UNIVERSITI PUTRA MALAYSIA

CHARACTERIZATION OF VACUOLAR PROCESSING ENZYME GENE FAMILY AND ITS EXPRESSION IN RESPONSE TO *Fusarium oxysporum* INFECTION IN *Musa acuminata* COLLA

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By

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November 2018

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Panama disease caused by *Fusarium oxysporum* f. sp. *cubense* tropical race 4 *(Foc*TR4*)* has been the major threat for global banana production. Countless mitigation approaches have been implemented to avert Foc infections previously which include utilising of chemical fungicide, improvement of cultural practices and eradication through physical measures. However, most of the attempts failed to provide a satisfactory outcome in field trials. Cultivation of resistant cultivars has been shown to be the most effective approaches in counteracting the Panama disease which could be generated *via* genetic modification or conventional breeding. Hence, understanding the molecular events during compatible and incompatible interaction of *Foc* with banana could provide valuable insight into the development of genetically modified resistant banana which is crucial for protection strategies against Panama disease. This research was undertaken to study the molecular characteristic of *Musa acuminata* vacuolar processing enzyme (MaVPE) – a cysteine proteinase that mediates programmed cell death during *Foc*TR4 infection. A total of seven *MaVPE* genes (designated as *MaVPE1* through *MaVPE7*) were successfully identified through systemic *in silico* analysis of DH-Pahang (AA group) banana genome. Phylogenetic study showed that MaVPEs could be divided into seed type or vegetative type. Quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR) further revealed that most of *MaVPE* genes expressions were induced in susceptible *M. acuminata* cv. Berangan after *Foc*TR4 infection, specifically at 1 and 2 days post inoculation (DPI). However, in resistant *M. acuminata* cv. Jari Buaya, *MaVPEs* expression remained at low level at all time points after inoculation with *Foc*TR4. The enzymatic caspase-1 activity of MaVPE also corroborated with the gene expression analysis. Interestingly, comparative proteomic profiling analysis

revealed an increase in the abundance of cysteine proteinase in inoculated susceptible *M. acuminata* cv. Berangan as opposed to cysteine proteinase inhibitors in resistant *M. acuminata* cv. Jari Buaya. Consistently, inhibition of MaVPE activity through caspase-1 inhibitor reduced vacuolar membrane disintegration and decreased lesion formation of *Foc*TR4 infected banana root. Further functional analysis using an *Arabidopsis VPE*-null mutant exhibited higher tolerance to *Foc*TR4 infections and decreased cell death incidence. Taken together, the findings suggest that VPE acts as a key molecule in modulating susceptibility response in *Foc*TR4-infected plant. This study could be the stepping stone for the development of silenced *MaVPE* banana *via* exploitation of gene editing technology. This would be useful for the production of *Foc*TR4-resistant banana cultivar in future.

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

PENCIRIAN KELUARGA GEN VPE DAN PENGEKSPRESANNYA KETIKA TINDAK BALAS DENGAN JANGKITAN *Fusarium oxysporum* DI DALAM *Musa acuminata* COLLA

Oleh

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Penyakit Panama, yang disebabkan jangkitan kulat *Fusarium oxysporum* f. sp. *cubense* "tropical race 4" *(Foc*TR4*)* merupakan salah satu kekangan utama bagi pengeluaran pisang global. Sebelum ini, pelbagai langkah-langkah yang telah dilaksanakan bagi mengawal penyakit Panama ini dari merebak termasuklah penggunaan racun kulat, penambahbaikan teknik tanaman dan pembasmian kulat melalui kaedah fizikal. Tetapi, hasil dari cubaan-cubaan tersebut gagal memberikan hasil yang memuaskan. Penanaman kultivar rintang telah menunjukkan yang ianya merupakan teknik yang paling efektif dalam megatasi masalah penyakit Panama dan ianya boleh dihasilkan melalui pengubahsuaian genetik atau pembiakan konvensional. Maka, pemahaman tentang aktiviti molekular ketika jangkitan *Foc* dengan pokok pisang rintang dan rentang boleh memberikan petunjuk berharga dalam penghasilan pisang rentang yang diubah suai secara genetic bagi melawan penyakit Panama ini. Kajian ini dijalankan untuk mengkaji ciri-ciri molekular "*Musa acuminata* vacuolar processing enzyme (MaVPE)" yang merupakan salah satu enzim "*proteinase cysteine*". Melalui kajian ini, MaVPE telah dikenalpasti sebagai enzim pengantara bagi pengaktifan program kematian sel semasa jangkitan *Foc*TR4. Tujuh *MaVPE* gen (dinamakan sebagai *MaVPE1* hingga *MaVPE7*) telah berjaya dikenalpasti melalui analisis "*in-silico"* tersusun dengan menggunakan pengkalan data genom pisang DH-Pahang (kumpulan AA). Analisis "*phylogenetik*" menunjukkan bahawa MaVPE yang dikenalpasti boleh dibahagikan kepada dua iaitu jenis benih atau jenis vegetatif. Analisis pengekspresan gen menggunakan kaedah tindak balas berantai polimerase kuantitatif masa sebenar (RT-qPCR) menunjukkan kadar pengekspressan gen-gen *MaVPE* meningkat di dalam pokok pisang Berangan (rentan), terutama pada satu dan dua hari selepas inokulasi (DPI). Manakala, di dalam pokok pisang Jari Buaya (rintang), pengurangan pengekspressan gen-gen *MaVPE* telah dikesan terutama pada satu hari dan dua hari selepas inokulasi. Tambahan pula, aktiviti enzim *"caspase-1"* juga selari dengan analisis ekspresi gen. Analisis proteomik perbandingan juga menunjukan peningkatan

proteinase cysteine dapat dikesan di dalam pokok pisang Berangan selepas inokulasi dengan *Foc*TR4 manakala peningkatan perencat "*proteinase cysteine*" dapat dikesan di dalam pokok pisang Jari Buaya selepas inokulasi dengan *Foc*TR4. Sejajar dengan itu, perencatan aktiviti MaVPE dengan menggunakan perencat "*caspase-1*" menunjukkan pengurangan perpecahan membran vakuol dan pengurangan pembentukan luka di dalam akar pokok pisang Berangan yang diinokulasi dengan *Foc*TR4. Analisis fungsian lebih lanjut di dalam *Arabidopsis thaliana VPE*-null mutan menunjukkan toleransi yang lebih tinggi terhadap inokulasi *Foc*TR4 dan pengurangan gejala penyakit dapat dilihat. Sebagai kesimpulan, kajian kami mencadangkan bahawa MaVPEs bertindak sebagai molekul utama dalam respon kerentanan pokok yang diinokulasi dengan *Foc*TR4. Kajian ini boleh dijadikan sebagai batu loncatan bagi pembangunan "*silenced MaVPE"* pisang melalui eksploitasi teknologi pengubahsuaian gen. Kajian ini akan bermanfaat bagi menghasilkan pisang rentang terhadap *Foc*TR4 pada masa hadapan.

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LIST OF ABREVIATIONS

A	Alpha
B	Beta
Δ	Delta
Γ	Gamma
°C	Degree celsius
%	Percentage
A_{595nm}	Optical density at wavelength 595 nanometer
µL	Microliter
µm	Micrometer
µmoles	Micromoles
aa	Amino acids
bp	Base pair
$CaCl_2$	Calcium chloride
cDNA	Complementary deoxyribonucleic acid
DNA	Deoxyribonucleic acid
DPI	Days post inoculation
DTT	Dithiothreitol
Foc	*Fusarium oxysporum* f. sp. *cubense*
G	Gram
kb	Kilobase
kDA	kilodaltons
L	Litre
M	Molar
MaVPE	*M. acuminata Vacuolar Processing Enzyme*
mL	Millilitre
mg	Milligram
mm^3	Cubic millimetre
mM	Millimolar
MS	Murashige and Skoog
nM	Nanomolar
PCR	Polymerase chain reaction
pI	Isoelectric point
PMSF	Phenylmethylsulfonyl fluoride
qPCR	Quantitative real time PCR
RT	Reverse transcription
VPE	Vacuolar Processing Enzyme

CHAPTER 1

INTRODUCTION

1.1 Background study

Bananas and plantains *(Musa* spp.) are listed as the fourth important food crops with global production of 138 million tons in 2010 (Bai et al., 2013). However, Panama disease caused by *Fusarium oxysporum* f. sp. cubense (*Foc*) is a major constraint in developing sustainable banana production. *Foc* Tropical Race 4 (*Foc*TR4) has raised concerns amongst banana producing countries as it was identified as the most virulent strain and it affected most of the banana cultivars (Jones, 2009). Currently, researchers have been focusing on detailed molecular mechanism in plant-pathogen interactions to counteract the problem with Panama disease which includes the mechanisms involved in programmed cell death (PCD).

Several studies have shown the involvement of PCD during *Foc*TR4 infection in banana plants (Bai et al., 2013; Dale et al., 2017; Paul et al., 2011). For instance, transcriptomic analysis of banana roots infected with *Foc*TR4 showed upregulation of PCD related genes in susceptible *M. acuminata* cv. Brazilian but down-regulated in resistant *M. acuminata* cv. Yueyoukang 1 (Bai et al., 2013). In addition, overexpression of *cell death abnormality gene 9* (*Ced9*); an anti-apoptotic gene from *Caenorhabditis elegans*, in banana have shown to increase resistance level towards *Foc*TR4 infection by preventing fungal-induced cell death and contributing to the maintenance of organelle homeostasis (Dale et al., 2017; Paul et al., 2011). In addition, Dickman et al. (2001) have also reported that alteration of pathways controlling PCD is a valid strategy to confer resistance to broad range of necrotrophic fungi.

Caspase has been known to play important roles in mediating PCD execution. In plants, a cysteine proteinase known as vacuolar processing enzyme (VPE) has been identified as a counterpart of caspase-1 (Jan & Andrabi, 2008). VPE is highly associated with cell suicidal strategy which initiates the vacuolar rupture of the cells and leads to proteolytic cascade of PCD. Previous study has shown that VPE could involve in both physiological development as well as plant immune response against biotic and abiotic stresses (Kinoshita et al., 1995; Kuroyanagi et al., 2005; Hatsugai et al., 2015). For example, both *AtVPEβ* and *AtVPEδ* from *Arabidopsis thaliana (A. thaliana)* were shown to be essential for the processing and maturation of seed storage proteins within protein storage vacuoles (Kinoshita et al., 1995; Nakaune et al., 2005). Moreover, *AtVPEγ* is expressed in vegetative organ and has critical roles in plant immune response by mediating vacuolar mediated PCD (Kinoshita et al., 1995; Kuroyanagi et al., 2005; Misas-Villamil et al., 2013; Rojo et al., 2004).

In plant pathogen interaction, VPE-mediated cell death could be progressed in a form of either VPE-dependent hypersensitive response (HR) cell death or VPE-dependent susceptible cell death (Hara-Nishimura & Hatsugai, 2011). VPE-dependent HR cell death is important for plant protection against pathogen as it inhibit the pathogen growth at the site of infections. Study in *Nicotania benthamiana* (*N. benthamiana*) showed that *VPE* deficiency prevented virus-induced hypersensitive cell death whereby the *VPE*-silenced plant failed to show vacuolar membrane disintegration and PCD symptoms which leads to increased virus proliferation in the plants (Hatsugai et al., 2004). However, in VPE-dependent susceptible cell death, different phenomenon was observed whereby VPE was found to promote pathogen invasion in susceptible host plant. For instance, study in *A. thaliana* showed that silencing all VPE genes in *A. thaliana* has successfully increased resistance towards mycotoxin fumonisin B1 (FB1) (Kuroyanagi et al., 2005). In *VPE*-null mutant plant, no lesion formation or vacuolar collapse were observed in the infiltrated Fumonisin B1 (FB1) leaves (Kuroyanagi et al., 2005). Taken together, VPE could act as a switch in mediating HR cell death to contain pathogen invasion or facilitate further spread of pathogen infections in the host plants.

VPE has shown to be involved in various plant immune response *via* activation of vacuolar cell death in response to biotic and abiotic stresses. However, to date no reports have been made on VPE genes family in banana Thus, this research was undertaken to identify and characterize *Musa acuminata VPE* (*MaVPE*) genes in banana genome as well as discovering the potential of *MaVPE* in mediating banana immune response against *Foc*TR4 infection *via* regulation of cell death mechanism. Taken together, this study will unravel the roles of *MaVPEs* in regulating cell death response against *Foc*TR4 which will be beneficial for addressing the problem with Panama disease.

1.2 Objectives

The objectives of present study were:

1. To identify and characterize *VPE* genes in *M. acuminata via* genome wide analysis.

2. To determine the expression pattern of *MaVPE* genes family in different parts of banana tissues and in response to infection with *Foc*TR4 *via* conventional reverse transcription polymerase chain reaction (RT-PCR) and quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR).

3. To examine the proteome changes in banana root upon infections with *Foc*TR4 *via* comparative proteomic profiling LC/MS-MS and investigate the probable mechanism of VPE during *Foc*TR4 infection.

4. To functionally characterize *VPE* genes upon infection with *Foc*TR4 *via* caspase inhibitor and *A. thaliana VPE*-null mutant studies.

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LIST OF PUBLICATIONS

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