progresses to the rhizome and eventually ends up in the pseudostem. The hyphae colonizes the xylem and blocks the uptake of water hence causes the wilting of the leaves leading ultimately to plant death (Ploetz, 2001). One of the challenges lies in the mode of cultivation of banana. Bananas are cultivated either through suckers or from tissue culture stock. Tissue culture is the preferred form for propagation since the sucker might have already been infected by *Foc* because the infected rhizome is usually symptomless (Stover, 1962). Tissue culture propagation provides advantages such as pathogen-free, uniform planting materials and shorter harvesting period. Nevertheless, vegetative propagated bananas have limited variation in their genetic base and hence are identically vulnerable. Cavendish, for example, was derived from a set of closely related clones which are now collectively susceptible and under attack of *Foc*TR4 (Ploetz et al., 2015; Stover & Buddenhagen, 1986). According

to Molina et al. (2009), both Cavendish and Berangan are ranked as very susceptible to *Foc*TR4.

Foc can survive up to 30 years in the soil even without a banana host, either by forming chlamydospores or infecting non-host species such as weeds (Waite, 1953; Schippers & Van Eck, 1981). No fungicide was proven to be effective in the field to date and soil fumigation, which is a useful soil pest control measure, failed to keep *Foc* at bay for long term. Currently, once the field is infected by *Foc*, susceptible cultivars are no longer suitable to be planted and must be replaced by a recalcitrant cultivar (Nel et al., 2007).

Foc is a hemibiotroph as it initiates the primary infection stage *in planta* as a biotroph but later turns into a necrotroph which eventually results in the death of the host plant, due to extensive wilting (Ghag et al., 2014). Biotrophs require a large repertoire of small secreted proteins to mediate the host plant cellular processes into favorable conditions (Meyer et al., 2017). Hence in hemibiotrophs, effector genes that are actively manipulating the host mechanism, are expected to be enriched in the early biotrophic phase. Whereas, in the later necrotrophic stage, fewer effectors are expected to be expressed. The interest of this study was to narrow down and identify those effectors which specifically work to manipulate host defense. Virulence factors facilitate the infection whereas the avirulence factor triggers the host defense system (Schneider & Collmer, 2010). In common, these effector proteins are typically small and are secreted by the pathogen in the host systems.

To date, only a handful of fungal effectors have had their functions elucidated whereas the majority still remains unresolved (Rep et al., 2004; Thatcher et al., 2012). Discernible characteristics of an effector protein are the presence of a N-terminal signal peptides for

secretion and cysteine residues enrichment. Signal peptides enable the protein to be secreted outside of the fungal cell whereas cysteine residues have been shown to form disulfide-bridges which help to stabilize the proteins tertiary structure against the host proteases (van den Burg et al., 2003). From the characteristics, fungal effectors can also be known as Small secreted cysteine-rich protein (SSCRP). Fungal effectors or SSCRP typically bear neither sequence homology with known proteins nor recognizable domain which has made difficult to be identified via conventional *in silico* approaches (Rep et al., 2005).

In contrast, a group of fungal effectors know as Secreted-in-xylem (SIX) have their homologs in different *forma speciales* (f. sp.) of *Fusarium oxysporum* predicted through sequence identity. Those exclusively present in a particular f. sp. could have more specialized function within a specific host. For instance, SIX3 (a.k.a Avr2) and SIX5 which are only found in *F. oxysporum* f. sp. *lycopersici* play essential roles of virulence in infecting its tomato hosts (Gawehns et al., 2014).

This study aims to identify *SIX* effector gene homologs as well as novel *SSCRP* from *Foc*TR4. The expression of selected effectors *in planta* during primary biotrophic stages were investigated since effectors are expected to be actively expressed. Early time points (48 and 96 hour-post-inoculation) were selected for investigation in this study. Amplification of selected *SSCRP* predicted using *in silico* pipelines were attempted at these time points. *SIX* effector genes expression level at these 2 time points were also investigated. It is hypothesized that the *SIX* effector genes and the selected *SSCRP* will be upregulated upon host contact and the early stages of infection. Tracing the expression level of potential effector genes serves as a prognosis of their roles in interfering with the host response mechanism. Further functional characterization on this potential set of

effectors can reveal the virulence factor(s) required for compatible *Foc*TR4-*Musa acuminata* interaction.

1.2 Objectives

The objectives of this study are,

1. To quantify the gene expression level of Secreted-in-xylem (*SIX*) effector genes in *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 (*Foc*TR4) strain C1HIR_9889 vegetative phase and *Foc*TR4 strain C1HIR_9889-*Musa acuminata* cv. Berangan early interaction.
2. To predict Small secreted cysteine-rich protein (SSCRP) in the genome of *Foc*TR4 strain C1HIR_9889.
3. To investigate the gene expression of *SSCRP* in *Foc*TR4 strain C1HIR_9889 vegetative phase and during *Foc*TR4 strain C1HIR_9889-*Musa acuminata* cv. Berangan early interaction.

CHAPTER 2: LITERATURE REVIEW

2.1 Banana and its diseases

Banana is an important cultivated fruit crop worldwide. In 2018, global production of 116 million tonnes of banana was recorded and Asia contributed 54.1 % of its production, with India as the largest producer (26.6%). According to Food and Agriculture Organization (FAO), Malaysia contributed to 0.6% of the banana produced in Asia in year 2018. Banana broadly refers to the fruit produced by herbaceous monocots of the genus *Musa.* Modern cultivated bananas are seedless and are mostly diploid (AA) or triploid (AAA, AAB & ABB) derived from *Musa acuminata* (A genome) or the hybrid with *Musa balbisiana* (B genome) (De Langhe et al., 2009; Simmonds & Shepherd, 1955). Edible bananas are usually sterile but can be vegetative propagated with ease (Simmonds, 1962). Banana caters various nutritional benefits to its consumer. Among a variety of fruits, banana is one of the high sources for potassium which a single banana contributes up to 23% of the daily potassium intake (Kumar et al., 2012). Apart from being a fruit crop, some *Musa* spp., such as *Musa paradisiaca*, is widely recognized as cooking bananas or plantains. Plantain constitutes major staple food of many African and Latin American countries. Banana fruit is an excellent dietary source of carbohydrates, fibers, vitamins, potassium, phosphorus and calcium (Ploetz, 2001).

Musa spp. are one of the oldest domesticated crops (Simmonds, 1962) and the process was first started in Southeast Asia (Perrier et al., 2011). Since most of the popular cultivars, such as Cavendish, are produced in large scale monoculture, the risk of the major banana productions being brought down by adapted pathogens is extremely high. According to Ploetz (2001), banana is highly susceptible to two fungal diseases, namely Black Sigatoka and Fusarium wilt, inflicted by *Mycosphaerella fijiensis* and *Fusarium*

oxysporum f. sp. *cubense* respectively. Black Sigatoka is a leaf spot disease which causes loss of banana yields of up to 50% or more. Moreover, the diseased plants produce fruits with premature ripening which seriously hampers the export of the banana (Ploetz, 2001). On the other hand, Fusarium wilt poses a much more serious threat to the industry that the infected plants are eventually wilted and dead. The soil, water and the banana rhizomes from the infected areas are highly contagious and capable of spreading the pathogens to previously Fusarium-free areas if uncontained (Ploetz, 2006).

2.2 Economic impact of banana Fusarium wilt

According to the Food and Agriculture Organization (FAO), banana is ranked eighth on the list of worldwide most important food crop and it is the fourth most important crop for the least developed countries. Globally, nearly 85% of the annual banana production was consumed locally and only less than 15% were exported (FAO, 2019). Production and export of banana are frequently being affected by the fact that banana is highly susceptible to diseases caused by various pathogens (fungi, bacteria, viruses and nematodes). Disease is the major biotic constraint faced by banana export trade. The first obvious setback traced back to the loss of cultivar Gros Michel to *Fusarium oxysporum* f. sp *cubense* Race 1 in 1950s (Ploetz et al., 2015). Currently, almost all bananas traded international and a considerable part of local consumed bananas are of Cavendish and other cultivars with no resistance towards *F. oxysporum* f. sp *cubense* Tropical Race 4 (*Foc*TR4). In early 1990s, the occurrence of *Foc*TR4 epidemic in Malaysia collapsed a then newly established Cavendish exportation effort which targeted the expanding East Asia and Middle East market in merely 2-year period (Molina et al., 2009).

Since the infection of the *Foc*TR4 is irreversible and untreatable for decades (Stover, 1962), its economic impact is profound and not restricted only to the losses related to

banana yields, but also results in the gradual losses of arable lands for banana (Joven, 2014). *Foc*TR4 is known to infect non-host species such as weeds. These asymptomatic species will act as a reservoir of inoculum which in turn spread to nearby uninfected lands and water sources (Schippers & Van Eck, 1981; Waite, 1953). Tengku Ab Malik et al. (2013) reported a country-wide survey carried out in year 2008-2009 in Malaysia and showed that around 883 hectares of banana plantation lands were infected with Fusarium wilt and cost the country's banana industry about USD14.1 million loss per annum. In the Johor state alone, an estimated 25.3% of banana plants were infected with *Foc* (Tengku Ab Malik et al., 2013). The disease incurs additional cost to producers and causes small holders to cease operation due to lack of sustainability (Ploetz et al., 2015). Also, Ploetz et al. (2015) stated diseased plants with lower yields and quality of fruit have significantly reduce the income of growers and adversely impacted the banana industry at both local and international levels.

2.3 *Fusarium oxysporum*

Kingdom: Fungi

Division: Ascomycota

Class: Sordariomycetes

Order: Hypocreales

Family: Nectriaceace

Genus: Fusarium

Species: *Fusarium oxysporum*

Fusarium oxysporum is a root-invading phytopathogenic fungus. It exists as conidiospores during non-infectious condition in soil (Fravel et al., 2003). More recently, *F. oxysporum* has also been found developing in a Korean zebrafish (*Danio rerio*)

culturing system. This is the first observation of *F. oxysporum* species complex *(Fo*SC*)* recorded in this vertebrate model organism (Kalatunga et al., 2016). There are also non-pathogenic isolates of *F. oxysporum*. Individual isolates of *Fo*SC usually elicits infection only on a certain host species, hence are subdivided into formae speciales (f. sp.) (Fourie et al., 2009) based on their host.

Despite previous assumptions that a f. sp. shares a common evolutionary origin, phylogenetic studies on *F. oxysporum* f. sp. *cubense* (*Foc*) found that the pathogenicity of this f. sp. on banana cultivar has, in fact, a polyphyletic nature (Czislowski et al., 2018; Fourie et al., 2009; Ploetz, 2006a). *F. oxysporum* isolates designated as f. sp. *cubense* clustered together with other f. sp. rather than forming a monophyletic clade when phylogenetic trees were constructed (Fourie et al., 2009). The f. sp. designation has more distinct impact in discriminating the avirulence/virulence gene products carried by the fungus (host specificity) than to determine phylogenetic relationship (Nimchuk et al., 2003).

There are a total of 24 vegetative compatibility groups (VCG) currently being recorded and are used systematically to categorize newly identified *Foc* isolates worldwide. The Race grouping system (i.e. race1, 2, 4, STR4 and TR4) has been shown to correlate to VCGs to a certain extent (Czislowski et al., 2018) and both are standard classifications used in reporting *Foc* isolates. The original Race 3 designation was found to infect only the *Heliconia* species and since excluded from the f. sp. *cubense* (Ploetz & Pegg, 2000).

VCG grouping accurately reflects the alleles present in the *vic* loci of an isolate and those belonging to the same VCG group have consistent phenotype. Even though VCG testers are useful in describing *Foc* isolates, it is a laborious diagnostic tool which takes

approximately 2 months to characterize a previously unidentified isolate (Czislowski et al., 2018). Some efforts are actively carried out to develop a more time efficient alternative for screening different *Foc* isolates, for instance, PCR-RFLP approach described by Fourier et al. (2009).

Seventeen out of the 24 recognized VCGs were found distributed in Asia with at least 8 *Foc* VCGs were present in Peninsular Malaysia and VCG 01213/16 was found to be the majority (Mostert et al., 2017). This VCG, reported by Mostert et al. (2017) studies, was also frequently found in other Asia countries, such as Indonesia, Philippines, China and Taiwan; while absent from the collections from Thailand, Vietnam, Cambodia, Bangladesh, India and Sri Lanka. It was found to infect 6 different banana cultivars cultivated in Peninsular Malaysia, namely Berangan (AAA), Mas (AA), Raja (AAB), Awak (ABB), Abu Keling (ABB) and Port Dickson (unidentified genome). Geographically, VCG 01213/16 dominated the infected plantations in West Coast of Peninsular Malaysia (Mostert et al., 2017).

2.4 Infection mode of *Fusarium oxysporum* f. sp. *cubense*

Fusarium oxysporum f. sp. *cubense* (*Foc*) is a hemibiotroph. *Foc* forms three types of spores throughout its full life cycle, namely, macroconidium, microconidium and dormant chlamydospore (Warman & Aitken, 2018). Macro- and microconidia involve in germination and infection whereas chlamydospores remain dormant and are infectious when in contact with compatible hosts (De Cal et al., 1997). At first, *Foc* conidia germinate under a suitable condition such as with nutrients supply or in host proximity. Hyphae are then form and colonization of the host root system is activated. (Li et al., 2011; Xiao et al., 2013) Invasive hyphae are capable to penetrate the root epidermis which suggested by Petre & Kamoun (2014). Initially, fungal hyphae colonize the xylem vessel

system of the root and progresses upwards into the rhizome and then the pseudostem (Guo et al., 2014). Obstruction of the xylem vessels by the fungal mass and the plug produced by the host immune responses eventually causes the death of the whole banana plant due to wilting (Lyons et al., 2015).

2.5 Current disease management approaches of banana Fusarium wilt

Unfortunately, there is yet an effective measure to treat or cure plants or soils infected with *Foc*TR4. The preventive measures taken by commercial plantations are to contain the infected materials in order to restrict the spread of the fungal spores to Fusarium-free areas (FAO, 2019). Chemical control approaches, such as fungicides and soil fumigations, were not efficient and have disadvantages of high cost and adversely affected the soils and environment (Siamak & Zheng, 2018).

Current efforts made in controlling Fusarium wilt of banana generally fall on biological control and establishment of new resistant banana cultivars towards *Foc* especially Tropical Race 4 (Dale et al., 2017; Siamak & Zheng, 2018). Biological control of *Foc* utilizes the antagonistic effect of other non-pathogenic microbes such as *Trichoderma* spp., *Pseudomonas* spp., *Bacillus spp*., some actinomycetes and non-pathogenic *F. oxysporum*. In general, the modes of control of the microbes are competition for the nutrients and niches, producing secondary metabolites which have antagonistic effect to the growth of *Foc*, promotion of host plant growth by altering the physiology and finally the induction of plant resistance of potential immune responses (Siamak & Zheng, 2018).

Also, some crops, *Allium tuberosum* for instance, were found to have potential suppression on the progression of banana Fusarium wilt and their effectiveness in halting

the wilt disease is under evaluation (Huang et al., 2012; Siamak & Zheng, 2018; Zhang et al., 2013). In a study from Wang et al. (2015), a two-year crop rotation of banana with pineapple showed that this system effectively suppressed Fusarium wilt incidence.

2.6 Plant defense response

The passive defense response of plants is the front-line against general pathogen invasion which consists of nonspecific physical or chemical barriers. The first level or active resistance is PAMP-triggered immunity (PTI) (Guo et al., 2009; Zipfel, 2008;). Pathogens which successfully overcome the passive defense will be halted by PTI since pathogens typically produce some conserved pathogen-associated molecular patterns (PAMP) that are easily recognized by plant host (Surico, 2013). Large number of pathogens will be derailed by both the passive defense and PTI. Eventually, there is a second level or specific resistance defined as Effector-triggered immunity (ETI) (Jones & Dangl, 2006).

ETI is thought to be mediated by a special group of genes encoding Resistance (R) proteins which function in pathogenic effectors recognition (Jones & Dangl, 2006; Rep et al., 2004). A small number of pathogens which able to produce specific effector proteins which are not recognized by the PTI will challenge the final gateway guarded by ETI. If unfortunately, the plant host does not possess the specific resistance genes required to recognize and halt the effector-triggered susceptibility, disease will then ensue. On the other hand, Ma et al. (2013) suggested plant host which possesses the cognate *R* genes will be able to percept the effector proteins (Figure 2.1). Defense response such as hypersensitive response (HR) and oxidative burst will be triggered to banish the pathogen, usually via necrosis of infected tissues (Jones & Dangl, 2006; Ma et al., 2013).

R genes play an important role in ETI and hence many efforts have been done in search for them in various economically important crop plants. Putative *R* genes have been identified in rice (Zhou et al., 2004), soybean (Graham et al., 2000), wheat (Seah et al., 2000) and banana (Pei et al., 2007). A typical class of *R* genes is the one which encode proteins with Nucleotide-binding site and leucine-rich repeat (NBS-LRR) domain. Pei et al. (2007) has identified 12 *R* gene analogues with NBS-LRR domain in banana. Whereas in genome of other plants, such as rice, 480 NBS-LRR class of *R* genes were estimated (Zhou et al., 2004). While in Arabidopsis, the number is of predicted *R* genes was around 150 (Meyers et al., 2003).

Avirulence (Avr) proteins or effectors have been identified in a variety of pathogens including fungal pathogens. They are usually secreted by the pathogen to aid colonization of the host cell and causes the disease (Hogenhout et al., 2009). Despite the efforts in cloning and characterization of the putative R genes in banana, to date, no interaction between the banana R protein and the Avr protein has been reported (Swarupa et al., 2014).

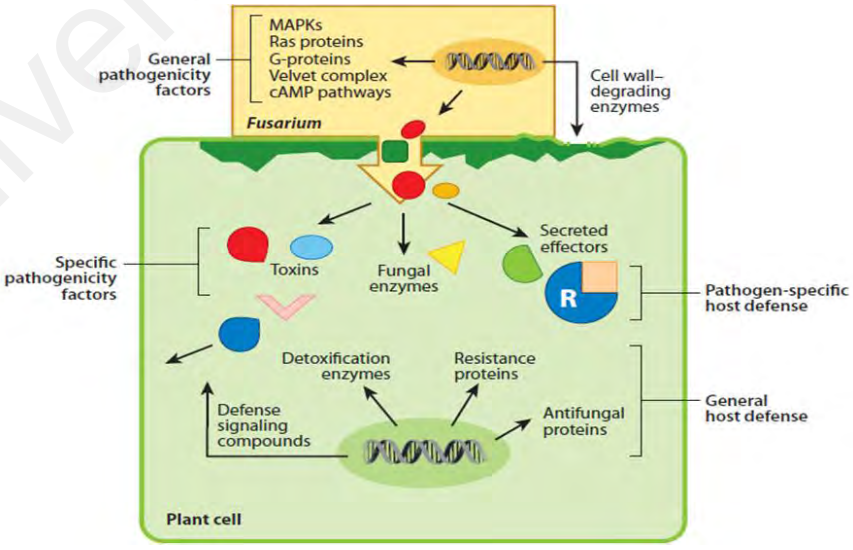

Figure 2.1: *Fusarium* sp. pathogenicity and plant host defense mechanisms. The plant host which possesses Resistance (R) proteins that confer pathogen-specific host defense will recognize the matched secreted effectors from a specific pathogen. Effector triggered immunity will be then activated by matched set of effector and R proteins (Ma et al., 2013).

2.7 Fungal secretome and Small secreted cysteine-rich protein (SSCRP)

Fungal pathogen secretes a repertoire of molecules to facilitate host colonization and manipulate host processes in favor of its infection. Generally, proteins secreted by the fungus at host contact, during host colonization and manipulate disease manifestation are collectively known as 'Secretome'. Effectors are a subset of secretome secreted inside plant host (a.k.a. *in planta*) either as apoplastic effector or cytoplasmic effector (host-translocated). Apoplastic effector targets host-pathogen interstitial space whereas the cytoplasmic effector is further translocated into the cytoplasm and targeted to various subcellular compartments (Rovenich et al., 2014; Win et al., 2012).

Apart from effectors, pathogenic fungus also secretes a number of enzymes for cell wall breaking, protection and also to acquire nutrients. For example, carbohydrate-active enzymes (CAZymes), oxidoreductases, proteases and lipases (Girard et al., 2013). The size of secretomes varies widely across fungi of different lifestyle and also between species (Kim et al., 2016). The classes of enzyme mentioned were further removed from the secretome in order to enrich the putative effectors since most known fungal effectors are lacking enzymatic activity (Kim et al., 2016).

Phytopathogenic fungi can be classified into nectrotrophs, biotrophs and hemibiotrophs. *Fusarium oxysporum* is considered as an hemibiotroph because it initiates the primary infection stage *in planta* as a biotroph but later turns into a necrotrophic stage which eventually resulted in the death of the host plant, due to extensive wilting (Lyons et al., 2015; Warman & Aitken, 2018).

Commonly, non-pathogenic and symbiotic fungi tend to produce less Small secreted proteins (SSPs) than their pathogenic counterparts. The secretomes of hemibiotroph and

nectrotroph consist of larger portion of enzymes than those of biotroph and symbiont and encode lesser SSPs (Kim et al., 2016). Necrotrophic fungi tend to produce least SSPs or effector proteins since they kill the host plant to obtain nutrients. On the other hand, biotrophs require a larger repertoire of SSPs to mediate the host plant cellular processes into favorable conditions (Meyer et al., 2017). SSPs are reported to function as species-specific effectors or have enzymatic functions (Kim et al., 2016).

Small secreted cysteine-rich protein (SSCRP) has emerged as a source of potential effectors involved in plant pathogenesis, symbiotic and saprophytic associations. Their roles ranged from induction of virulence (Gawehns et al., 2014; Thatcher et al., 2012), determination of host specificity (Lievens et al., 2009; Meldrum et al., 2012), suppression of immune response (Gonzalez-Cendales et al., 2015; Qi et al.,2016), modulataion of defense signaling pathways (Kim et al., 2016) that elicit host defense responses such as hypersensitive response (HR) to trigger resistance in compatible host (Houterman et al., 2009; Rep et al., 2004).

2.8 Secreted-in-xylem (SIX) as fungal effector proteins

Generally, effector proteins can be further categorized into two types. When the effector able to override the PAMP-triggered immunity (PTI) or Effector-triggered immunity (ETI) and leading to disease, it is considered as a virulence factor. Whereas, an effector which can be recognized by PTI or ETI and subsequently triggering the hypersensitive response (HR), is considered as an avirulence factor/protein (Rep et al., 2005; Surico, 2013). Virulence factor facilitates the infection whereas the avirulence factor triggers the defense system via Resistance (R) gene recognition (Schneider & Collmer, 2010). In common, these effector proteins are typically small and are secreted by the pathogen in/into the host tissues.

The proteins secreted in/into host cell during the course of infection are not necessary always targeting to host proteins. There are evidences suggests that some fungal effectors secreted *in planta* might not target host proteins but to offer protection against host defense. For instance, An Avr4 protein from *Cladosporium fulvum* binds to the chitins of its own cell wall, probably to confer protection towards the chitinase of the host plant (Rep et al., 2005).

Only the function of a few fungal effectors in virulence/avirulence have been elucidated and the role of a large part of effectors still remains unknown (Rep et al., 2004; Thatcher et al., 2012). Up to now, the only known characteristics of putative fungal effector proteins are small, cysteine-rich and secreted *in planta* (therefore, presence of signal peptide in N-terminus) (Rep et al., 2004).

The first avirulence factor *F. oxysporum*, Secreted-in-xylem 1 (SIX1) (Rep et al., 2004) was found secreted into the xylem sap of the host. Since then, 11 others SIX proteins were identified (Houterman et al., 2007; Lievens et al., 2009; Schmidt et al., 2013). Apart from triggering the defense response of the compatible host, other biological functions of SIX proteins remained unclear. It is very likely that these group of effector proteins play a significant role in host colonization, at least at some time point or in certain hosts (Laugé & De Wit, 1998).

Several knock-out constructs of SIX proteins demonstrated their roles in *F. oxysporum* virulence. Reduced virulence was observed in *F. oxysporum* f. sp. *lycopersici* in the study of Gawehns et al. (2014), when the *SIX6* gene was substituted by a hygromycin resistance cassette. Whereas in another study, SIX1 protein was classified as avirulence factor. Its loss failed to elicit immunity mediated by I-3 resistance gene in tomato (Rep et al., 2004).

SIX proteins do not possess any sequence homology to previously annotated proteins hence their functions remained unclear (Rep et al., 2005). Though it is possible to identify SIX protein homologs in different f. sp. of *F. oxysporum*, some are exclusively present in a particular f. sp. inferring a specialized function in a specific host. For instance, Avr 2 and SIX5 were only found in *F. oxysporum* f. sp. *lycopersici* (*Fol*) which infects tomato.

SIX genes were first thought exclusively present in *Fol* (Lievens et al., 2009). Most of the effector-R gene protein pairs were identified and characterized in tomato and also, later in Arabidopsis. Avr2 (also known as SIX3) secreted by *Fol* is found to trigger plant defense response through recognition by an *R* gene, *I-2*, which presents in the resistant cultivar. However, a point mutation in the *Avr2* breaks the I-2 recognition and the mutant failed to trigger immunity even in resistant cultivars (Houterman et al., 2009).

2.9 Identification and functional characterizations of fungal effector proteins

Fungal effector proteins typically bear neither sequence homology with known proteins nor recognizable domain which has made it difficult to be functionally characterized via *in silico* approaches (Jones & Dangl, 2006; Rep et al., 2005; Sonah et al., 2016). A few discernible characteristics such as presence of N-terminal signal peptide for secretion and cysteine residues enrichment reveal effector proteins generic functions (Thomma et al., 2005). Signal peptide enables the protein to be secreted outside of the fungal cell whereas cysteine residues have been shown to form disulfide-bridges which help to stabilize the proteins tertiary structure against the host proteases (van den Burg et al., 2003). An effector protein Avr4 from *Cladosporium fulvum* was predicted having a chitin binding domain based on disulfide bonding pattern and was proved to bind chitin experimentally (van Esse et al., 2007). However, in most cases, protein structure alone was not adequate in dissecting their possible functions (Rep et al., 2005).

Knock-out experiment is a frequent approach to check the role of the effector in virulence or avirulence. For instance, in the studies by Gawehns et al. (2015), infections done with *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) knock-out strains for these effector genes: Avr2, Avr3, Six5, and Six6, respectively, resulted in changes in xylem sap compositions when each compared with the xylem sap of tomato plants infected with wild type *Fol*. However, this knowledge is still unable to reveal the molecular mechanism or function of a particular effector. Hence, it is of utmost importance to identify the molecular target(s) of secreted effector proteins in order to elucidate its specific molecular function (Rep et al., 2005).

Duplessis et al. (2011) identified a pool of 1184 small secreted proteins whose length not more than 300 amino acids and possess no transmembrane domains in their study on *Melampsora larici-populina*, a leaf rust fungus. In a subsequent study of Petre et al. (2015), 20 candidate effector proteins of *M. larici-populina* were selected and expressed *in planta*. The candidates are enriched in haustorial-expressed proteins. Coimmunoprecipitation targeting GFP-fusions protein were used to purify the GFP-fusion effector proteins and their associating proteins from *Nicotiana benthamiana*. Six hundred and six (606) interacting protein partners from *N. benthamiana* were found and 138 proteins, which interact with only one effector, were considered as specific. Six candidate effector proteins were found to be subcellularly localized such as into chloroplast, mitochondrion and nucleus. Whereas few effectors from oomycetes have been shown to localize into nuclear compartment, Petre et al. (2015) was the first study to demonstrate localization of fungal effectors into plant organelles.

2.10 Models of protein-protein interactions during pathogenesis

Plant disease is defined as the reduction in ability of a plant or its part to perfom normal functions (such as transportation of water or nutrients) when interfered by a pathogen (Surico, 2013). According to Ferreira et al. (2007), a plant–pathogen interaction can be compared to an open warfare. The main arsenal is proteins synthesized by both organisms. There are several hypotheses on how plant and pathogenic proteins interact with each other during the course of infection. First, the gene-for-gene relationship developed by Flor (1971) postulates that there are pairs of matching genes existing in a pathogen and its host plant which mediate the pathogen infection (gene-for-gene hypothesis).

The pathogenic gene is known as an Avirulence (*Avr*) gene and its correspondent in the host is known as Resistance (*R*) gene. Then protein encoded by *R* gene able to interact and recognize the invading pathogenic Avr proteins, which then lead to the host immunity responses, such as hypersensitive response and localized cell death. On the other hand, another alternative, known as Guard hypothesis, suggests that R proteins do not interact directly with the Avr proteins. Rather, recognition signal of Avr proteins by another group of "guardee" proteins trigger the R proteins and lead to immunity responses (Dangl et al., 2001). Flor's hypothesis suggests an effector interact only with a single host protein whereas Dangl et al. (2001) postulates a specific pathogenic effector can actually target multiple host proteins.

More recently, a refined model of Guard hypothesis has been proposed by van der Hoorn & Kamoun (2008) and was termed Decoy model. It further describes a potential strategy deployed by the plant host, which is to have a decoy protein that evolved to be highly similar with the guardee protein. The decoy protein itself does not possess any function in triggering disease manifestation or resistance, but solely functions as a

perception medium of effector for the R protein (van der Hoorn & Kamoun, 2008). Nevertheless, in either hypotheses, physical interaction between the pathogenic proteins and host receptor proteins is always essential to trigger the host defense mechanism (Figure 2.2).

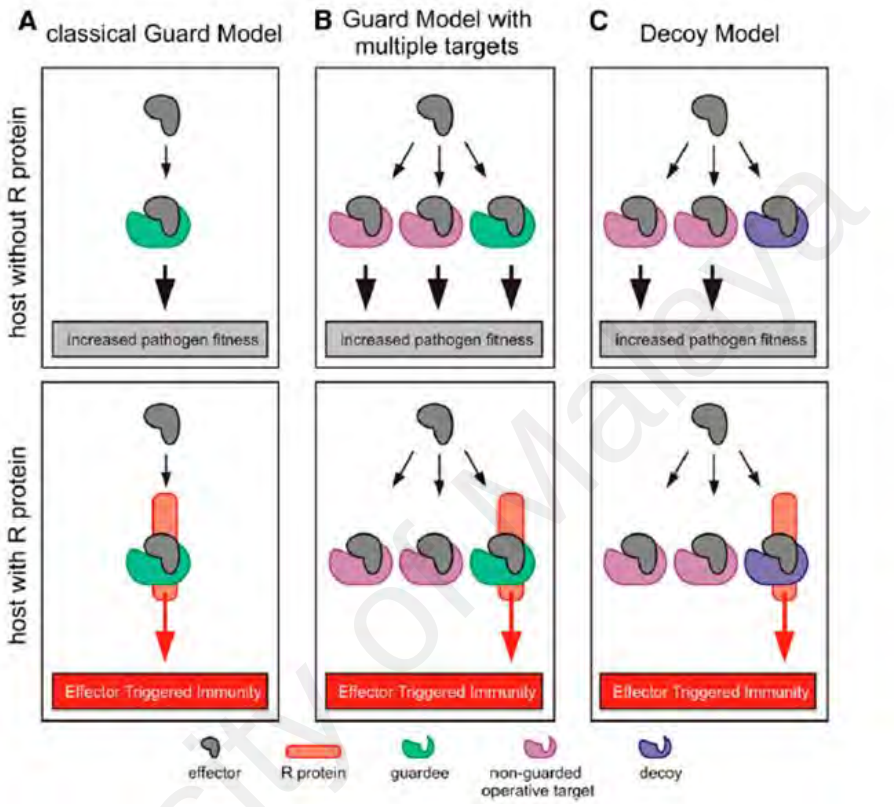

Figure 2.2: Comparisons of Guard and Decoy models. The classical Guard Model (A) is contrasted with a modified Guard Model in which the effector targets multiple plant proteins (B) and the Decoy Model (C). Effectors are depicted in gray, operative effector targets in purple, guardee in green, decoy in blue, and the R protein in orange (van der Hoorn & Kamoun, 2008).

CHAPTER 3: MATERIALS AND METHODS

3.1 Plant material, fungal strain and inoculation method

One month old tissue cultured plantlets of *Musa acuminata* cv. Berangan (AAA), an *Foc*TR4 susceptible cultivar, were purchased from Granatech Sdn. Bhd. (Kuala Lumpur, Malaysia) The plantlets were hardened on sterile 1:1 clay: coarse vermiculite for 2 months. *Foc*TR4 strain C1HIR_9889 (VCG type 01213/16), an isolate from Kuala Terengganu, Terengganu, Malaysia was used in this study. Agar plugs with pure culture of *Foc* mycelia were placed on Potato Dextrose Agar (PDA) (BD Difco™, USA) supplied with 50µg/mL streptomycin with and without a nylon membrane placed on top of the agar. The *Foc* culture was allowed to grow on the PDA for 7 days. Mycelia were harvested from the agar or nylon membrane using a sterile spatula and used for fungal inoculation and total nucleic acid isolation.

The artificial inoculation was done using double tray method (Baharum et al., 2018). Twenty healthy plantlets (10 to 15cm in height) were used. A control (mock inoculation) and 3 time points, namely 0 hpi (hour-post-inoculation), 48 hpi and 96 hpi, were performed in this study. Spores suspension in Potato Dextrose Broth (PDB) (BD Difco™, USA) with a concentration of 106 spores/mL was used in all 3 inoculation time courses. Root of 10 *M. acuminata* cv. Berangan plantlets were immersed into the spores suspension and allowed to sit for 2 hours. The remaining 4 plantlets were immersed into autoclaved distilled water as a control. After 2 hours, all plantlets were retrieved. Three plantlets retrieved from the spores suspension were randomly selected to represent the 0 hpi group. The remaining were replanted into autoclaved soil until the designated harvesting time points (48 hpi and 96 hpi). The harvested root samples were rinsed and then freeze dried in liquid nitrogen. All root samples were stored in -80°C freezer until nucleic acid extraction.

3.2 Total nucleic acid extraction protocol

Total nucleic acid were extracted from samples using a CTAB (hexadecyl-trimethyl-ammonium bromide) method modified from Doyle and Doyle (1987). The modified lysis buffer consists of 100 mM Tris-Cl (pH8.0) (Promega, USA), 25 mM EDTA (pH8.0) (Promega, USA), 2 % (w/v) CTAB (Nacalai Tesque, Japan), 2 M NaCl (Merck, Germany) and 2 % (w/v) PVP-10 (Sigma-Aldrich, USA). Two percent (v/v) of β-mercaptoethanol (Sigma-Aldrich, USA) was added to the lysis buffer prior to the extraction. Mycelia were ground to a fine powder (abbreviated as 'mycelial powder' hereafter) in liquid nitrogen with a sterile mortar and pestle and transferred to sterile microcentrifuge tubes. Two mL prewarmed CTAB lysis buffer was added to microcentrifuge tubes to resuspend the fine powder (250 mg, 100 mg or 50 mg). Samples were mixed vigorously until all powder dissolved in the buffer and then incubated at 65 °C for 10 minutes. The remaining steps were carried out under cool conditions (≤ 4 °C).

Chloroform: isoamyl alcohol (24: 1) (VWR Life Science, USA) extraction was carried out twice followed by pure chloroform extraction. All centrifugation steps were conducted at 16000 x g for 15 minutes. Aqueous phase collected from the last step were added with either 2.5 volumes of absolute ethanol or to a final concentration of 2.5 M Lithium chloride (LiCl) (Merck, Germany), then kept at -20 °C overnight. Samples were centrifuged at 16000 x g for 10 minutes to recover pellets, supernatant was discarded. Pellets were washed with 500 µL of 70 % ethanol twice, air dried and resuspended in nuclease-free water. The same extraction protocol was used for mock-inoculated and *Foc*-infected banana samples. Commercial RNeasy plant mini kit (QIAGEN, Germany) was used to extract nucleic acids from 100 mg of mycelia powder according to manufacturer's instructions to serve as comparison to the optimized extraction method.

3.3 Total nucleic acid measurement and quality control

Integrity and concentration of the total nucleic acids were tested with agarose gel electrophoresis and Nanophotometer (Implen GmbH, Germany). In general, A260/A280 ratio of ~1.8 for DNA and ~2.0 for RNA indicated the samples are free of protein contaminations. While DNA and RNA with a A260/A230 ratio of 2.0 and above are considered free of common contaminants such as humic acid and phenolic compound. A nylon membrane was placed on top of potato dextrose agar (PDA) to facilitate the collection of mycelia as described in Schumann et al. (2013). In this study, total nucleic acids isolated from 100 mg of mycelia powder (PDA with nylon membrane) were used for further nuclease treatment. Total nucleic acids from infected roots were treated according to the schematic diagrams (Figure 3.1) to produce DNA and RNA used for further PCR and qRT-PCR assessments. All samples were stored at -80 °C until nuclease treatments.

3.4 DNase I and RNase A treatments

Total nucleic acids were treated with DNase I (New England Biolabs, UK) to obtain DNA-free RNA. Ten µg of total nucleic acids were treated with either 2 units of DNase I for LiCl-precipitation or 4 units of DNase I for ethanol-precipitated samples. Samples were incubated at 37 °C for 1 hour. One volume of phenol: chloroform (1: 1, pH 4.5) was added to treated-RNAs and mixtures were centrifuged for 15 minutes. The treated-RNAs were precipitated overnight at -20 °C with LiCl to a final concentration of 2.5 M.

For RNase A treatment, total nucleic acid were treated with 50 µg/mL of RNase A (Vivantis Technologies Sdn. Bhd., Malaysia) and incubated for an hour at 37 °C. One volume of phenol: chloroform: isoamyl alcohol (125: 24: 1, pH 6.7) (VWR Life Science, USA) was used to purify the RNase-treated DNAs and centrifuged for 15 minutes. The

DNAs were precipitated overnight at -20 °C using 0.1 volume of 3 M sodium acetate (pH 5.2) (Merck, Germany) and 2 volumes of absolute ethanol (J-Kollins, UK).

RNA and DNA pellets were air dried and resuspended in 20 µL of nuclease-free water. Integrity and concentration were determined with agarose gel electrophoresis and Nanophotometer (Implen GmbH, Germany). All centrifugations were conducted at 16000 x g at 4 °C. The optimized extraction protocol was illustrated in Figure 3.1.

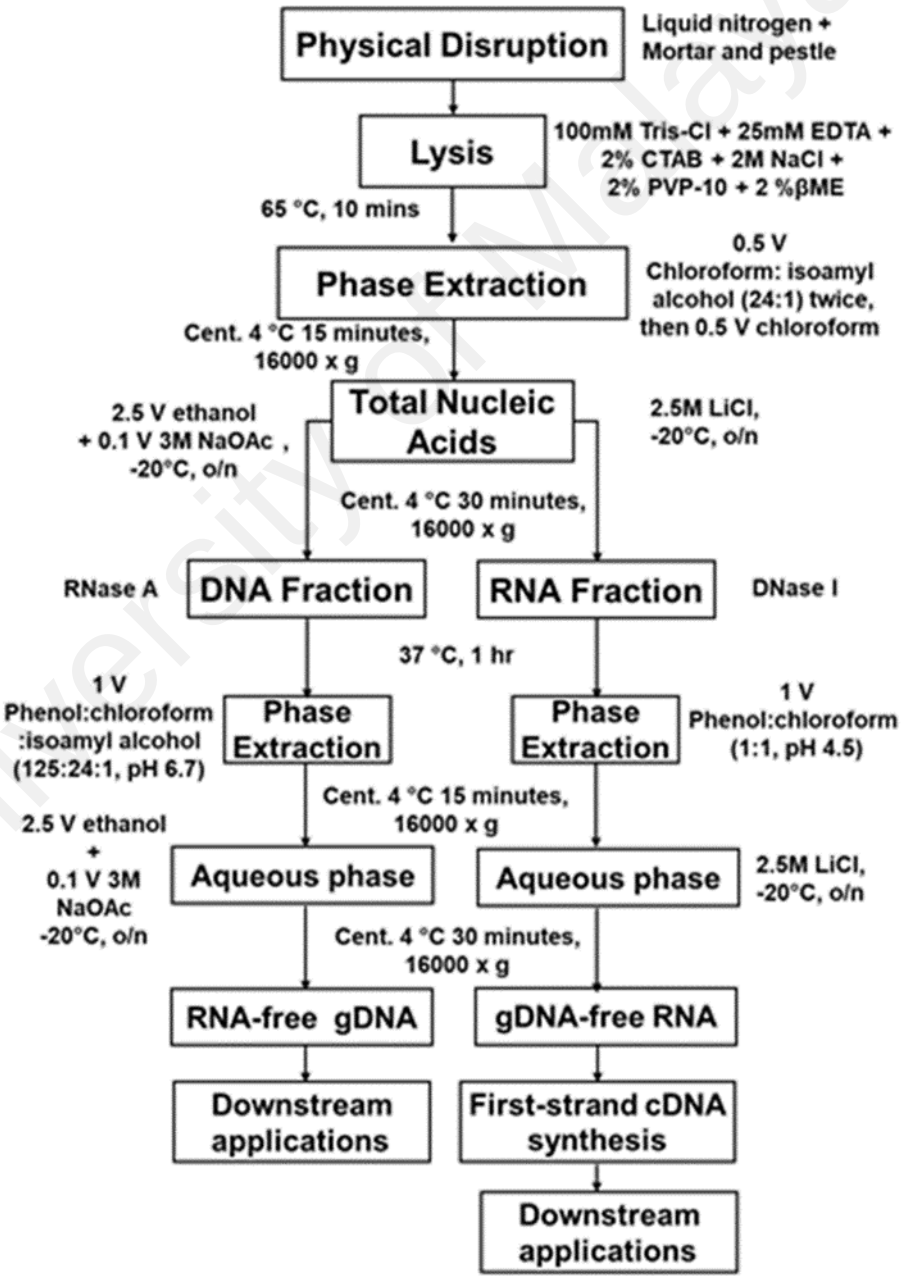

Figure 3.1: Schematic diagram of optimized CTAB-based method. V, volume; cent., centrifuge; o/n, overnight; mins, minutes; hr, hour.

3.5 Polymerase chain reaction (PCR) and quantitative reverse transcriptase PCR (qRT-PCR) analyses

FoTEF1α-F and FoTEF1α-R primers (Table 3.1) were used to check the amplifications of *Foc*TR4 translation elongation factor 1α (*FoTEF1α*), the endogenous control, in 4× diluted cDNA samples and no reverse transcriptase (no RT) controls of *Foc*TR4 vegetative mycelia and inoculated banana roots. The PCR reaction was set up with, 1 µL of template, 0.02 U of OneTaq DNA polymerase (New England Biolabs, UK), 1 X buffer, 0.2 µM dNTPs, 0.25 µM of FoTEF1α-F and FoTEF1α-R primers and nuclease free water in a 10 µL reaction volume. The thermocycling profile used was 94 °C for 2 mins for initial denaturation; followed by a 40-cycle of denaturation at 94 °C for 15 seconds, annealing at 65 °C for 30 seconds and extension at 72 °C for 30 seconds. The final extension was at 72 °C for 10 minutes. All 10 uL of the PCR products were loaded on 1.5 % agarose gel to check the amplifications. The absence of target amplicons from no RT controls serves as verification for efficient gDNA removals by DNase I.

The same test was run on the Applied Biosystems™ QuantStudio™ 12K Flex Real-time PCR System. The qRT-PCR was set up with 1 µL of cDNA template, 1X SensiFAST Lo-ROX mix (Bioline Reagent Ltd., UK), 0.1 µM of FoTEF1α-F and FoTEF1α-R primers and nuclease free water in a 10 µL reaction volume. The quantitative cycles for cDNA samples and no RT controls of *Foc*TR4 vegetative mycelia and inoculated banana roots serves as verification for efficient gDNA removals by DNase I (amplification signal later than 35 cycles for no RT control).

3.6 Secreted-in-xylem (*SIX*) effector genes identification from whole genome sequences of *Foc*TR4 C1HIR_9889

Local BLAST searches against *Foc*TR4 C1HIR_9889 assembled genome (Accession no. GCA_001696625.1) were carried out using Secreted-in-xylem (*SIX*) genes sequences from *Fusarium oxysporum* f. sp. *lycopersici* (*Fol SIX* genes) (Accession numbers: *SIX1*, GQ268948.1; *SIX2*, GQ268949.1; *SIX4*, GQ268951.1; *SIX6*, FJ755835.2; *SIX8*, FJ755837.1; *SIX9*, KC701447.1; SIX13, KC701451.1) to identify *FocSIX* genes. The Expectation value (E value) for BLASTN was set to be 1.0 E-80 for a hit to be considered significant. On the other hand, TBLASTN was also used for those gene sequences which returned in no significant hits using BLASTN. *Fol SIX* translated amino acid sequences were BLAST against the assembled genome (i. e. TBLASTN) and the E value for significiant hits was set to be 1.0 E-30. All matched *SIX* gene sequences with significant hits mentioned above were manually located and annotated as of contigs numbers in the assembled genome of *FocTR4* strain C1HIR_9889 and named *FocSIX* genes.

3.7 Sequence analysis of Secreted-in-xylem (*SIX*) effector genes

The exon-intron organization of 11 *SIX* gene homologues (3 isoforms for *SIX1,* 3 isoforms for *SIX9* and 2 isoforms for *SIX13*) was analyzed using Gene Structure Display Server (GSDS 2.0) (http://gsds.cbi.pku.edu.cn/index.php) (Hu et al., 2015). All *SIX* gene homologous sequences obtained from the genome were translated into amino acids. Amino acid alignment of the *SIX* genes were done with BioEdit version 7.2.5 (Hall, 1999). Gene trees of two *SIX* gene homologues with isoforms, which are *FocSIX1* and *FocSIX13* from *Foc*TR4 and other forma speciales were constructed with MEGA 7.0 (Kumar et al., 2016) using neighbour-joining method (bootstraps value = 1000). The amino acid sequences were also subjected to SignalP 4.0 (Petersen et al., 2011) for N-

terminal signal peptide prediction. Cysteine residues in the mature peptide were calculated for each *FocSIX* gene.

3.8 Relative quantification of *FocSIX* effector genes

Primer Blast NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used to screen for possible non-specific amplification from *Musa* spp. since transcripts from *Musa* spp. are the most prominent contaminants in the infected root cDNA samples. PCR Primer Stats (hosted by Sequence manipulation suite at http://www.bioinformatics.org/sms2/pcr_primer_stats.html) was used to exclude possible primer dimer-forming primer sequences and 3' hairpin structures in order to increase the primer efficiency during quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). qPCR primers were designed for all *FocSIX* genes. Three pair of primers were designed to for 3 *FocSIX1* isoforms. The priming sites were carefully selected to discriminate 3 copies of *FocSIX1* by the insertion and deletion sites, INDELs, present in each isoform. All 3 copies of *FocSIX9* found in *Foc*TR4 C1HIR_9889 genome have 100 % identical nucleotide sequences hence a single qPCR was carried out to quantify the expression. *FocSIX13* primer pair used in this study was designed based on the conserved sequences from both *FocSIX13a* and *e* copies. Hence, the final number of *FocSIX* genes being quantified by qRT-PCR was eight.

To check the primer efficiency, standard curves for the primers for FoTEF1α and 8 *FocSIX* genes (Table 3.1) were determined with 5 serial dilutions (100 ng to 0.01 ng) of *Foc* DNA on the Applied Biosystems™ QuantStudio™ 12K Flex Real-time PCR System. The reaction specificity of the PCR reaction was monitored by melting curves. The qPCR was set up with 100 ng of *Foc* DNA, 1X SensiFAST Lo-ROX mix (Bioline Reagent Ltd., UK), 0.1 µM of forward and reverse primers (Table 3.1), and nuclease free water in a 10

μL reaction volume. The standard curve was plotted using quantitative cycle (Cq) and $\log_{10}$ of DNA concentrations. Primers FoTEF1α was then used to quantify the expression of *Foc* translation elongation factor 1α in cDNA of infected banana roots and served as normalization.

Table 3.1: Primer sequences for qRT-PCR of *Fusarium oxysporum* f. sp. *cubense* (*Foc*) Secreted-in-xylem (*SIX*) genes.

Primer	Sequence (5' to 3')	Melting Temp.	Amplicon size
FoTEF1α-F	TCGGCTACAACCCCAAGGCTG	62.0 ºC	120 bp
FoTEF1α-R	CGGACTTGATCTCACGCTCCCA	61.0 ºC	
FocSIX1a-F	CAAGACCAGGCAACGAGGC	59.0 ºC	128 bp
FocSIX1a-R	ACGCTACGATAATTGACCGCC	57.4 ºC	
FocSIX1b-F	GGGAGTGTCCCAGATAACAGTG	57.0 ºC	92 bp
FocSIX1b-R	CGTCTCGGTCTGAACACTATCG	56.9 ºC	
FocSIX1c-F	CCAGAGGGGCAGGCTCAG	60.8 ºC	96 bp
FocSIX1c-R	GTAGACTTGTCCGTGGTAGGCGAC	60.8 ºC	
FocSIX2-F	CTCAAAAGCATTCTCCAGGCTACA	57.2 ºC	98 bp
FocSIX2-R	CATATCGGGATCGGCTTCAACGA	58.9 ºC	
FocSIX6-F	CACTCCTTGCAACTCAGGCGA	59.8 ºC	112 bp
FocSIX6-R	ATCCGGGTCAGTTCTCCACGA	60.2 ºC	
FocSIX8a-F	GGAGGGACATCGAACGTGCTT	59.6 ºC	92 bp
FocSIX8a-R	TTCACCTCACCCGGCATGATCT	60.6 ºC	
FocSIX9-F	AATCATCCTTACAACTGGGCTTCC	57.1 ºC	102 bp
FocSIX9-R	GATAGTGCATTGCCCCATCTGGTA	58.9 ºC	
FocSIX13-F	TGATCAGCCTCCTAGCGTCGAA	59.9 ºC	92 bp
FocSIX13-R	AGCTTTACCGAGAGCTCGTCCA	60.1 ºC	

cDNA was synthesized from 2 μg of DNA-free total RNA of vegetative *Foc* mycelia and infected roots using the SuperScript IV™ First Strand Synthesis System (Thermo Fisher Scientific, USA) according to manufacturer's instructions. FoTEF1α-F and FoTEF1α-R primers were used to quantify the expression of *Foc*TR4 translation elongation factor 1α (*FoTEF1α*), the endogenous control, in 4× diluted cDNA of *Foc* vegetative mycelia and inoculated banana roots.

FocSIX genes expression were quantified using respective primers set listed in Table 3.1. qPCR amplicons were collected after the reaction and purified with PCR purification

kit (Mahcery-Nagel, Germany). The purified products were outsourced to Apical Scientific Sdn. Bhd. for sequencing to verify the target amplicons. The relative gene expressions were calculated using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

3.9 Statistical analysis

The range of *FocSIX* genes fold change was calculated according to the Applied Biosystems instruction manual (Applied Biosystems, USA). Mean C_T and standard deviation (σ) of each time point for *FoTEF1α* and *FocSIX* genes were calculated using Microsoft Excel.. ΔC_T was calculated by subtracting mean C_T *FoTEF1α* from mean C_T *FocSIX*. The standard deviation of ΔC_T ($\sigma_{\Delta CT}$) was calculated with following formula:

$$\sigma_{\Delta CT} = \sqrt{(\sigma_{SIX}^2 + \sigma_{TEF1\alpha}^2)} \quad (1)$$

For each time point, the $\sigma_{\Delta CT}$ for experimental groups (*Foc*, 0 hpi, 48 hpi and 96 hpi) were incorporated into $\log_2$ fold change and fold change range. Multiple T- tests were done on the ΔC_T of each gene to check the significance of upregulation. The false discovery rate (FDR) procedure was adopted to correct p-values for multiple comparisons. Genes with $p < 0.01$ were accepted as differentially expressed.

3.10 *in silico* pipeline for Small secreted cysteine-rich protein (SSCRP) prediction

A computational pipeline (Figure 3.2) was designed to predict the secretome of *Foc*TR4. Fusarium genome sequences (Accession numbers for *Foc*TR4_II5, GCA_000149955.2 and *Foc*TR4 C1HIR_9889, GCA_001696625.1) and predicted protein sequence data were downloaded from the Fusarium Comparative Sequencing Project, Broad Institute of Harvard and MIT (http://www.broadinstitute.org/annotation/genome/Fusarium_group/MultiHome.html). The predicted proteins of *Foc*TR4 strain, C1HIR_9889 (Shetty et al., unpublished) were

also subjected to secretome analyses and compared with that of the reference *Foc*TR4_II5 strain. The putative secreted proteins were predicted by the classical secretion prediction pipeline that consists of series of prediction algorithms followed by annotation of proteins. For local installation, SignalP v4.0 (Petersen et al., 2011) and TMHMM v1.2 (Krogh et al., 2001) stand-alone software programs were downloaded from the CBS prediction servers (http://www.cbs.dtu.dk/services/). A stand-alone copy of the program Phobius (v1.01) was downloaded from http://phobius.sbc.su.se/ (Kall et al., 2007). Analyses in ProtComp prediction server (www.softberry.com) and YLoc web server (Briesemeister et al., 2010) were executed remotely using HTTP client. The WoLF PSORT software was downloaded from http://www.wolfpsort.org and MultiLoc2 program (Blum et al., 2009) was downloaded from http://abi.inf.uni-tuebingen.de/Services/ website. Custom Perl scripts were used to link the different programs and parse the output from component programs.

The performance of the computational framework was tested with following positive datasets of secreted proteins with experimental evidence: BaCelLo dataset (Pierleoni et al., 2006) and *Fusarium oxysporum* f. sp. *lycopersci* dataset of Secreted-in-xylem proteins (Schmidt et. al., 2013). The two datasets were combined to obtain a curated database of 205 proteins. Two proteins in the BaCelLo dataset (>BUBL_PENBR and >RNA1_ASPPL) lack start codon methionine were not detected by SignalP 4.0 program. To assess the quality of predictions in individual predictors and the computational pipeline, Matthews correlation Secretome Repertoire of *Foc*TR4 coefficient was calculated. Based on the performance with positive datasets, cut-off values for parsing the raw output from the individual sub-cellular predictors were determined. In WoLF PSORT, 'extr value' threshold was set to >15 for parsing the putative secreted proteins; whereas in YLoc and MultiLoc2, high-resolution prediction option was used and

extracellular protein predictions were filtered with probability threshold values of 0.4 and >40 % respectively. In the case of ProtComp, integral predictions with flags "extracellular secreted" and "membrane-bound extracellular secreted" were separately parsed. Finally, *Foc*TR4 proteins which were predicted to be extracellulary secreted in at least two of the predictors were combined to compile a final secretome.

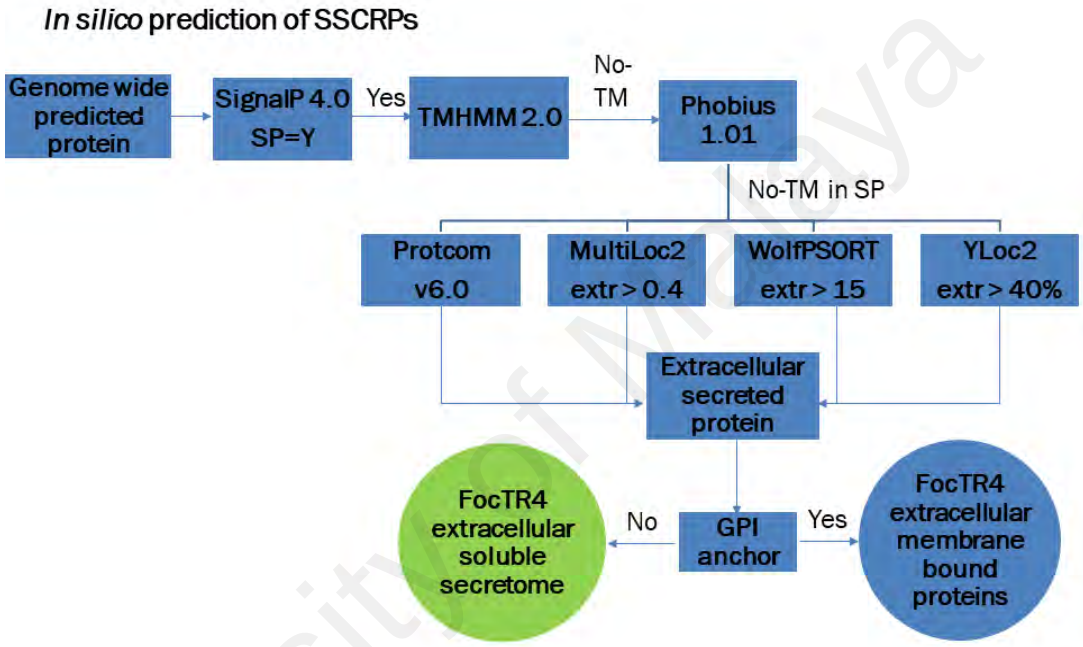

Figure 3.2: The computational framework used for the prediction of *Fusarium oxysporum* f. sp. *cubense* tropical race 4 (*Foc*TR4) extracellular secretome. SP, signal peptide; Y, present; TM, transmembrane domain; extr, extracellular. The threshold values used for extracellular prediction are indicated below the subcellular predictors**.**

The putative extracellular proteins linked to membranes with GPI anchors were filtered using a webserver known as FragAnchor (Poisson et al., 2007) and hosted at http://navet.ics.hawaii.edu/~fraganchor/NNHMM/NNHMM.html and biGPI web program(Eisenhaber et al., 2007). The remaining non-membrane bound putative extracellular proteins were screened for cysteine richness. The putative protein with less than 300 amino acids while more than 5 % cysteine compositions were selected and known as putative Small secreted cysteine-rich protein (SSCRP) in this study. The pool of the putative SSCRP was then subjected to NCBI Protein BLAST server hosted at

https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome and PHI-base BLAST (http://phi-blast.phi-base.org/).

3.11 Reverse transcriptase PCR (RT-PCR) for Small secreted cysteine-rich protein (SSCRP) prediction

FoTEF1α was used as the endogenous control for gene expression for selected *SSCRP* genes. The PCR reaction was set up with 1 µL of 4× diluted cDNA template, 0.02 U of OneTaq DNA polymerase (New England Biolabs, UK), 1 X buffer, 0.2 µM dNTPs, 0.1 µM of forward and reverse primers (Table 3.2) and nuclease free water in a 10 µL reaction volume. The thermocycling profile used was 94 °C for 2 mins for initial denaturation; followed by a 25-cycle of denaturation at 94 °C for 15 seconds, annealing at 65 °C for 30 seconds and extension at 72 °C for 30 seconds. The final extension was at 72 °C for 10 minutes. All 10 uL of the PCR products were loaded on 1.5 % agarose gel to check the amplifications.

Table 3.2: Primer sequences for RT-PCR of *Fusarium oxysporum* f. sp. *cubense* (*Foc*) Small secreted cysteine-rich protein (*SSCRP*) genes.

Primer	Sequence (5' to 3')	Melting Temp.	Amplicon size
FoTEF1α-F	TCGGCTACAACCCCAAGGCTG	62.0 ºC	120 bp
FoTEF1α-R	CGGACTTGATCTCACGCTCCCA	61.0 ºC	
Foc9889_2126-F	ACGTCACCCACAAGTATGTTGCT	58.9 ºC	103 bp
Foc9889_2126-R	TTGAATCCAGGAGGGACACAGTA	57.4 ºC	
Foc9889_5756-F	TGATCTGCCGTCTAGCTGGATTC	58.5 ºC	109 bp
Foc9889_5756-R	GGGGCATTCTGATGGAATAGGTC	57.4 ºC	
Foc9889_1356-F	CCTATCAGGAAGACGGCCAAACT	58.8 ºC	101 bp
Foc9889_1356-R	ACCTCCTTTCCAATGGTGCAGA	58.8 ºC	
Foc9889_10106-F	TTCGCTCATCAATCGCTTCTGG	57.6 ºC	106 bp
Foc9889_10106-R	GGGGCGTTTTTGCTCTTGTCAT	58.6 ºC	
Foc9889_11412-F	CTACCTCTTTCCTCGTCGCTGCT	60.5 ºC	81 bp
Foc9889_11412-R	AGGTCTCGGGACAATCAGTAGGG	59.9 ºC	

CHAPTER 4: RESULTS

4.1 Total nucleic acid isolation

The ratio of mycelia powder mass to CTAB lysis buffer was critical for the reproducibility of the *Foc* nucleic acid isolation. In this study, 100 mg of mycelia powder resuspended in 2 mL of CTAB lysis buffer has resulted in a clean separation of cell debris from the aqueous phase (ii in Figure 4.1b). However, 250 mg of mycelia powder collected from PDA resuspended in 2 mL CTAB buffer failed to separate after centrifugation (i in Figure 4.1b). The resulting lysates were extremely viscous which trapped the buffer and hindered the following phase separation. Hence, 100 mg of mycelia powder to 2 mL lysis buffer was the optimal ratio for *Foc* nucleic acid isolation.

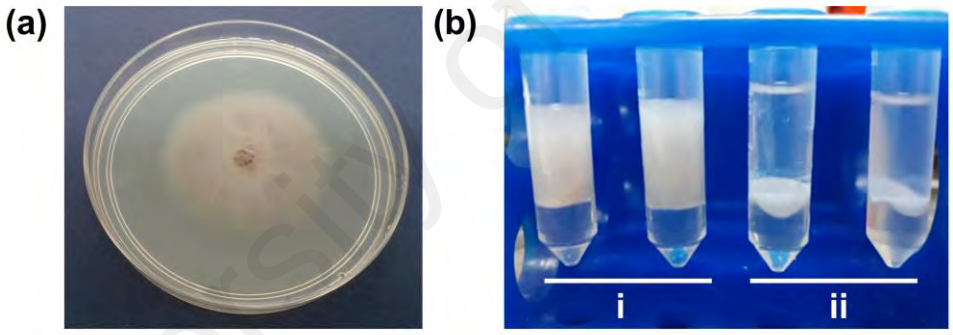

Figure 4.1: *Fusarium oxysporum* f. sp. *cubense* TR4 (*Foc*TR4) culture and RNA extraction. (a) *Foc*TR4 vegetative culture on PDA; (b) Phase separation of *Foc*TR4 RNA extraction, (i) 250 mg, (ii) 100 mg.

When the RNA was precipitated using ethanol, the RNA bands (28S and 18S rRNA) were not pronounced on agarose gel while high amount of DNA can be observed (EtOH lanes in Figure 4.2). To investigate the effect of precipitation agents on the quality of RNA, another attempt was made to precipitate the RNA with lithium chloride (LiCl). With LiCl, RNA of good integrity was precipitated except the sample with 50 mg mycelial powder collected directly from PDA (Lane 10: PDA (P)/ 50 mg/ LiCl in Figure 4.2).

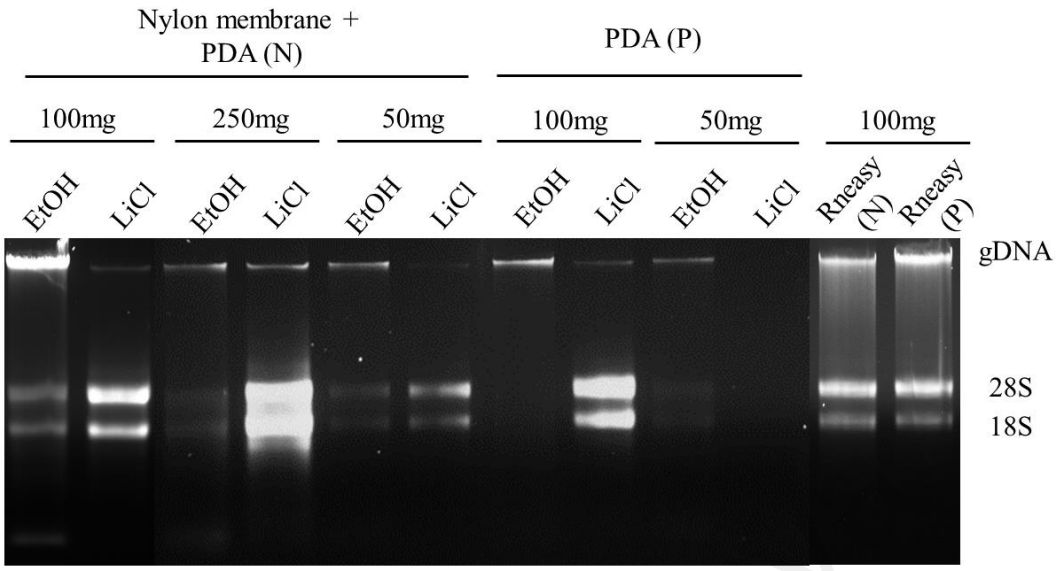

Figure 4.2: Total nucleic acid of *Foc* extracted with different ratios of mycelia powder to 2 mL lysis buffer. EtOH, ethanol precipitated; LiCl, lithium chloride precipitated; RNeasy(N), mycelia grown on PDA with nylon membrane overlay and extracted with plant RNeasy kit; RNeasy(P), mycelia grown directly on PDA and extracted with plant RNeasy kit.

Total RNA yield from 100 mg of *Foc* mycelia powder extracted using the commercial kits was 1.6 µg. It was much lesser than the 10.6 to 28.2 µg yielded from our CTAB-based method (Table 4.1). For both samples extracted with plant RNeasy kit, either from mycelia grown on PDA with nylon membrane overlay or directly on PDA, heavy smearings present due to incomplete removal of contaminants (Figure 4.2). Also, low A260/230 ratio (0.659) was also observed for sample collected directly from PDA (Table 4.1). Commercial kit offers less optimizations for difficult sample type while our method allows modifications for different sample types. For example, the method developed in this study allow us to use optimized sample mass for higher and purer RNA yield. Presence of impurities was indicated by the low A260/230 readings (Table 4.1) and smearing of the RNA bands observed on gel (Figure 4.2). This further limited the use of commercial kit for sample types used in this study.

The spectrometric readings of the nucleic acids extracted with different ratio of mycelial powder to buffer and 2 different precipitating agents were shown in Table 4.1.

The CTAB-based buffer capable of isolating the RNA from mycelia grown on the membrane as well as directly on agar. When precipitated with 2.5 M lithium chloride, RNA with good values of A260/280 (2.064 ± 0.021) and A260/230 (1.937 ± 0.076) was obtained (Table 4.1).

Table 4.1: Amount and spectrometric readings of *Foc* RNA isolated with the optimized CTAB-based method. Two mL of lysis buffers were added to every samples during extraction.

Culture media	**Precipitation Reagent**	**Mycelial Powder Mass (mg)**	**Amount of RNA (µg)/100 mg**	**$A_{260/280}$**	**$A_{260/230}$**
PDA*	-	250	-	-	-
PDA	Absolute ethanol	100	10.6	1.912	1.207
PDA	2.5M LiCl	100	12.5	2.088	1.975
PDA	Absolute ethanol	50	2.5	1.986	1.521
PDA**	2.5M LiCl	50	-	-	-
PDA	RNeasy plant mini kit	100	1.6	2.022	0.659
Nylon membrane + PDA	Absolute ethanol	250	9.6	1.937	1.469
Nylon membrane + PDA	2.5M LiCl	250	12.6	2.055	1.986
Nylon membrane + PDA	Absolute ethanol	100	28.2	1.965	1.529
Nylon membrane + PDA	2.5M LiCl	100	23.4	2.050	1.850
Nylon membrane + PDA	Absolute ethanol	50	3.2	2.009	1.733
Nylon membrane + PDA**	2.5M LiCl	50	-	-	-
Nylon membrane + PDA	RNeasy plant mini kit	100	1.6	1.966	1.683

* Failed to separate at first chloroform isoamyl alcohol phase separation.
** Resulting pellets after precipitation were unrecoverable

In the case of infected root samples, A260/230 readings from ethanol-precipitated nucleic acids are always below 1.0, despite multiple chloroform-isoamyl alcohol or even phenol: chloroform extractions were performed. Even though ethanol is very efficient at precipitating nucleic acids, unfortunately, it also works unselectively in precipitating impurities such as polyphenol and humic acid as reflected by low A260/230 readings

(Table 4.2). But, when 2.5 M lithium chloride was used for the precipitation, the A260/230 values improved drastically to above 2.0 (Table 4.2). This indicated the isolated RNA was free of above-mentioned impurities. Genomic DNA contamination were successfully removed after digestion of DNaseI while the RNA bands (28S, 18S and 5S) remained intact (Figure 4.3).

Table 4.2: Spectrometric readings of nucleic acids isolated from infected banana roots precipitated with absolute ethanol and 2.5 M lithium chloride.

Root samples	Ethanol-precipitated		LiCl-precipitated	
	A260/280	**A260/230**	**A260/280**	**A260/230**
Mock	2.327 ± 0.217	0.705 ± 0.090	1.986 ± 0.041	2.369 ± 0.067
0HPI	2.359 ± 0.079	0.577 ± 0.015	1.997 ± 0.012	2.412 ± 0.019
48HPI	2.268 ± 0.160	0.590 ± 0.024	2.021 ± 0.019	2.380 ± 0.016
96HPI	2.191 ± 0.104	0.645 ± 0.036	2.082 ± 0.069	2.097 ± 0.422

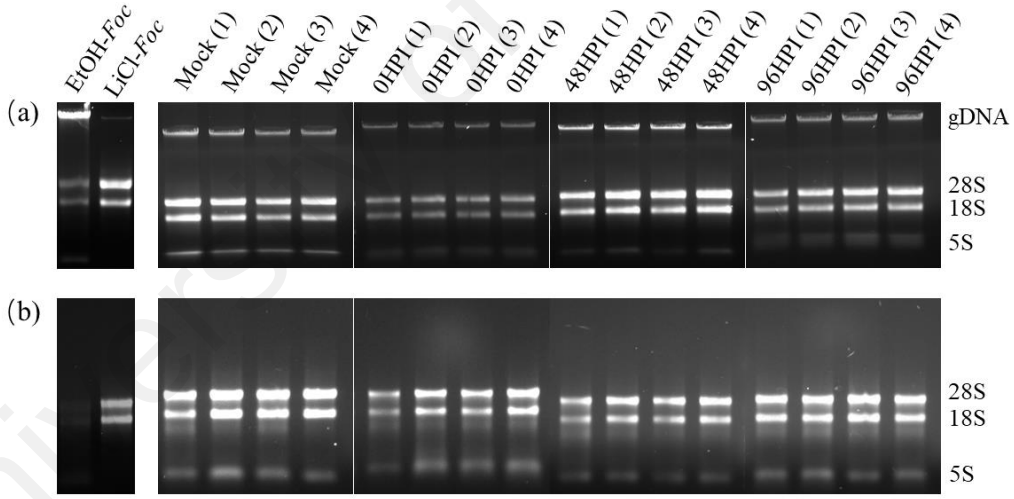

Figure 4.3: RNA of *Foc*TR4 vegetative mycelia and infected banana roots. (a) before DNaseI digestion (b) after DNaseI digestion. Two microliters (µL) of RNA was loaded to each lane.

4.2 PCR and qRT-PCR analyses

Foc nucleic acid samples isolated from 100 mg mycelial powder were further digested with DNase I to yield DNA-free RNA. Two units of DNase I was sufficient to remove genomic DNA (gDNA) from the LiCl-precipitated total nucleic acid as there were no amplification from the FoTEF1α primers for no reverse transcriptase samples (no RT

lanes in Figure 4.4). Both cDNA synthesized from LiCl-precipitated and ethanol-precipitated *Foc* RNA showed a single amplicon of 120 bp when FoTEF1α gene-specific primers were used. The amplicon was absent in both no reverse transcriptase (no RT) controls and mock inoculated sample (Figure 4.4).

However, when qRT-PCR was used to check the gDNA contamination, the ethanol-precipitated no RT controls showed amplification signal even though substantial amount of DNase I (up to 4 units for 10 µg of nucleic acids) was used to digest the co-extracted gDNA. In contrast, no qRT-PCR amplification signal was detected on LiCl-precipitated no RT controls (Appendix B). This showed that *Foc* RNA extracted with this CTAB-based method, when coupled up with LiCl, was compatible with downstream applications such as reverse transcription, polymerase chain reaction (PCR) and quantitative reverse transcriptase PCR (qRT-PCR).

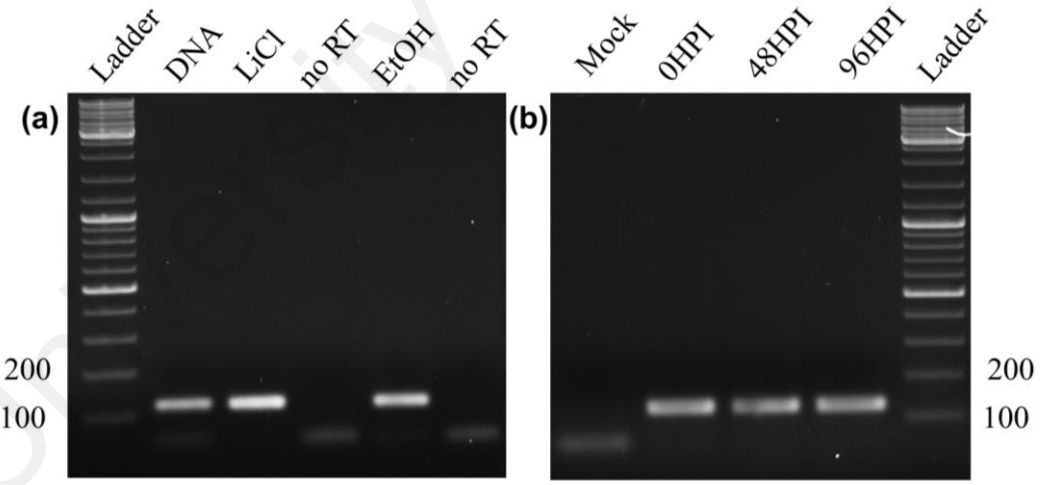

Figure 4.4: PCR amplification of cDNAs with FoTEF1α primers. (a) Amplifications from *Foc* cDNA synthesized with RNA precipitated with either lithium chloride (LiCl) or ethanol (EtOH). Ladder- O' Gene ruler DNA ladder mix (Thermo Fisher Scientific, USA); no RT- No reverse transcriptase control (b) Infected banana root cDNA. Mock- Mock inoculated root. The target amplicon is 120 bp long.

4.3 Secreted-in-xylem (*SIX*) effector genes in *Foc*TR4 C1HIR_9889 genome

Local BLAST searches against *Foc*TR4 C1HIR_9889 assembled genome were carried out using Secreted-in-xylem (*SIX*) genes sequences from *Fusarium oxysporum* f. sp.

lycopersici (*FolSIX* genes) to identify *FocSIX* genes (Table 4.3). *SIX1, 2, 4, 6, 8, 9* and *13* were found presence in the genome either as single copy or multiple copies.

Table 4.3: Secreted-in-xylem (*SIX*) gene homologues identified in *Fusarium oxysporum* f.sp. *cubense* Tropical Race 4 (*Foc*TR4) strain C1HIR_9889.

Gene name	Location [1]	*Fol* [2]	Identity to *Fol* sequence	Length	Start codon	Query sequence
FocSIX1a	MBFV01000417.1, 99-914+	*SIX1* (*AVR3*)	83 %	816 bp	yes	Rep et al., 2004 (GQ268948.1)
FocSIX1b	MBFV01001340.1, 1799-2638+	*SIX1* (*AVR3*)	81 %	840 bp	yes	Rep et al., 2004 (GQ268948.1)
FocSIX1c	MBFV01000683.1, 464-1297+	*SIX1* (*AVR3*)	84 %	834 bp	yes	Rep et al., 2004 (GQ268948.1)
FocSIX2 [3]	MBFV01000960.1, 34308-34967−	*SIX2*	70 %	672 bp	yes	Rep et al., 2004 (GQ268949.1)
FocSIX4	MBFV01000714.1, 11137-11831−	*SIX4* (*AVR1*)	83 %	695 bp	no	Houterman et al., 2007 (GQ268951.1)
FocSIX6	MBFV01000714.1, 12452-13105−	*SIX6*	69 %	654 bp	yes	Gawehns et al., 2014 (FJ755835.2)
FocSIX8a	MBFV01000340.1, 3799-4319−	*SIX8*	92 %	521 bp	yes	Lievens et al., 2009 (FJ755837.1)
FocSIX9.1 [3]	MBFV01000430.1, 1495-1794−	*SIX9*	61 %	333 bp	yes	Schimdt et al., 2013 (KC701447.1)
FocSIX9.2 [3]	MBFV01000573.1, 1667-1999+	*SIX9*	61 %	333 bp	yes	Schimdt et al., 2013 (KC701447.1)
FocSIX9.3 [3]	MBFV01000614.1, 1051-1383−	*SIX9*	61 %	333 bp	yes	Schimdt et al., 2013 (KC701447.1)
FocSIX13a	MBFV01001186.1, 5618-6559−	*SIX13*	93 %	942 bp	yes	Schimdt et al., 2013 (KC701451.1)
FocSIX13e	MBFV01001225.1, 1388-2323+	*SIX13*	96 %	936 bp	yes	Schimdt et al., 2013 (KC701451.1)

[1] Location of the gene in contig of *Foc*TR4 strain C1HIR_9889. + - sense strand; − - antisense strand

[2] *Fusarium oxysporum* f. sp. *lycopersici SIX* genes

[3] Homologues identified using TBLASTN of *Fol* SIX protein sequences instead of BLASTN

SIX1 homologues in *Foc*TR4 C1HIR_9889 were designated as *FocSIX1a*, *FocSIX1b* and *FocSIX1c*. *FocSIX2, FocSIX4, FocSIX6* and *FocSIX 8* were present in single copy. Sequence of *FocSIX4* found in the genome has it start codon mutated. The only start codon found in the sequence has an open reading frame, if functional, code for only 53 amino acids. Unlike *FocSIX4*, homologue of *SIX6*, *FocSIX6*, has a start codon with an open reading frame of 654 nucleotides. Both *FocSIX4* and *FocSIX6* were found in the same contig in the genome of *Foc*TR4 C1HIR_9889. *FocSIX4* was predicted to be a pseudogene by Czilowski et al. (2018). Hence, it was not included in the subsequent gene expression analysis.

All three copies of *SIX9* are identical in genome but each copy of *FocSIX13a* has 6 bases insertion when compared with *FocSIX13e* (Figure 4.5 and Table 4.4). All 3 copies of *SIX9* homologues found in the genome were identical hence were designated as *FocSIX9.1*, *FocSIX9.2* and *FocSIX9.3* based on the contigs they were located. Most *FocSIX* genes were intronless except for *FocSIX8* and *FocSIX13* with 2 and 1 introns, respectively (Figure 4.5).

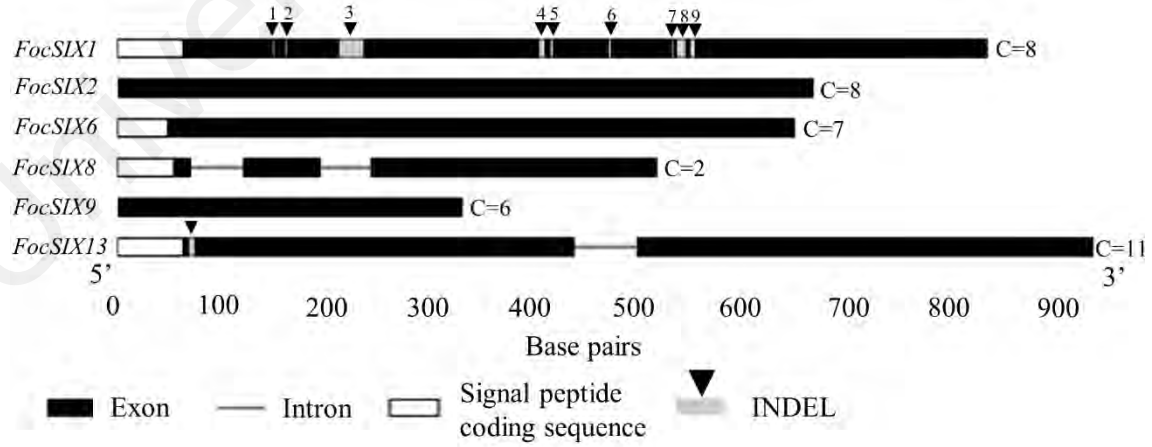

Figure 4.5: Gene structures of *FocSIX1*, *2*, *6*, *8*, *9* and *13*. C represents number of cysteine residues in the respective predicted mature peptide.

The identities between *FocSIX1a*, *b* and *c* gene copies were 83.0 % (*ab*), 88.3 % (*ac*) and 81.1 % (*bc*) (Figure 4.6b). The alignment for 3 copies of *FocSIX1* revealed altogether

9 insertion/deletion sites (INDELs) and the INDELs sequences were shown in Table 4.4. Sequence analysis of *FocSIX13a* and *e* in *Foc*TR4 C1HIR_9889 revealed a 6 nucleotides insertion/deletion (INDEL) and 64 SNP (*FocSIX13a* and *e* shared 92.6 % gene identity) resulted in 22 changes in the amino acids (Table 4.4).

Table 4.4: INDEL sequences found in different copies of *FocSIX1* and *FocSIX13* in the genome of *Foc*TR4 C1HIR_9889.

Gene	Position	Copy	Indel sequence
FocSIX1	151	*FocSIX1b*	+G
	162-163	*FocSIX1b*	+CA
	214-237	*FocSIX1b*	+TACGATAGTGTTCAGACCGA GACG
	407-412	*FocSIX1c*	+CTAACC
	418-420	*FocSIX1a/FocSIX1c*	+GCA/+GCG
	474-476	*FocSIX1a/FocSIX1b*	+CGA/+CGT
	535-536	*FocSIX1b/FocSIX1c*	+C/+CA
	540-548	*FocSIX1a/FocSIX1c*	+C/+ GCCTACCAC
	553-557	*FocSIX1c*	+AAGTC
FocSIX13	69-74	*FocSIX13a*	+AGAGGT

Since *FocSIX1* genes were intronless, all INDELs caused change in the peptide sequences. Phylogeny tree for *FocSIX1* genes constructed using amino acid sequences from Australian isolates (Czislowski et al., 2018), *Foc*TR4_II5 and *Foc*TR4 C1HIR_9889 showed that 3 copies of FocSIX1 clustered into 3 distinct subtrees and each subtree can be associated with different *Foc* races (Figure 4.6a). In the first subtree, FocSIX1c was clustered together with other SIX1 homologues identified in *Foc* Race 4 isolates whereas in the third and fourth subgroups, FocSIX1a and FocSIX1b are clustered with SIX1 homologues identified in other *Foc* Tropical Race 4 isolates.

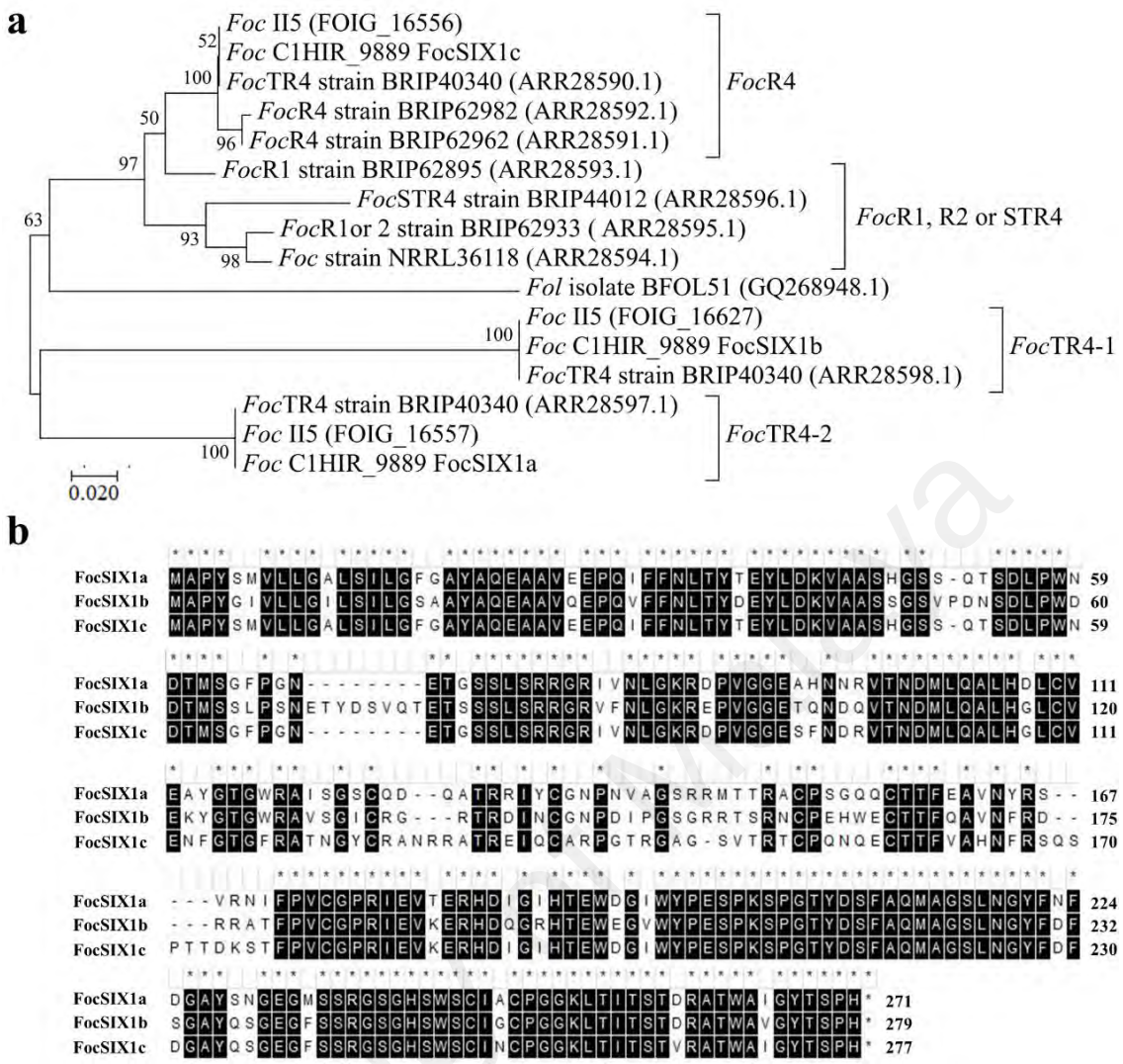

Figure 4.6: Phylogenetics relationships of *Fusarium oxysporum* f. sp. *cubense* (*Foc*) SIX1 amino acid sequences. The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths represent the number of amino acid substitutions per site. (a) Phylogenetic tree of *Foc* SIX1 protein sequences. (b) Alignment of 3 copies of FocSIX1 proteins. R1- Race 1; R2-Race 2; R4- Race 4; STR4- Subtropical race 4; TR4- Tropical race 4.

The two copies of *FocSIX13* genes identified in this study shared 92.6 % homology (Figure 4.7b). When aligned with the sequences reported in Czislowski et al. (2018), the *FocSIX13* sequences showed high homology to *a* and *e* variants hence were named accordingly as *FocSIX13a* and *FocSIX13e* (Figure 4.7a).

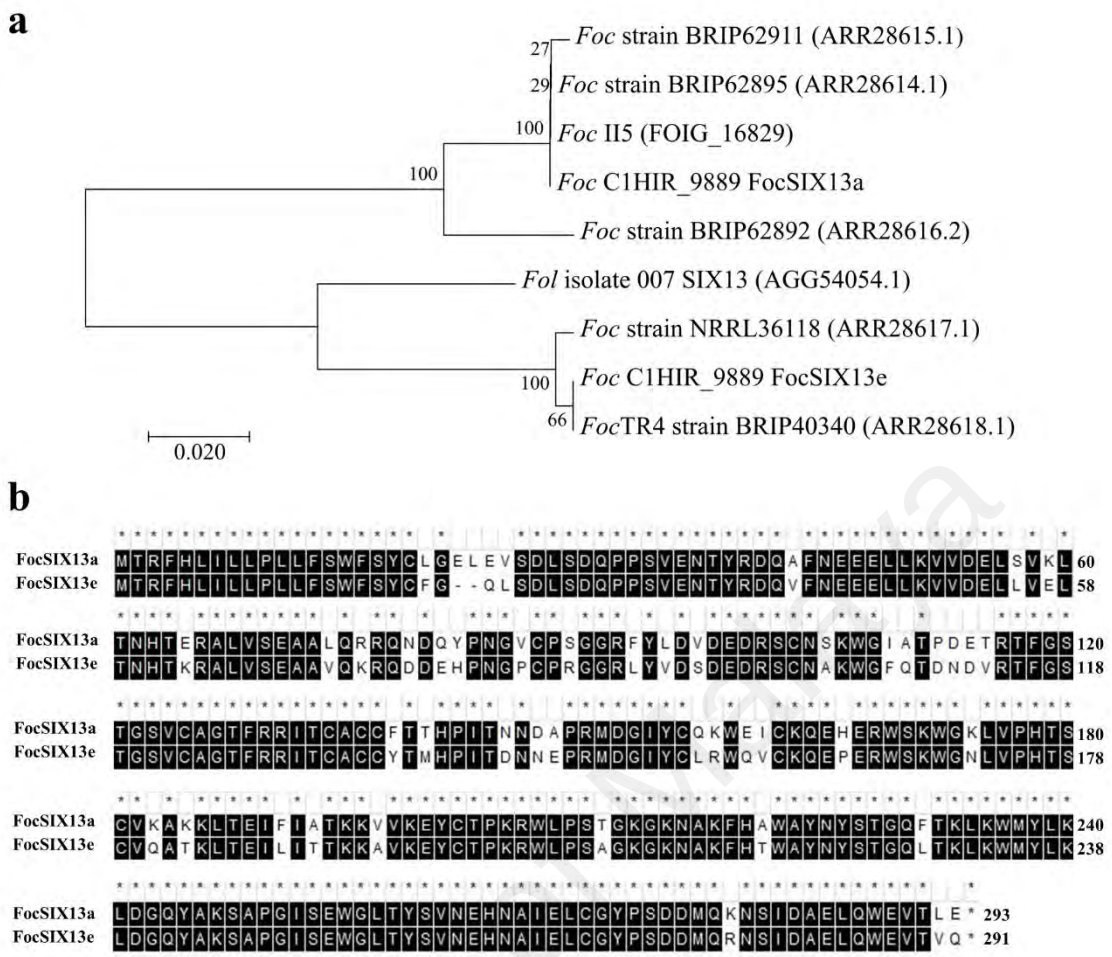

Figure 4.7: Phylogenetics relationships of *Fusarium oxysporum* f. sp. *cubense* (*Foc*) SIX13 amino acid sequences. The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, The tree is drawn to scale, with branch lengths represent the number of amino acid substitutions per site. (a) Phylogenetic tree of *Foc* SIX13 protein sequences. (b) Alignment of FocSIX13a with FocSIX13e proteins.

4.4 Relative gene expression of *FocSIX* effector genes

The presence of all eight *SIX* homologues in the *Foc*TR4 C1HIR_9889 genome was first verified using polymerase chain reaction (Appendix D). Then, their expressions in the *Foc*TR4 vegetative mycelia and infected root samples at 0-, 48- and 96 hpi was quantified through quantitative real-time PCR.

In order to quantify the *FocSIX* effector genes expression levels in the the mentioned stages, SYBR-based relative quantification was employed. In this study, the reference

gene used to normalize the effector transcripts was the eukaryotic translation elongation factor 1-alpha (*TEF1α*) in *Foc*TR4 (FoTEF1α). *TEF1α* was found to be the most stable reference gene for *Fusarium graminearum* among 15 candidates (Kim and Yun, 2011; Niño-Sánchez et al., 2015; Chen et al., 2016).

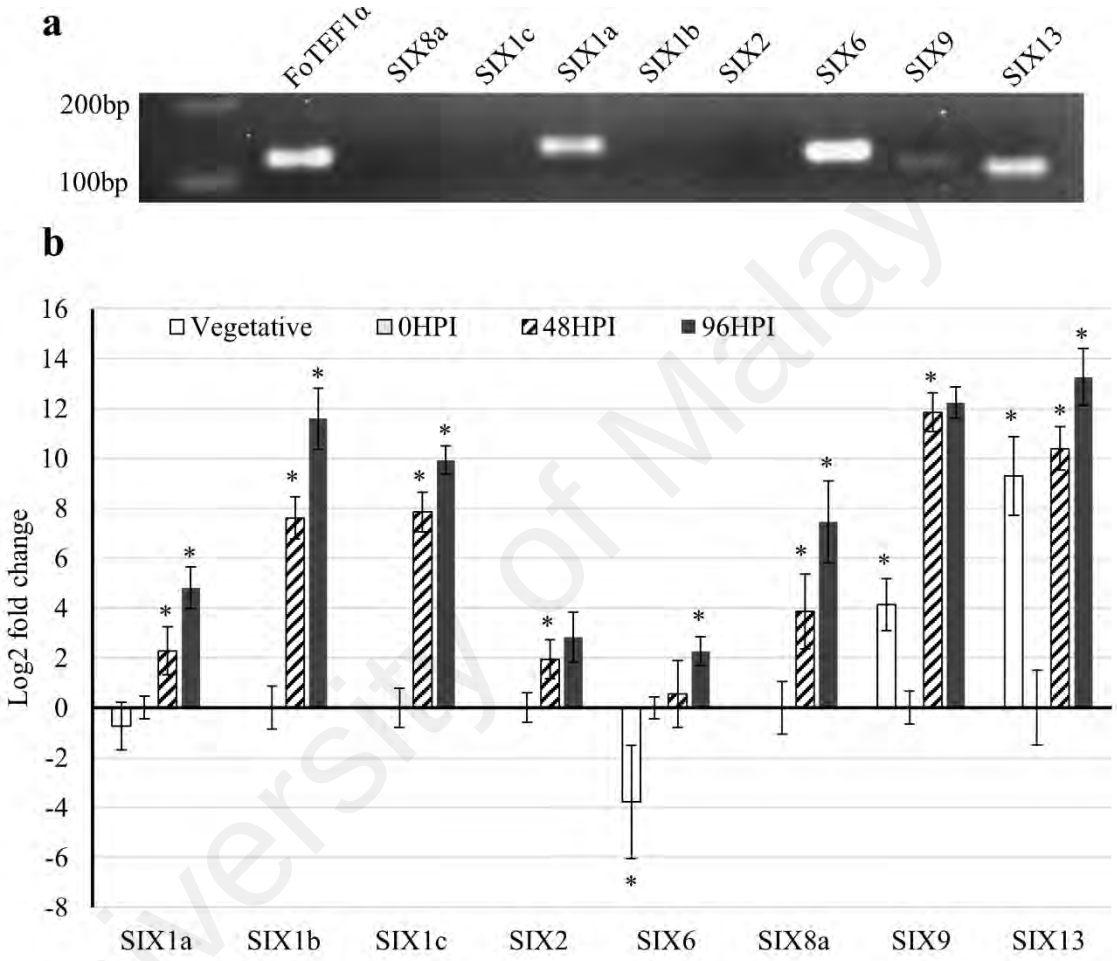

Figure 4.8: *Fusarium oxysporum* f. sp. *cubense* Secreted in xylem *(FocSIX)* effector genes expression analyses. (a) RT-PCR of *FocSIX* genes expression in vegetative mycelia samples. (b) qRT-PCR of *FocSIX* genes *in planta* at 48- and 96 hpi versus 0 hpi (control). For transcripts expressed in *Foc*TR4 vegetative mycelia, expression was also compared to the control. 0 hpi were chosen as a control group since not all *FocSIX* genes were expressed in vegetative mycelia. Mean ± SD for three biological replicates and three technical replicates are shown. Expression is relative to that of *Fusarium oxysporum* f. sp. *cubense TEF1α (*FoTEF1α*)*.
*Difference in expression between times is significant at $p < 0.01$

qPCR amplicon from *FocSIX* genes were sequenced and analyzed and all amplicons matched with the targeted *FocSIX* sequences (Appendix E). *Fusarium oxysporum*-specific endogenous control, FoTEF1α was successfully quantified in the cDNA from

*Foc*TR4 vegetative mycelia and inoculated banana roots. Figure 4.8 shows the summary of the results. Fold changes *FocSIX* gene transcripts were calculated relative to the FoTEF1α. Four out of eight *FocSIX* transcripts (*FocSIX1b*, *FocSIX1c*, *FocSIX2* and *FocSIX8a*) were undetectable in *Foc*TR4 vegetative mycelia grown on PDA (Figure 4.8a). At 48- and 96 hpi, *FocSIX1b*, *FocSIX1c* and *FocSIX8a* were significantly upregulated *in planta* when compared with 0 hpi control. *FocSIX1b* was significantly upregulated for an average of 195 folds and 3082 folds, at 48- and 96 hpi respectively while *FocSIX1c* was upregulated at 48 hpi for an average of 230 folds and 978 folds at 96 hpi. Lastly, *FocSIX8a* was upregulated on average for 15 folds at 48 hpi and 175 folds at 96 hpi (Table 4.5). In contrast, *FocSIX2* showed only weak upregulation at 48- (an average of 3.8 folds) and 96 hpi (average of 7.8 folds) (Figure 4.8b; Table 4.5).

On the other hand, *FocSIX1a*, *FocSIX 6*, *FocSIX9* and *FocSIX13* transcripts were all detectable in both vegetative mycelia and infected roots. *FocSIX9* and *FocSIX13* were strongly upregulated upon host contact, an average of 3692 folds and 1342 folds respectively. *FocSIX1a* showed no significant differential expression between vegetative mycelia and 0 hpi control and only weak upregulations *in planta*. Whereas *FocSIX6* was the only one among eight *FocSIX* genes tested that significantly downregulated in vegetative phase (compared with 0 hpi control, an average of 14 folds) but upregulation *in planta* at 48 hpi was insignificant. Interestingly, for multi-copy genes (i.e. *FocSIX9* and *FocSIX13)*, both were highly expressed at vegetative mycelia than control 0 hpi banana, meanwhile also highly upregulated *in planta*.

Table 4.5: Log_2 fold change and fold change of *Fusarium oxysporum* f. sp. *cubense* Secreted-in-xylem (*FocSIX*) genes.

Time / Gene	*Foc* vegetative mycelia		0 hpi		48 hpi		96 hpi	
	Log_2 FC	FC[1]	Log_2 FC	FC[1]	Log_2 FC	FC[1]	Log_2 FC	FC[1]
FocSIX1a	-0.74 ±0.95	0.3-1.2	0 ±0.46	0.7-1.4	2.28 ±0.96	2.5-9.4	4.81 ±0.84	16-50
FocSIX1b	ND	ND	0 ±0.86	0.6-1.8	7.61 ±0.85	108-352	11.59 ± 1.22	1323-7179
FocSIX1c	ND	ND	0 ±0.78	0.6-1.7	7.84 ±0.80	132-400	9.93 ±0.58	656-1458
FocSIX2	ND	ND	0 ±0.59	0.7-1.5	1.94 ±0.78	2.2-6.6	2.84 ±0.99	4-14
FocSIX6	-3.78 ±2.28	0-0.4	0 ±0.44	0.7-1.4	0.54 ±1.34	0.6-3.7	2.26 ±0.57	3.2-7.1
FocSIX8a	ND	ND	0 ±1.05	0.5-2.1	3.86 ±1.50	5-41	7.45 ±1.65	56-549
FocSIX9	4.13 ±1.03	9-36	0 ±0.65	0.6-1.6	11.85 ±0.78	2145-6316	12.24 ±0.63	3126-7459
FocSIX13	9.29 ±1.57	211-1858	0 ±1.50	0.4-2.8	10.39 ±0.87	733-2465	13.26 ±1.14	4451-21616

FocSIX genes *in planta* at 48- and 96 hpi versus 0 hpi (control). For transcripts expressed in *Foc*TR4 vegetative mycelia, expression was also compared to the control. ND- Not determined; FC- Fold change. **Bold gene names** were host-induced and highly expressed *in planta*.

In this study, expressions of *FocSIX* genes were categorized into two major groups. Firstly, *FocSIX1b*, *FocSIX1c*, *FocSIX8a* which exhibited a strong host-induced characteristic while *FocSIX2* expression weakly responded to host induction. In contrast, the second group of *FocSIX* genes was expressed constitutively both in vegetative mycelia and *in planta*. They were then further divided into 2 patterns where *FocSIX1a* and *FocSIX6* showed constant upregulation after host contact whereas expressions of multi-copy genes *FocSIX9* and *FocSIX13* were significantly inhibited upon host contact but bounced back to an upregulatory trend at 48 hpi.

4.5 Small secreted cysteine-rich protein (SSCRP) *in silico* prediction and RT-PCR verification

A set of 101 sequences were curated from 1,111 secreted protein-encoding genes predicted using computational pipeline. One hundred and one (101) putative secreted

proteins were selected as they fulfilled the criteria of less than 300 amino acids and with cysteine content of more than 5 % in translated amino acids sequence. All these genes were termed Small secreted cysteine-rich protein (SSCRP). The SSCRP were then subjected to NCBI BLAST and PHI-base BLAST for *in silico* characterization.

Table 4.6: NCBI BLAST of the predicted Small secreted cysteine-rich protein (SSCRP) with a hit of 40 % identity and above.

Gene	Accession No.	Protein description	Species
Foc9889_578	XP_009254666.1	NLS2	*Fusarium pseudograminearum*
Foc9889_1356	AKQ62707.1	Elicitor protein	*Fusarium oxysporum*
Foc9889_1767	RGP72594.1	Proteinase inhibitor Kazal	*Fusarium longipes*
Foc9889_2126	OTA04651.1	SSCRP protein	*Trichoderma parareesei*
Foc9889_3001	XP_008601985.1	Bacteriodes thetaiotaomicron symbiotic chitinase	*Beaureria bussiana* ARSEF2866
Foc9889_3485	OPB40918.1	SSCRP protein	*Trichoderma guizhouense*
Foc9889_4083	OTA08033.1	SSCRP protein	*Trichoderma guizhouense*
Foc9889_4485	RFN49322.1	Small secreted protein	*Fusarium sp*. FIESC 12
Foc9889_4489	RFN49334.1	Small secreted protein	*Fusarium sp.* FIESC 12
Foc9889_4870	CBF85125.1	TPA: MBL2-like secreted peptide, putative 65	*Aspergillus nidulans* FGSC A4
Foc9889_4883	OBW67868.1	NIF-domain-containing protein	*Aureobasidium pullulans*
Foc9889_4999	AAD18059	MHP1	*Magnaporthe oryzae*
Foc9889_5042	AGF29844.1	Putative pathogenicity protein	*Fusarium oxysporum*
Foc9889_5756	AEN19352.1	Tox1	*Fusarium virguliforme*
Foc9889_6032	EMT72495.1	Pectate lyase F	*Fusarium oxysporum* f. sp. *cubense* race4
Foc9889_6205	XP_024704121.1	Fibronectin type III domain protein	*Aspergillus steynii* IBT 23096
Foc9889_6650	KPA40768.1	Gelatinase b	*Fusarium langsethiae*
Foc9889_7041	EXL97925.1	Pectate lyase F	*Fusarium oxysporum* f. sp. *cubense* tropical race 4 54006
Foc9889_8015	RFN55365.1	cdp-alcohol phosphatidyltransferase protein	*Fusarium langsethiae*

Table 4.6, continued.

Foc9889_8837	EXL92596.1	Ribonuclease T2 family protein	*Fusarium oxysporum* f. sp. *cubense* tropical race 4 54006
Foc9889_8922	KPA44778.1	Surface protein sp1 protein	*Fusarium langsethiae*
Foc9889_9064	EMT67793.1	Glutamyl-tRNA (Gln) amidotransferase subunit A	*Fusarium oxysporum* f. sp. *cubense* race 4
Foc9889_9065	ACN87967	Secreted-in-xylem 5	*Fusarium oxysporum*
Foc9889_9104	XP_018252237.1	Pectate lyase	*Fusarium oxysporum* f. sp. *lycopersici* 4287
Foc9889_9596	RGP70091.1	High-affinity glucose transporter	*Talaromyces islandicus*
Foc9889_10106	AKQ62707.1	Elicitor protein	*Fusarium oxysporum*
Foc9889_11412	XP_001546261	BcCFEM1	*Botrytis cinerea*
Foc9889_11663	KZL70461.1	Hydrolytic enzyme protein	*Colletotrichum tofieldiae*
Foc9889_12042	OTA00542.1	SSCRP protein	*Trichoderma parareesei*
Foc9889_12240	XP_023423610.1	Related to hydrophobin	*Fusarium fujikuroi* IMI 58289
Foc9889_12318	XP_009262868.1	NPS19	*Fusarium pseudograminearum*
Foc9889_13835	XP_023433066.1	Long chronological lifespan protein 2	*Fusarium fujikuroi* IMI 58289
Foc9889_13952	AAO16869.1	Hydrophobin 3	*Fusarium verticillioides*
Foc9889_14650	RFN46888.1	d-alanyl-d-alanine carboxy peptidase	*Fusarium sp.* FIESC 12
Foc9889_15235	EMT72523.1	Bypass of stopcodon protein 6	*Fusarium oxysporum* f. sp. *cubense* race 4
Foc9889_15263	AAN76355.1	Hydrophobin	*Fusarium verticillioides*
Foc9889_15275	CVK96721.1	Related to glucoamylase precursor	*Fusarium mangiferae*

Among 37 putative SSCRP which BLAST-X analyses returned with a known protein hit of 40 % identity and above (Table 4.6), four (*Foc*9889_4999, *Foc*9889_12240, *Foc*9889_13952 and *Foc*9889_15263) were highly identical towards hydrophobin proteins. In this study, both *Foc*9889_15263 and 4999 were found with a class II hydrophobin domain (Appendix F), each shared 65 % and 43 % identity to *MHP1* gene respectively (Table 4.7). Three pectate lysase genes (*pel)* were also identified from the list of putative SSCRP with high significant levels. Based on sequences identity,

*Foc*9889_7041 was classified as *FocpelA-Like* whereas *Foc*9889_6032 and 9104 were named *FocpelD-Like1* and *FocpelD-Like2*. All 3 *pel-Like* genes have a conserved domain belongs to the pectate lyase superfamily (Appendix F).

Table 4.7: PHI (Plant Host Interaction)-base BLAST of the predicted Small secreted cysteine-rich protein (SSCRP).

Gene	PHI accession	Gene name (gene function)	Mutant phenotype	Pathogen Species	Host (Disease)
Foc9889_4999	458	*MHP1* (Hydrophobin)	Reduced virulence	*Magnaporthe oryzae*	*Oryza sativa* (Rice blast)
Foc9889_15263	458	*MHP1* (Hydrophobin)	Reduced virulence	*Magnaporthe oryzae*	*Oryza sativa* (Rice blast)
Foc9889_6032	180	*pelD* (Pectate lyase)	Reduced virulence	*Nectria haematococca*	*Pisum sativum* (Root rot)
Foc9889_9104	180	*pelD* (Pectate lyase)	Reduced virulence	*Nectria haematococca*	*Pisum sativum* (Root rot)
Foc9889_7041	179	*pelA* (Pectate lyase)	Reduced virulence	*Nectria haematococca*	*Pisum sativum* (Root rot)
Foc9889_8837	811	*MGG_10510* (Hypothetical protein)	Reduced virulence	*Magnaporthe oryzae*	Oryza sativa (Rice blast)
Foc9889_9065	5286	*SIX5* (Effector)	Effector (plant avirulence determinant)	*Fusarium oxysporum*	*Solanum lycopersicum* (*Fusarium* wilt)
Foc9889_5756	3703	*FvTox1* (Toxin)	Reduced virulence	*Fusarium virguliforme*	*Glycine max* (Sudden death syndrome)
Foc9889_11412	7594	*BcCFEM1* (Putative secretory protein)	Reduced virulence/ unaffected pathogenicity	*Botrytis cinerea*	*Phaseolus vulgaris* (Grey mould fungus)

The 101 SSCRP were also subjected to Plant Host Interaction (PHI)-base BLAST-P analysis. From both NCBI BLAST-X and PHIB BLAST-P results, *Foc*9889_9065 was

found to match to *SIX5* effector gene. It was not identified from the genome of *Foc*9889 when queried with known *SIX5* sequence from *Fol.* Another SSCRP*, Foc9889_11412,* was found to match to *Botrytis cinerea BcCFEM1*, which is involved in the virulence of bean grey mould disease. Lastly, *Foc9889_5756* was a homologue to *FvTox1* that is involved in soybean foliar sudden death syndrome (Table 4.7).

Subsequently, *5 SSCRP* gene were selected based on the BLAST-X and PHIB BLAST-P hits to elicitor and SSCRP proteins from other species with no known function. Their early expressions were profiled with RT-PCR.

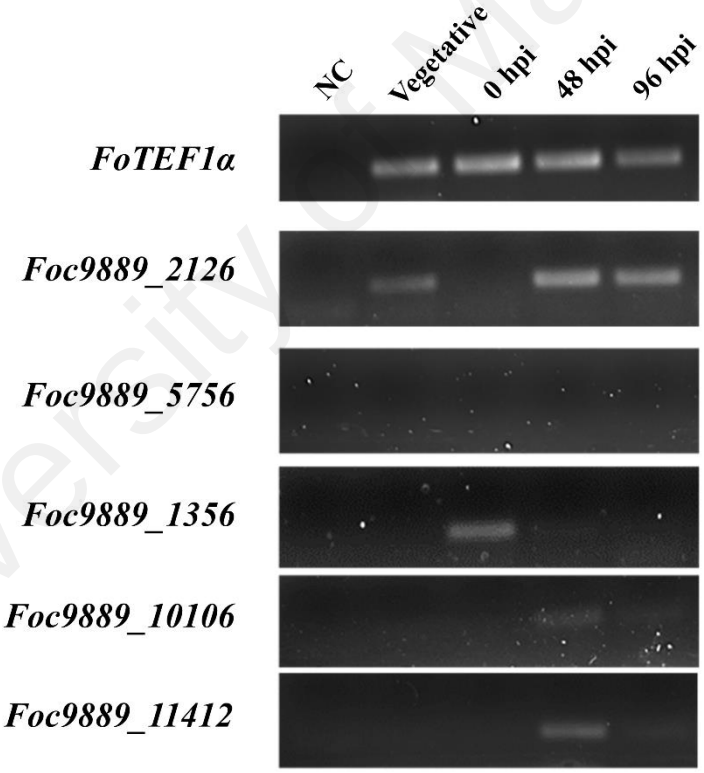

Figure 4.9: Semi quantitative reverse transcriptase-PCR (RT-PCR) of selected Small secreted cysteine-rich protein (*SSCRP*) genes. FoTEF1α is the endogenous control in *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4.

Interestingly, among 5 profiled SSCRP, the expression of *Foc*9889_1356 was highly induced at 0 hpi but ceased at following time points. In contrast, *Foc9889_2126* expression was not detected at 0 hpi, while *Foc9889_10106* and *Foc9889_11412* were

upregulated at 48 hpi. In contrast, transcripts from *Foc9889_5756* was undetectable at all the time points investigated (Figure 4.9).

CHAPTER 5: DISCUSSION

Investigation of *in planta* early pathogenic gene expression is extremely challenging. Since the pathogen, *Fusarium oxysporum* f. sp. *cubense* (*Foc*) and banana root were inseparable, a large amount of plant RNA contamination interfered with the fungal gene quantification. The added complexity was many of the effectors are of critical functions during the early stages of infection, where the fungal cells (hence, RNA) were presented in lowest quantity (Anderson et al., 2016). To overcome the situation, an RNA extraction protocol which ensured high RNA yields was highly desirable.

For RNA extraction from filamentous fungi, TRIzol is routinely used since guanidium salt is an excellent RNase denaturant (Chomczynski & Sacchi, 2006; Cox, 1968). However, TRIzol and other related products such as TRI Reagent consist of high proportion of phenol and guanidium thiocyanate (30 – 60 %), which are hazardous towards experimenter's heatlh. Hexadecyltrimethylammonium Bromide (CTAB) is a relatively safer alternative to TRIzol (Abu Almakarem et al., 2012). The chloride concentration in CTAB lysis buffer was increased to more than 2 M and therefore effectively prevented polysaccharide precipitation (Fang et al., 1992). While previous attempts in utilizing CTAB-based buffer for filamentous fungi RNA extraction failed (Islas-Flores et al., 2006; Sánchez-Rodríguez et al., 2008), this study showed that CTAB lysis buffer was suitable for isolating DNA and RNA both from *Foc* mycelia grown on solid media and banana roots.

It was found that the ratio of mycelia powder mass to CTAB lysis buffer was critical for the reproducibility of *Foc* nucleic acid extraction. Filamentous fungal nucleic acids extractions are often compromised by its rich content of carbohydrates (i.e.

polysaccharides) (Sάnchez-Rodríguez et al., 2008). The resulting lysate tends to be extremely viscous, which traps the buffer and hinders phase separation. In this study, 100 mg of mycelia powder resuspended in 2 mL of CTAB lysis buffer resulted in a clean separation of cell debris from the aqueous phase, whereas 250 mg of mycelia powder collected from PDA resuspended in 2 mL CTAB buffer failed to separate after centrifugation. Hence, 100 mg of mycelia powder to 2 mL lysis buffer was chosen as the optimal ratio for *Foc* nucleic acid extraction.

The use of using LiCl as precipitant reduces co-precipitation of DNA, proteins, polyphenolics and carbohydrates (Barlow et al., 1963; Rubio-Piña and Zapata-Pérez 2011). Ethanol, on the other hand, though not efficient in excluding above-mentioned contaminants, is more efficient in precipitating RNA of smaller species. The activity of cellular RNases (a protein) severely affects the quality of extracted RNA. Lithium chloride (final concentration 2.5 M) precipitation followed by a phenol-chloroform (1: 1, pH 4.5) extraction after DNase I treatment effectively slowed down the RNA degradation process. Since lithium chloride does not effectively precipitate DNA and proteins and, any potential degradation caused by endogenous RNases can be minimized (Barlow et al., 1963; Sambrook & Russel, 2001). Moreover, phenol acts to disrupt protein structures and hence helps in denaturing RNases (Abu Almakarem et al., 2012).

In majority of environmental samples, such as infected roots retrieved from soil in this study, are prone to contamination with a myriad of inhibitory compounds. DNase I activities in removing genomic DNA (gDNA) from RNA samples co-extracted with high level of inhibitors have proven to be incomplete (Lim et al., 2016). According to Lim et al. (2016), DNAse I treatment is considered inefficient when amplification signal of the RNA sample with no reverse transciptase added in a qRT-PCR reaction is being detected

earlier than 35 cycles. It is critical to completely eliminate residual gDNA in RNA samples when conducting gene expression analyses considering that as low as 0.002 % of gDNA can contribute to as high as 60000 copies of false transcripts in a qRT-PCR assay (Lim et al., 2016). In the current optimized extraction protocol, total nucleic acid that have been subjected to DNase I digestion resulted in intact RNA bands. In addition, digestion with only 2 units of DNase I was sufficient to remove residual gDNA from the LiCl-precipitated nucleic acids.

The whole genome of *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 (*Foc*TR4) has been sequenced and deposited in GenBank by two independent groups. The first was from the *Foc*TR4 isolate II5 (Accession number: GCA_000149955.2) and followed by the deposition of *Foc*TR4 isolate C1HIR_9889 (Accession number: GCA_001696625.1). A practical approach to identify potential effectors from the genome is via literature mining. Secreted-in-xylem (SIX) proteins are a group of validated effectors identified from the xylem sap of *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*)-infected tomato (Gawehns et al., 2014; Houterman et al., 2007; Lievens et al., 2009; Rep et al., 2004; Schimdt et al., 2013). The final interface of the *Foc*-banana pathosystem is in the xylem, proteins which are secreted into the plant host xylem vessel, must have critical functions such as to break down or halt the host's immune response and turn the earlier biotrophic phase of the infection into a necrotrophic phase. Biotrophic pathogens attack and colonize plants as a food source, and the xylem is situated just next to the phloem. Although phloem is the vessel with rich sugar content, but the osmostic pressure is also considerably higher than the xylem and this makes the penetration of the fungal hyphae into this space more difficult. Hence, colonization of the xylem is a reasonable alternative for an invading fungus (Yadeta & Thomma, 2013).

Additionally, extra energy is always needed for the fungus to secrete the proteins into host. Thus, extracellularly secreted proteins must play an essential role in manipulating the host system, favoring the colonization of the pathogen. It is valuable to characterize SIX proteins in order to deduce the pathway(s) they attempt to manipulate or interrupt. Extracellular proteins are expected to aid in host colonization. If the secreted pathogenic proteins do not help in the process of infection, their presence will only burden the pathogen by offering the plant host an opportunity to detect and defend against the invasion. Natural selection would not favor the existence/production of such proteins during host colonization (Laugé & De Wit, 1998), so *SIX* proteins should be put high on the watch list when studying *Foc*-banana molecular interaction during pathogenesis.

Homology searches against the whole genome assembly were done to identify the presence of *SIX* gene homologues in *Foc*TR4 C1HIR_9889 genome. The copy number of each *SIX* genes present in different forma speciales varies. Horizontal transfer of *SIX* genes to other strains has been observed and described (Ma et al., 2010). *SIX8* presents as a multi-copy gene in *Fol* (Schmidt et al., 2013), 2 copies in *Foc* Subtropical Race 4 (STR4) whereas only a single copy of *SIX8*, namely *FocSIX8a,* has been identified in *Foc*TR4 C1HIR_9889 genome. Czislowki et al. (2018) reported that there was a total of 5 variations of *SIX8* present in the *Foc* isolates of various vegetative compatibility group (VCG) types spanning STR4 and TR4 isolates. In contrast, *SIX8* was absent in both Race 1 and Race 2 isolates which are less virulent (Czislowki et al., 2018). Moreover, the presence of *SIX8a* but not *SIX8b* across STR4 and TR4 isolates (Fraser-smith et al., 2014) suggests a potential conserved role of *FocSIX8a* in the virulence of Race 4 isolates. Interestingly, 2 introns were found in *FocSIX8a* while most of the *SIX* genes are intron-less. Alternative splicing (AS) is an important cellular process in fungi (Grützmann et al., 2014). About 50 % of multi-exonic genes in *Verticillium dahliae* were found to possess

alternatively spliced mRNA isoforms (Jin et al., 2017). The multi-exons feature of *FocSIX8a* suggested a possibility of AS and isoforms. However, alternative splicing is less investigated for multi-exonic *SIX* genes found in *Fusarium oxysporum*.

Local BLAST of *SIX* genes to the *Foc*TR4 C1HIR_9889 genome identified 3 copies of *SIX9.* Similarly, 2 copies of *SIX13* were present in the genome of the isolate. The presence of different copies of *SIX* genes infer the diversity of effectors in the *F. oxysporum* isolates collected from different geographical areas and the ability of fungus to evolve rapidly for better colonization of native plant hosts (Czislowki et al., 2018). SIX proteins are also present in many forma speciales (f. sp.) of *F. oxysporum*. Those that are consistantly present in different f. sp. are considered as core-effectors as their conservation throughout evolutionary lineage implies a conserved function or effector targets (Gawehns et al., 2014). Intensive research on the SIX effectors and other novel effectors provides evidence of the effector's repertoire that plays a role in determining host-specificity of different f. sp. of *F. oxysporum* (van Dam et al., 2016).

Despite several studies agreeing on the potential of SIX effector suites in discriminating host specificity and further differentiation of the races/VCGs in the same f. sp. (Li et al., 2013b; Singh & Kapoor, 2018), the presence of the effector genes in the soil isolates of *F. oxysporum* does not always correspond to the pathogenicity of the isolate. In a study of Jelinski et al. (2017), 2 isolates tested positive for a full suite of effectors were found to be non-pathogenic after a tomato pathogenicity assay. Therefore, an immediate downstream process, the expression and activation of *SIX* genes is an important indicator to investigate the difference in pathogenicity. Jelinski et al. (2017) found that *SIX* genes in both non-pathogenic isolates only have a few SNPs when compared with a pathogenic strain that possessed the same SIX effector suites. This

suggests if the *SIX* genes expression is varied, the mutations will most probably be at the promoter regions or originated from the deficiency in their transcription factors. Since genomic markers such as *EF1α* and *SIX* genes have been used for diagnosis of the presence of *Foc* in the soil, simply linking the genomic marker to the pathogenicity is possibly misleading in some cases. The presence of the effector gene homologues in the genome alone was not sufficient to determine its functionality during pathogenesis. Therefore, this study screened for the expression of the *SIX* effector genes in the banana root inoculated with *Foc* at 0, 48 and 96 hour-post-inoculation (hpi).

Quantitative reverse transcriptase PCR revealed several *SIX* genes that were highly up-regulated at 48 and 96 hpi. Among all the *SIX* genes analysed, *FocSIX1b* and *FocSIX1c*, and *FocSIX8a* expressions were induced by the presence of host cells since the transcripts were non-detectable in the axenic vegetative culture but highly expressed *in planta* where expressions were detected as early as 48 hpi. *SIX1* homolog in *Fusarium oxysporum* f. sp. *conglutinans* has been shown to be required for full virulence by knock-out analysis (Thatcher et al., 2012). A recent study showed that *FocSIX1a* is required for full virulence towards Cavendish banana (Widinugraheni et al., 2018) but functions in virulence of *FocSIX1b* and *c* are unknown. In comparison, *FocSIX2* shows a very weak expression *in planta* in this study. This is in accordance to Gawehns et al. (2014) who reported that *FolSIX2* did not show any significant association in eliciting plant responses in *Fol*-tomato pathosystem. A *FolSIX2* knock-out strain infecting tomato has the similar xylem sap proteome comparable with wild-type inferring that absence of *FolSIX2* did not impair *Fol* virulence.

Since FolSIX protein is found in the xylem sap of infected tomato plant, it was expected to find all *FocSIX* genes displaying an upregulation pattern *in planta*. FolSIX6

protein was first identified from tomato plants 2.5 weeks after initial inoculation. But Gawehns et al. (2014) showed that *FolSIX6* transcript was detectable as early as 96 hpi. Effectors such as FolSIX6 interfere with cell death and modulate immune response of a plant (Gawehns et al., 2014; Guo et al., 2009; Stassen & Van den Ackerveken, 2011). The qRT-PCR results in this study also suggest that *FocSIX6* has potential functions in modulating the plant immune response as its expression steadily increased as infection progressed from 0 hpi to 96 hpi which supports its indispensable role during *in planta* infection. However, *FocSIX6* was also found to be expressed in *Foc* vegetative mycelia, suggesting that it is needed for the free-living stage of *Foc* and not exclusively host-induced.

According to Gawehns et al. (2014), *FolSIX8* was found to be expressed in infected tomato roots from 4 days-post-inoculation (dpi) onwards and expression gradually increased up to 15 dpi. *FolSIX8* accelerated cell death responses in *Nicotiana benthamiana* when expressed transiently and its effector was highly homologous to *Arabidopsis thaliana* TOPLESS protein. *SIX8* is the only *SIX* gene that was exclusively found in *Foc* Race 4, where 2 copies were found in subtropical race 4 (STR4) isolates and a single copy found in Tropical race 4 (TR4) isolates (Czislowski et al., 2016; Fraser-Smith et al., 2014). This strongly suggests the role of *SIX8* in the pathogenicity or host-specificity for the *Foc*TR4 isolate. *SIX8* accelerates the onset and development of *Inf1*-mediated cell death, but not of *R* gene mediated cell death, generally in 24 hours (Gawehns et al., 2014). *FocSIX8a* was highly up-regulated *in planta* at 48- and 96 hpi. This is similar to reports from the investigation of *Foc*TR4 infection mode by Li et al. (2013a) that stated that by 48 hpi, *Foc*TR4 hyphae were found to penetrate the vascular tissue of banana root. This study showed that the transcript of *FocSIX8a* gene was barely detectable in the vegetative mycelia but was highly upregulated in infected root tissues.

This indicates that the expression of *FocSIX8a* was induced upon contact with host tissues. The expression can be detected as early as 48 hpi whereas it remained undetectable at 0 hpi. According to Gawehns et al. (2014), *FolSIX8a* amino acid composition was more similar to *FocSIX8b* rather than *FocSIX8a*. However, since *FocSIX8a* is a single copy gene that is specific for TR4 isolates, the function of *FocSIX8a* is expected to be similar with that of *FolSIX8a*. Its characteristic expression after banana host contact at 48 hpi serves as potential marker for that could serve as an extremely important indicator for preventive diagnostic measures during early onset of *Foc*TR4 infection in banana.

SIX13, as a more recent identified member of the *SIX* genes repertoire, is less characterized and its function is largely unknown. In this study, *FocSIX13* expression was highly upregulated at 96 hpi (9809 folds) when compared to the 0 hpi control. This corresponds to the observation reported by Thatcher et al. (2017) where *FomSIX13* expression *in planta* was found to be 1 million times higher than observed *in vitro*. Also, no significant expression of *FocSIX13* at 0 hpi control was observed when compared with vegetative mycelia, but then an upregulation at 48 hpi suggesting this gene was inhibited at early host interaction. The *FocSIX13* primer pair used in this study was designed based on the conserved sequences from both *FocSIX13a* and *e* copies.

The findings in this study suggests members of Group I genes, *FocSIX1b*, *FocSIX1c* and *FocSIX8a,* were induced in a compatible interaction since *Musa acuminata* cv. Berangan is a highly susceptible host to *Foc*TR4. They have potential to be used as marker genes for early diagnosis of a compatible interaction and future work should focus on the induction of these 3 genes in an incompatible interaction i.e. resistant banana cultivars.

Lastly, the observed standard deviations of *SIX* genes fold change was relatively larger than conventional single organism gene expression studies. Similar results were observed in other *in planta* gene expression studies of phytopathogens, such as *Magnaporthe oryzae* (Yang & Li, 2012) and *Rhizoctonia solani* (Anderson et al., 2016). Moreover, Anderson et al. (2016) compared both the *in planta* soil-based and *in vitro* petri dish-based infection assays and found the *in vitro* assay successfully reduced the standard deviations. This suggests that the proportion of the host RNA from *in planta* infection samples interfered with the consistency of pathogenic gene detection to a certain extent and caused higher variations. In the case of *F. oxysporum*, an *in vitro* assay is yet being developed and the results suggest the need to establish an assay with lower standard deviations as demonstrated in Anderson et al. (2016) to enhance the accuracy and reproducibility of similar studies.

Apart from effector homology search, *Foc*TR4 secretome predicted by genome-based approach was used to identify effector candidates. Effector genes must encode extracellular or secreted proteins. A typical effector encodes small proteins of less than 300 amino acid residues (Duplessis et al., 2011). Next, encoded protein will also have a rich cysteine content and carry a N-terminal secretion signal most of the times (Van den Burg et al., 2003). Cysteine-rich protein can be defined as protein with more than 5% cysteine residues found in the mature peptide (Krijger et al., 2014). Due to the selection criteria, candidate effectors selected were therefore known as Small secreted cysteine-rich protein (SSCRP). The number of cysteine residues encoded by putative SSCRP in this study stretches from 4 to 21 where the highest percentage of cysteine content scores a 12.3%, as found in *Foc9889*_1767. Cysteine residue is well known for its disulphide-bridge forming ability. The disulphide bridge formed between 2 cysteine residues confers stability to the tertiary protein structure, which is vital for extracellular protein to

withstand host proteases repertoire (Rep, 2005). Therefore, cysteine richness has long been associated with pathogenic effectors.

Generally, prediction of fungal effectors was principally based on a relatively broad set of afore-mentioned criteria. This is because individual fungal effectors from a species do not appear to share significant sequence similarity, due to rapid mutations in effector and host adaptation (Sperschneider et al., 2015). Hence, *in planta* expression of selected SSCRP was incorporated as a validation step immediately downstream of the *in silico* selection. *in planta* induced gene expression is an important additional criterion in effectors prediction pipeline (Saunders et al., 2012).

The *in silico* prediction pipeline used in this study successfully predicted 1,111 putative secreted proteins. Among them, 101 secreted protein-encoding genes were curated based on cysteine richness and protein size. While this pipeline is currently following the standards for predicting potential effectors, it was restricted to the Small secreted cysteine-rich protein (SSCRP). While other effectors which have relatively lower cysteine residue and larger in size were excluded by the pipeline (Sperschneider et al., 2015). For example, it was found that the majority of *FocSIX* genes were present in the initial pool of predicted secretome but further selection steps with cysteine richness and peptide size exclusion did not retain the *FocSIX* genes. This was because most of the *SIX* genes except *FocSIX9* had relatively lower cysteine residues than the cutoff point. It was evident that not all effectors have high cysteine richness, nevertheless it did not negate the potential of SSCRP as competitive effector candidates (Qi et al., 2016; Shcherbakova et al., 2016).

in silico prediction yielded a large amount of effector candidates that exceeding capacity of experimental validation. In order to narrow down the pool, RNAseq for the transcriptome of *in planta* infected samples can be applied to the downstream selection pipeline. For a reported study of nematode effectors prediction, only ~15 % from the predicted secreted proteins were found to be upregulated *in planta* and hence ~85 % of the predicted proteins were excluded as likely effector candidate. This approach successfully narrowed down the pool of effector candidate to a feasible size (Gardner et al., 2018). While in this study, the selection criteria with 5 % cysteine residue and 300 amino acids as cut off length greatly reduced the putative effectors pool to ~10 % from the original secretome.

The BLAST results of SSCRP returned with several classes of proteins that are potentially involved in *Foc* and banana pathogenesis. Among them are hydrophobins, pectase lyase and Tox1 proteins. Hydrophobins are exclusively found in filamentous fungi and are known to be presented in outer conidial surface or hyphal wall of many ascomycetes and basidiomycetes (Sunde et al., 2008). Their roles involve surface attachment, interaction of the fungal spores with the host during infection (Leger et al., 1992; Talbot et al., 1996) and might help in preventing host recognition in triggering immunity (Aimanianda & Latge, 2010). According to Guo et al. (2014), a hydrophobin gene from *Foc* Race 4 that is related to *MHP1* of *Magnaporthe grisea* was greatly upregulated at 48 hpi. Conversely, it was suppressed in *Foc* Race 1, suggesting that it may specifically function in pathogenesis between the *Foc* Race 4 and banana. MHP1 protein of *M. grisea* is essential in plant colonization as it is involved in appressoria formation. Its expression is significantly upregulated *in planta*, especially at the later stage of infection (Kim et al., 2005). It is interesting to investigate the potentials and

functions of *MHP1* gene in *Foc* infection since *F. oxysporum* is a non-appressorium forming fungal pathogen (Pérez-Nadales & Di Pietro, 2011).

Another class of protein, pectate lyase (pel) is involved in fungal infection as its function is to degrade the carbohydrate barrier of the host. Pectate lyase was recognized as one of the virulence factors as its absence resulted in reduced virulence of the fungus *Nectria hematococca* (Rogers et al., 2000). *pelA* and *pelD* are two classes of inducible PLs, with *pelD* was observed to be induced only *in planta*. Also found is *Foc9889_4870*, is a potential MBL2-like secreted peptide-encoding gene. MBL2-like proteins are often involved in recognition, attachment, adhesion and appressoria formation (Kulkarni et al., 2003; Nakkeeran et al., 2016).

FvTox1 is a single copy gene which cause foliar sudden death syndrome (SDS)-like effect on soybean (*Glycine max*) leaves (Brar et al., 2011; Li et al., 1999). Like *Foc*, *F. virguliforme* is also a root-invading pathogen. Attempts at isolating the fungal cell from the diseased leaves were in vain suggesting that the pathogen never progresses to the plant shoot (Roy et al., 1989; Rupe, 1989). The proteinaceous toxin, Tox1, released by *F. virguliforme* into soybean root during infection is the causal agent of the foliar SDS-like effect (Brar et al., 2011). Brar et al. (2011) showed that *FvTox1* expressed in infected root at 10 dpi but did not provide the expression information earlier than that. It is plausible that *Foc9889_5756* expression will be upregulated at a later stage of infection (> 96 hpi) since banana Fusarium wilt also exhibits a similar foliar wilting phenotype.

In a report by Czislowski et al. (2018), *SIX5* was absent in the genome of all investigated *Foc* VCG types. *SIX* genes were hypothesized to be horizontally transferred within *Foc* and *SIX* homologues in various races or VCG types will slowly mutate and

resulted in cultivar-specific pathogenicity (Czislowski et al., 2018; Ma et al., 2010). SIX5 was identified as one of the genuine effectors in *Fol* which triggered *I2* immunity in tomato (Houterman et al., 2015). Hence it has high possibility to mutate in *Foc* in order to avoid recognition by the cognate R gene which might also be present in the banana host.

In recent years, several sets transcriptomic data of banana cultivars infected with *Foc* have been published (Bai et al., 2013; Li et al., 2012; Li et al., 2013a; Wang et al., 2012). The transcriptome analyses mainly focused on the responses of banana cultivars towards the *Foc* infection. In the transcriptomic approach, *Foc* transcripts are present in very low amounts and are overwhelmed by the host transcripts. Many of the SSCRP did not show differential expression with robust statistical significance due to the extremely low transcript number below the detection limit of RNAseq.

In contrast, PCR-based expression analyses have an advantage in enhancing the detection limit by amplifying the limited template for transcripts with very low copies. In this study, the expression changes of the *Foc*TR4 was analysed during the early stage of infection at 48- and 96 hpi. For five selected SSCRP genes, the expressions were validated with RT-PCR. The inoculation was carried out *in vivo* and mimic the natural infection mode of *Foc* towards banana root. Out of five SSCRP validated by RT-PCR, three showed clear activation at 48 hpi while the other two were not detected or inhibited at 0 hpi. *Foc9889_1356* which shared significant identity with an elicitor protein-coding gene was expressed at 0 hpi. Elicitor proteins plays a role in inducing and triggering plant defense responses. Elicitor proteins have been found in a variety of pathogen such as viruses, bacteria, oomycetes and fungi (Liu et al., 2016). An elicitor protein found in *Phytophthora colocasiae* showed highest expression at the mycelium stage but was not

found expressed in the spores (Mishra et al., 2010). *Foc9889_1356* expression pattern is similar with pectate lyase coding gene (Cho et al., 2015) rather than elicitor protein but conserved domain finding failed to identify it as a pectate lyase. Further characterization is needed to confirm the identity of *Foc9889_1356*. Another SSCRP gene, *Foc9889_2126,* showed similar expression patterns with *FocSIX9* and *FocSIX13* where the expressions were clearly inhibited at 0 hpi but were again upregulated at 48 hpi and 96 hpi. They were also expressed in both vegetative mycelia and infected banana roots.

Apart from literature mining and extracellular SSCRP prediction methods used in this study, a feature which proved valuable in predicting novel effectors is the presence of miniature inverted-repeat transposable elements (MITEs) at the promoter region. Schmidt (2013) reported that miniature IMPALAs (mimps), a class of MITEs, were identified in the promoter region upstream of several *FolSIX* genes. The study showed that the occurrence of this motif at promoter region is useful for prediction of novel effectors. In future, *in silico* fungal effector prediction should take into consideration of this newly discovered feature and combining the carefully curated RNAseq data to generate a more refined pool of effector candidates.

CHAPTER 6: CONCLUSION

In this study, the presence of Secreted-in-xylem (*SIX*) effector genes were verified in the *Foc* Tropical Race 4 (*Foc* TR4) genome. Gene expression of all the eight *FocSIX* genes were characterized during the early pathogenic interaction of banana cv. Berangan and *Foc* through quantitative real-time PCR. All *FocSIX* genes were upregulated during early pathogenic interaction but were further categorized into 2 groups based on the expression patterns. Members of Group I showed characteristics of host-induced expression patterns and can serve as potential biomarkers for early detection of *Foc*TR4 infection. In addition, a computational pipeline was applied to predict a pool of *Foc* effector candidates known as Small secreted cysteine-rich protein (SSCRP). This *in silico* prediction pipeline successfully predicted 101 putative SSCRP, and *in planta* expression of five predicted SSCRP were validated through RT-PCR. Four out of five SSCRP were found to be expressed *in planta*. Genomic features of the investigated effector genes were also examined in this study. The results suggest the potential virulence functions of this set of genes in the pathogenic interaction of banana and *Foc*. Future work should focus on functional characterization of the SSCRP and FocSIX encoding genes in both susceptible and resistant banana.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

PUBLICATIONS:

1. **Poon, N. K.** & Teo, C. H. (2019). Fusarium wilt disease of banana: Current development of Fusarium resistant banana. *Open Access Journal of Microbiology & Biotechnology*, *4*(1), Article#000134.

2. **Poon, N. K.**, Teo, C. H. & Othman, R. Y. (2019). Differential gene expression analysis of Secreted in Xylem (*SIX*) genes from *Fusarium oxysporum* f.sp. *cubense* tropical race 4 in *Musa acuminata* cv. Berangan and potential application for early detection of infection. *Journal of General Plant Pathology*, *86*, 13-23.

3. **Poon, N. K.**, Othman, R. Y., Mebus, K. & Teo, C. H. (2019). Optimization of CTAB-based RNA extraction for *in planta Fusarium oxysporum* f. sp. *cubense* gene expression study. *Sains Malaysiana*, *48*(10), 2125-2133.

PAPER PRESENTED:

1. **Poon, N. K.**, Teo, C. H. & Othman, R. Y. (2017). *Study on candidate effector genes of Fusarium oxysporum f. sp. cubense Tropical Race 4 (FocTR4) in Musa acuminata var. Berangan.* Paper presented at International Conference on Crop Improvement 2017 (ICCI 2017), 8-10th November 2017, Johor, Malaysia.