UNIVERSITI PUTRA MALAYSIA

GENETIC DIVERSITY AND EXPRESSION ANALYSES OF SECRETED IN XYLEM EFFECTOR GENES OF Fusarium oxysporum f. sp. cubense ISOLATED FROM PENINSULAR MALAYSIA

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By

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Faculty : Agriculture

Banana is a commercially important fruit in the majority of tropical and subtropical countries. Banana plantations cover nearly 35,000 hectares of land, accounting for approximately 24% of Malaysia's total fruit production, with Johor plantations accounting for approximately 32% of the nation's banana production. The *Fusarium* wilt pathogen, *Fusarium oxysporum* f. sp. *cubense* (Foc) is a major cause of disease in the banana industry, limiting banana production in Malaysia and causing significant economic losses. Currently, the majority of locally grown bananas (Berangan, Rasthali, Mas) and plantains used for cooking or processing (Raja, Abu, and Awak) have been found to be susceptible to Foc-TR4. To date, AFLP, RAPD, ISSR and SSR markers are extensively used in PCR-based molecular characterization of Foc. Although these markers are effective in characterizing Foc, they all have their own limitations. An effector gene known as *Secreted in Xylem* (*SIX*) has been explored to broaden the molecular diagnostic toolbox for Foc. The main objective of this study was to identify expression of *SIX* genes in banana plantlet during infection with the Foc-TR4. This will be an important step in understanding the pathogenicity response of the plant and will help to develop any disease management strategies. In this study, the virulence potential of 27 isolates of Foc of banana based on pathogenicity test and the presence of *SIX1*, *SIX7* and *SIX8* genes were determined. All of 27 Foc 'Tropical Race 4' (TR4) (VCG 01213/160) isolates were obtained from Biological Control Laboratory, Department of Plant Protection, University Putra Malaysia (UPM). All isolates were pathogenic towards *Musa acuminata* Berangan AAA at different severity levels under greenhouse condition. Pathogenicity assays indicated that the level of aggressiveness varies between isolates. The Foc-TR4 isolates were screened for the presence of three *SIX* genes (*SIX1, SIX7* and *SIX8*) and the genetic differentiation of Foc-TR4 was evaluated by PCR analysis using three specific primers (*SIX1, SIX7* and *SIX8*). Among these three *SIX* genes, *SIX1* and

SIX8 genes were detected in all 27 Foc-TR4 isolates from Peninsular Malaysia. The presence of *SIX1* and *SIX8* in Foc-TR4 suggested these genes were likely involved in pathogenicity on banana. Phylogenetic analysis using *SIX1* and *SIX8* sequences showed that they were related to TR4 isolates (VCG 01213/16) from Australia and Indonesian with bootstrap values of 94% and 100%, respectively. There was no variation observed in the *SIX1* and *SIX8* sequences since all 27 isolates clustered in the same clade. Virulence level do not correlate with the existence or lack of these genes. Highest and lowest disease severity values (80.73% and 10.42%) on banana plantlet were selected. The T30 and T36 isolates were found to be highly aggressive and weakly aggressive, respectively. Both T30 and T36 isolates were analyzed by qRT-PCR analyses for the expression of *SIX1* and *SIX8* genes to elucidate whether these genes play a role in the pathogenicity response of Foc-TR4 on *Musa acuminate* cv. Berangan plantlets during early and late of infection stages. Expression analyses of *SIX1* and *SIX8* were constructed from banana roots collected after 0, 2, 4, 8, 12, 15, 20- and 30-day post-infection (dpi). The *SIX1* and *SIX8* genes were expressed after beinginduced by the host and showed higher expression at 8 dpi and 12 dpi respectively in both T30 and T36 isolates. From these results, it can be concluded that highly expressed virulence-associated genes, *SIX1* and *SIX8* can be used as markers for the assessment of Foc-TR4 virulence.

Abstrak tesis yang dikemukakan kepada Senat Universiti Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains

KEPELBAGAIAN GENETIK DAN ANALISIS EKSPRESI EFEKTOR GEN *SECRETED IN XYLEM Fusarium oxysporum* f. sp. *cubense* DIISOLASI DARI SEMENANJUNG MALAYSIA

Oleh

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Pisang adalah salah satu buah yang penting di kebanyakkan negara tropika dan subtropika. Ladang pisang meliputi hampir 35,000 hektar tanah, merangkumi sekitar 24% dari keseluruhan pengeluaran buah di Malaysia dengan ladang di Johor menyumbang sekitar 32% daripada pengeluaran pisang negara. Layu *Fusarium* yang di sebabkan oleh patogen *Fusarium oxysporum* f. sp. *cubense* (Foc) adalah salah satu penyakit utama dalam industri pisang, yang mengehadkan pengeluaran pisang di Malaysia dan menyebabkan kerugian ekonomi yang ketara. Pada masa ini, sebilangan besar pisang yang ditanam (Berangan, Rastali, Mas) dan pisang yang digunakan untuk memasak atau memproses (Raja, Abu, Awak) didapati rentan terhadap Foc-TR4. Sehingga kini, penanda SSR, ISSR, AFLP dan RAPD digunakan secara meluas dalam pencirian Foc di peringkat molekul berasaskan PCR. Walaupun penanda ini berkesan dalam pencirianFoc, tetapi penggunaannya masih terbatas. Gen efektor yang dikenalisebagai *Secreted in Xylem (SIX)* telah diterokai untuk perluaskan diagnostik molekul Foc. Objektif utama kajian ini adalah untuk mengenal pasti pengekspresan gen *SIX* pada tanaman pisang selepas dijangkiti dengan Foc-TR4. Ini akanmenjadi langkah penting dalam memahami tindak balas kepatogenan tanaman dan dapat membantu membangunkan strategi pengurusan penyakit. Dalam kajian ini, potensi kevirulenan bagi 27 pencilan Foc pisang berdasarkan ujian kepatogenan dan kehadiran gen *SIX1, SIX7* dan *SIX8* telah ditentukan. Kesemua 27 pencilan Foc 'Tropical Race 4' (TR4) (VCG 01213/160) diperoleh dari MakmalKawalan Biologi, Jabatan Perlindungan Tanaman, Universiti Putra Malaysia (UPM). Kesemua pencilan adalah patogenik terhadap *Musa acuminata* Berangan AAA pada tahap keterukan penyakit yang berbeza di dalam rumah hijau. Ujian kepatogenan menunjukkan bahawa tahap keagresifan antara pencilan adalah berbeza. Pencilan Foc-TR4 disaring untukmengetahui kehadiran tiga gen *SIX* (*SIX1, SIX7* dan *SIX8*) dan pembezaan genetik Foc-TR4 dinilai melalui analisis

PCR menggunakan tiga primer khusus (*SIX1, SIX7* dan *SIX8*). Di antara ketiga-tiga gen *SIX* ini, gen *SIX1* dan *SIX8* dikesan pada kesemua 27 pencilan Foc-TR4 dari Semenanjung Malaysia. Kehadiran *SIX1* dan *SIX8* dalam Foc-TR4 menunjukkan bahawa gen ini berkemungkinan terlibat dalam kepatogenan pada tanaman pisang. Analisis filogenetik pada kawasan jujukan *SIX1* dan*SIX8* menunjukkan bahawa mereka berkait rapat dengan pencilan TR4 (VCG 01213/16) dari Australia dan Indonesia dengan nilai bootstrap masing-masing 94% dan 100%. Tiada variasi yang diperhatikan dalam jujukan *SIX1* dan *SIX8* kerana kesemua 27 pencilan berada di dalam kelompok yang sama. Tahap kevirulenan tidak berkait dengan kewujudan atau kekurangan gen ini. Nilai keterukan penyakit yang paling tinggi dan paling rendah (80.73% dan 10.42%) dipilih untuk kajian seterusnya. Pencilan T30 dan T36 masing-masing didapati paling agresif dan paling lemah. Kedua-dua pencilan T30 dan T36 dianalisa berdasarkan analisis qRT-PCR untuk mengetahui tahap pengekspresan gen *SIX1* dan *SIX8* bagi melihat sama ada gen ini berperanan dalam tindak balas kepatogenan Foc-TR4 pada *Musa acuminata* cv. Berangan semasa peringkat awal dan akhirjangkitan. Analisis pengekspresan gen *SIX1* dan *SIX8* diambil daripada akar pisang selepas diinokulasi dengan Foc-TR4 pada 0, 2, 4, 8, 12, 15, 20- dan 30 hari (dpi). Gen *SIX1* dan *SIX8* terekspres selepas diaruh oleh perumah dan menunjukkan aras pengekspresan yang tinggi pada hari ke lapan selepas inokulasi bagi pencilan T30 dan hari ke-12 selepas inokulasi bagi pencilan T36. Daripada hasil kajian ini, dapat disimpulkan bahawa gen *SIX1* dan *SIX8* berkait dengan kevirulenan dan dapat digunakan sebagai penanda untuk penilaian tahap kevirulenan Foc-TR4.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirements for the Degree of Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

x g	Gravitational Constant
ANOVA	Analysis of variance
BLAST	Basic local alignment system tool
cDNA	Complimentary deoxyribonucleic acid
CFU	Colony forming unit
DNA	deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium bromide
ITS	Intergenic space
MEGA	Molecular evolutionary genetics analysis
MgCl2	Magnesium chloride
NCBI	National Centre for Biotechnology Information
PDA	Potato Dextrose Agar
PCR	Polimerase Chain Reaction
qRT-PCR	Real-time reverse transcription polymerase chain reaction
RNA	Ribonucleic acid
RNase	Ribonuclease
Rpm	Nanogram
nm	Rotation per minute
SIX	Secreted in xylem
rpm	Rotation per minute
TAE	Tris-acetate EDTA
TBE	Tris HCL-boric acid-EDTA

TEF1-α	Translation elongation factor one alpha
Taq	*Thermas aquaticus*
TM	Melting temperature
TUB2	Beta Tubulin
USDA	United States Department of Agriculture

CHAPTER 1

INTRODUCTION

1.1 Background of study

Bananas are an extremely important crop in Asia and the Pacific, both as a staple food and as a major export commodity for many developing countries. In Malaysia, banana is ranked second, after durian, as the most cultivated fruit crop in Malaysia, accounting for about 24% of total fruit production, primarily in Johor, Pahang, Sarawak and Sabah (MOA, 2019). Banana production in Malaysia is a lucrative business account for more than 330,000 metric tons of banana production and such enormous scale has generated revenue of approximately USD8 million of export value (FAO, 2014). Approximately 50% of locally cultivated bananas are AAA subgroups, Pisang Berangan and Cavendish type (Mohamad Roff et al., 2012). This crop has become an important commodity in Malaysia, with Cavendish types and Pisang Berangan planted on a commercial scale for both the domestic and export markets.

However, like many other crops, banana is also subjected to the attack of the fungal pathogen. Indeed, one of the main problems threatening the banana industry today both locally and globally is the banana *Fusarium* wilt disease. Most edible banana cultivars in Malaysia were reported to be susceptible to *Fusarium* wilt, caused by the soil inhibiting fungus, *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 (Foc-TR4) which has caused an estimated total loss of US$ 14.1 million in Malaysia alone (Aquino et al., 2013; Ploetz, 2015). The retail price of bananas has risen from RM1.53 per kg in 2000 to RM 4.25 per kg in 2018 because of the Foc-TR4 outbreak (Tumin and Shaharuddin, 2019). There is also a low genetic diversity between plantations due to selective planting of common cultivars (Fourie et al., 2011). This monoculture practice is always a predisposing factor for pest and disease outbreaks (Ploetz, 2015).

Currently there are no effective measures to control this disease as the nature of the pathogen is a soil-borne, mutate easily and a vascular inhabitant. Foc-TR4 can be easily spread from one farm to another by moving infected planting materials between farms, transporting human fungal propagules, farm tools, vehicles, irrigation as well as flood waters (Dita et al., 2018). Before suitable management strategies are implemented to control *Fusarium* wilt, a thorough understanding of their genetic diversity and virulence is essential.

1.2 Problem statements and significance of study

Understanding the biology of Foc-TR4 isolates is necessary prior to disease management (Czislowski et al., 2018). Generally, traditional pathogenicity test was used to distinguish pathogenic and non-pathogenic isolates under greenhouse condition and through molecular methods based on standard approaches. To date, most genetic diversity studies of Foc, using common SSR, ISSR, AFLP or RAPD markers did not show correlation between the phylogenetic data and virulence level of Foc. Since aggressiveness components are defined by quantitative traits, markers derived from fungal virulence or effector genes might present an attractive alternative as genetic marker. Hence, one of the necessary preparations in overcoming the problem is to discover more fungal effectors genes from pathogenicity traits. Advances in diversity evaluation methods have shown that the currently defined race structure does not sufficiently reflect the extent of genetic and phenotypic variation in Foc (Ploetz and Pegg, 2000a; Dita et al., 2010; Fourie et al., 2011).

Secreted in Xylem (SIX) genes were the first effector identified in *Fusarium oxysporum* f. sp. *lycopersici* (Fol), a causal agent of *Fusarium* wilt of tomato (Ma et al, 2010). The molecular characteristics and presence of *SIX* genes in Foc-TR4 isolates from Malaysia have not been studied in detail. To date, PCR assays and subsequent amplicon sequences are performed using a housekeeping gene primer such as the *Translation Elongation Factor (TEF1-α*) to investigate the phylogenetic relationship among locally isolated Foc and other isolates. The ability of *SIX* genes to be used as genetic markers in the phylogenetic analysis has not yet been evaluated. Reliable pathogenicity-based markers are required to distinguish between the phylogenetic data and the virulence levels of Foc isolates. Pathogenicity testing is therefore needed to understand the biology of local isolates prior to the implementation of any disease management strategies. It is therefore important to examine whether *SIX1, SIX7* and *SIX8* genes can clearly determine the genetic relationship between Foc-TR4 isolates in Peninsular Malaysia. These three genes were chosen based on previous reports that the presence or absence of these genes is capable of recognizing Foc in bananas. Numerous effector genes which express small, secreted proteins, such as *SIX1*, are specifically enhanced during infection (Rep et al., 2005). The expression of *SIX1* and *SIX8* was evaluated using qRT-PCR in order to study the circumstances requiring the use of Foc effectors and to identify the host signal that induces the development of these proteins. These expression patterns can be used as standards in pathogenicity studies, which are normally used to assess a plant's response to infection. These *SIX* gene could be used as a diagnostic tool to detect the presence of *Fusarium* wilt in farms. It can assist farmers in protecting their crops from *Fusarium* wilt and eliminating it at an early stage of infection. Bananas of high quality will open up more market opportunities for our agricultural products. Aside from that, investigating the role of known pathogenicity-related genes involved is a necessary step toward understanding how resistant plants are produced. Data derived from this study will serve as an invaluable genomic reference that will further our understanding of the molecular activities that occur specifically in banana plants during their early and late response to Foc-TR4. Potential effector genes can also be used

as DNA markers in the development of durable disease resistance strategies (Gibriel et al., 2016). The invention identifies gaps in current knowledge about the ability of *SIX* genes to function at the early and late stages of infection and provide insights into future research that can be pursued to improve our understanding of the functions of *SIX* genes.

1.3 Research objectives

The aim of this research was to address the identified research problems with the following research objectives:

1. To identify pathogenic variation and detect the presence of *SIX* genes of *F.oxysporum* f. sp. *cubense* Tropical Race 4 (Foc-TR4).
2. To determine the phylogenetic relationships between Foc-TR4 isolates using *SIX* genes.
3. To investigate the expression profile of *SIX* genes in Foc-TR4 isolates at different time-point through quantitative real-time PCR analyses (qRT-PCR).

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