UNIVERSITI PUTRA MALAYSIA

EXPRESSION OF SELECTED PATHOGENESIS-RELATED PROTEINS AT EARLY STAGE OF *Ganoderma boninense* INFECTION IN *Elaeis guineensis* Jacq. SEEDLINGS

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REDZYQUE RAMZA BIN RAMLI

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirement for the Degree of Master of Science

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DEDICATION

This thesis is dedicated to all I love, especially my dearest Mummy, Lijah Zainab for her endless support, love throughout my whole life. Also to my dear brothers and sisters, Ramiza, Ridza, Ramzan, Rudynata, Ruhaniza, Ruzaimi and Robaeha Ramza for their continuous support in terms of moral and financial in this long and adventurous journey. Last but not least, this is my tribute to my inspiring late Dad, Ramli, for everything he provided and supported me until he passed away. I remembered his jokes and laugh like it was yesterday. InshaAllah, I hope the best in life provided for my family and friends and hopefully my research will benefit others eventually.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

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Chairman : Profesor Siti Nor Akmar Abdullah, PhD
Faculty : Institute of Tropical Agriculture and Food Security

Basal stem rot (BSR) is the most devastating oil palm disease caused by *Ganoderma boninense*. It is only evident when the infection has progressed by 60–70% which is too late to cure the palm. Studies on early defense responses are required to provide insightful information on the initiation of defense signaling networks upon recognition of pathogen. Thus, information on early interactions of plant-pathogens is crucial to allow screening for detection of the potential threat of BSR, especially on young palms. This study was conducted to isolate and detect the presence of *G. boninense* within infected oil palm seedlings, and to identify the differentially expressed genes (DEGs) which related to pathogenesis (PR) as well as to analyse PR genes expression in oil palm roots as a result of oil palm-*G. boninense* interaction. Oil palm seedlings were artificially infected with *G. boninense* inoculums. Deoxyribonucleic acid (DNA) samples were taken from infected palms (T1) and *G. boninense* pure culture for detection using polymerase chain reaction (PCR), multiple sequence alignment (MSA) and basic local alignment search tool (BLAST) via NCBI. Polymerase chain reaction (PCR) and nested PCR produced amplicon with the expected size of 200 base pairs (bp) for 3 days post-inoculation (DPI) and *G. boninense* pure culture, as well as 100 bp for 7 and 11 DPI. MSA showed the presence of the conserved sequence and ≥95% identity generated via BLAST for all amplicons of T1 samples and *G. boninense* pure culture. Ribonucleic acid (RNA) samples were extracted from the root tissues at different periods (0, 3, 7, 11 DPI) and used for ribonucleic acid sequencing (RNASeq) analysis. DEGs were identified. DEGs analyses displayed that several PR proteins were expressed as a result of the defense mechanism of oil palm against *G. boninense* including *chitinase 1 isoform X3* (*EgChi1X3*), *germin-like 8-14* (*EgGer8*), *glu S. griseus protease inhibitor* (*EgGluP*), *glucan endo-1,3-beta-glucosidase 3-like* (*EgGlu3*) and *subtilisin-like protease SBT1.9* (*EgPro1.9*). These genes were searched for primary structural homology via BLAST, based on size and exon count. Homology searches for selected PR genes with their respected PR groups generated. The conserved domain for each PR gene was analysed to determine their functions in terms of plant defense. MSA was done and generated a phylogenetic tree for each PR gene to check their related grouping based on the conserved domain. MSA generated ≥ 90% of primary

structural homology among selected genes with their respected PR groups. Based on conserved domains, each PR gene function was determined which is related to plant defense. *EgChi1X3* may be involved in the production of elicitor compounds and the degradation of fungal chitins. *EgGer8* is probably involved in oxidative degradation of oxalate and preventing plant cell wall hydrolysis. *EgGluP* may be involved in promoting inhibition of pathogen proteolytic enzymes. *EgGlu3* is possibly related directly to the activity of degradation and rendering fungal cell walls susceptible to plant responses and cell lysis. *EgPro1.9* may be involved in extracellular protein secretion and mature peptide degradation. The defense response obtained from DEG analyses showed immense upregulation of *EgChi1X3*, *EgGer8* and *EgGluP* with an average of 12, 3 and 32-fold higher than control at 3 DPI. While *EgGlu3* and *EgPro1.9* significantly expressed with 218 and 6-fold at 11 DPI when compared to control. The phylogenetic tree showed that the selected PR genes were clustered together with date palm due to probable motif similarity and sequence homology. Based on this study, these selected PR genes will be potential candidates in developing biomarkers for early phase detection of *G. boninense* infection.

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

EKSPRESI PROTEIN-BERKAITAN PATOGENESIS YANG TERPILIH PADA PERINGKAT AWAL SERANGAN *Ganoderma boninense* TERHADAP ANAK POKOK *Elaeis guineensis* Jacq.

Oleh

REDZYQUE RAMZA RAMLI

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Reput pangkal batang (BSR) adalah penyakit kelapa sawit yang paling membinasakan disebabkan *Ganoderma boninense*. Ianya hanya jelas kelihatan apabila jangkitan telah mara sebanyak 60 hingga 70 peratus yang mana sudah terlambat untuk mengubati palma tersebut. Kajian ke atas tindak balas pertahanan awal diperlukan agar dapat memberi maklumat yang mendalam tentang permulaan rangkaian isyarat pertahanan apabila patogen dikenalpasti. Oleh itu, maklumat interaksi awal tumbuhan-patogen adalah penting untuk membolehkan saringan dilakukan untuk mengesan potensi ancaman BSR terutama pada pokok kelapa sawit muda. Kajian ini dijalankan untuk mengasingkan dan mengesan kehadiran *G. boninense* dalam anak pokok kelapa sawit yang dijangkiti dan mengidentifikasi gen yang terekspres dengan ketara (DEGs) berkaitan patogenesis (PR) dan juga menganalisa ekspresi gen PR dalam akar pokok kelapa sawit hasil dari interaksi kelapa sawit dengan *G. boninense*. Anak pokok kelapa sawit telah dijangkiti secara buatan menggunakan inokulum *G. boninense*. Sampel asid dioksiribonukleik (DNA) telah diambil dari pokok yang dijangkiti (T1) dan kultur tulen *G. boninense* untuk proses pengesanan menggunakan tindak balas rantai polimerase (PCR), penjajaran jujukan berbilang (MSA) dan pencari alatan tempatan asas (BLAST) melalui NCBI. PCR dan PCR bersarang menghasilkan amplikon dalam saiz jangkaan iaitu 200 pasangan bes (bp) untuk 3 hari-selepas-inokulasi (DPI) dan kultur tulen *G. boninense*, serta 100 bp untuk 7 dan 11 DPI. MSA menunjukkan kehadiran jujukan terabadi dan persamaan sebanyak ≥95% dihasilkan melalui BLAST untuk semua amplikon dari sampel T1 dan kultur tulen *G. boninense*. Sampel asid ribonukleik (RNA) diekstrak daripada tisu akar pokok pada tempoh masa yang berbeza (0, 3, 7, 11 DPI) dan digunakan untuk analisa penjujukan asid ribonukleik (RNASeq). DEGs telah dikenalpasti. Analisis DEG menunjukkan beberapa protein PR diekspres berpunca daripada mekanisme pertahanan kelapa sawit terhadap *G. boninense* termasuk *chitinase 1 isoform X3* (*EgChi1X3*), *germin like 8-14* (*EgGer8*), *glu S. griseus protease inhibitor* (*EgGluP*) , *glucan endo-1,3-beta-glucosidase 3-like* (*EgGlu3*) dan *subtilisin-like protease SBT1.9* (*EgPro1.9*). Homologi struktur utama gen-gen tersebut dihasilkan melalui BLAST, berdasarkan saiz dan jumlah ekson. Pencarian homologi bagi gen PR yang terpilih dengan kumpulan PR masing-masing dihasilkan. Domain terabadi

bagi setiap gen PR dianalisa untuk memahami fungsi mereka dalam pertahanan tumbuhan. MSA telah dilakukan dan menghasilkan pokok filogeneik bagi setiap gen PR untuk memeriksa pengelompokan berdasarkan domain terabadi. MSA menghasilkan sebanyak ≥ 90% homologi struktur utama dikalangan gen terpilih dengan kumpulan PR masing-masing. Berdasarkan domain terabadi, setiap fungsi gen PR ditentukan berkaitan dengan pertahanan tumbuhan. *EgChi1X3* mungkin terlibat dalam penghasilan sebatian elisitor dan degradasi kitin kulat. *EgGer8* mungkin terlibat dalam degradasi oksidatif oksalat dan menghalang hidrolisis dinding sel tumbuhan. *EgGluP* mungkin terlibat dalam mempromosikan penghambatan enzim proteolitik patogen. *EgGlu3* mungkin berkait langsung dengan aktiviti degradasi dan menjadikan dinding sel kulat mudah terdedah kepada tindak balas tumbuhan dan lisis sel. *EgPro1.9* mungkin terlibat dalam rembesan protein ekstraselular dan degradasi peptida matang. Tindakbalas pertahanan yang diperoleh dari analisis DEG menunjukkan kawal naik *EgChi1X3*, *EgGer8*, dan *EgGluP* yang tinggi dengan purata sebanyak 12, 3 dan 32 kali ganda lebih tinggi daripada kawalan pada 3 DPI. Sedangkan *EgGlu3* dan *EgPro1.9* diekspres secara ketara dengan 218 dan 6 kali ganda pada 11 DPI apabila dibandingkan dengan kawalan. Pokok filogenetik yang dihasilkan menunjukkan gen PR yang terpilih telah dikelompokkan bersama dengan pokok kurma disebabkan berkemungkinan mempunyai kesamaan motif dan homologi jujukan. Berdasarkan kajian ini, Gen PR yang terpilih berpotensi sebagai calon dalam membangunkan bio-penanda untuk fasa awal jangkitan *G. boninense*.

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

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LIST OF ABBREVIATIONS

ABA	Abscisic acid
AFLP	Amplified fragment length polymorphism
Avr	Avirulence
BSR	Basal Stem Rot
BLAST	Basic Local Alignment Search Tool
bp	Base pair
CDNA	Complementary DNA
CHI	Chitinase
cm	centimetres
CRK	Cysteine-like protein kinase
CTAB	Cetyltrimethyl ammonium bromide
CWDE	Cell wall degradation enzyme
DEG	Differentially expressed gene
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
DPI	Days post-inoculated
DSI	Disease severity index
EDTA	Ethylenediaminether tetra-acetic acid
Eg	*Elaeis guineensis*
ELISA	Enzyme-linked immunosorbent assay
E-nose	Electronic nose
EST	Expressed sequence tag
ETI	Effector-triggered immunity
FC	Fold Change
FFB	Fresh fruit bunch
FISH	Fluorescence in situ hybridization
g	Relative centrifugal force
GanoDROP	*Ganoderma* and Disease Research for Oil Palm
gDNA	genomic DNA
GER	Germin
GLP	Germin-like protein
GLU	Glucanase
GPI	Glycosylphosphatidylinositol
H2O2	Hydrogen peroxide

HPLC	High-performance liquid chromatography
HR	Hypersensitive response
ID	Identification
ITS	Internal transcribed spacer
JA	Jasmonic acid
kGy	kiloGray
LRR	Leucine-rich repeat
LTP	Lipid transfer protein
LTPG	GPI-anchored lipid transfer protein
MAMP	Microbe-associated molecular pattern
MIP	Molecularly Imprinted polymer
mM	MilliMolar
MPOB	Malaysian Palm Oil Board
mRNA	Messenger RNA
MSA	Multiple sequence alignment
NaCl	Sodium chloride
NBD	Nucleotide-binding domain
NBS-LRR	Nucleotide-binding site leucine-rich repeat
NCBI	National Center for Biotechnology Information
NGS	Next Generation Sequencing
nsLTP	Non-specific lipid transfer protein
OxO	Oxalate oxidase
PAMP	Pathogen-associated molecular pattern
PCD	Programmed cell death
PCI	Phenol-chloroform isoamyl alchohol
PCR	Polymerase chain reaction
PDB	Potato dextrose broth
PI	Protease inhibitor
PR	Pathogenesis-related
PRR	Pattern recognition receptor
PTI	Pattern-triggered immunity
PTK	Protein kinase
PVP	Polyvinyl pyrolidone
qPCR	Quantitative Polymerase chain reaction
R	Resistance
RAMS	Random amplification of microsatellite

RAPD	Random amplification of polymorphic DNA
rDNA	ribosomal DNA
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RNA-seq	Ribonucleic acid sequencing
ROS	Reactive Oxygen Species
RP	Resistance protein
RWB	Rubber wood block
SA	Salicylic acid
SAR	Systemic Acquired resistance
SBT	Subtilase
TE	Tri-EDTA
TIR	Toll/interleukin 1 receptor
USR	Upper stem rot
µL	Microliter
µM	Micromolar

CHAPTER 1

INTRODUCTION

Oil palm (*Elaeis guineensis* Jacq.), is mainly grown in Southeast Asia which contributes approximately 90% of the world's palm oil production. Palm oil is accredited as one of the prime sources of edible oil, the raw material for oleochemicals and precursors for biodiesel fuel (Stichnothe et al., 2014; Kurnia et al., 2016). The total export revenue of oil palm products for Malaysia in 2018 was RM67.49 billion (MPOB, 2018). Therefore, due to the increasing demand for palm oil annually, Malaysia has expanded from 5.81 million hectares in 2017 to approximately 5.85 million hectares in 2018 (MPOB, 2018). To obtain a sustainable oil palm industry without harming the environment and ecosystem, certain criteria are required such as establishing land allocation, ecological service mapping, conserving ecological integrity, ecosystem health, adaptability, network, raising awareness, better management practices, zero-waste milling technology, minimal production gaps, planting disease-resistant and high-yielding variety, alternate technologies and sources as well as less deforestation on acceptable land (Khatun et al., 2017).

Oil palm is vulnerable to stem rot diseases, including upper stem rot (USR) and basal stem rot (BSR) (Tisné et al., 2017). *G. boninense* which caused BSR, become economically problematic when the disease affects 10-20% of the palm and at the end of standard planting cycle of 25-year, the losses could reach up to 30-70%, which lead to several other problems such as substantial fresh fruit bunch (FFB) losses, early replantation and wasted resources (Cooper et al., 2011). *G. boninense* is considered to be the most prevalent fungal disease due to the huge economic losses of oil palm plantations reported of RM225 million to RM1.5 billion a year (Arif et al., 2011). These outbreaks easily occur due to *Ganoderma* ability to propagate through direct root contact and basidiospores. The detailed processes are still unresolved (Pilotti et al., 2002; Rees et al., 2012).

Ganoderma spp. is known to degrade plant cell wall components including lignin, resulting in white rot to oil palm (Paterson, 2007). Among all species of *Ganoderma*, *G. boninense* is the most epidemic towards oil palms (Susanto et al., 2005). BSR infection occurs indiscriminately in all growth stages of oil palm including mature and young palms. The manifestation of infection occurs more rapidly and drastic in earlier growth phases (Susanto et al., 2005). However, during an early stage of infection, the infected oil palm appears to be symptomless and the first symptom that can be observed usually on foliage part when the progression of infection already reaches 60 to 70% (Chong et al., 2017). At this stage, the infected young palms normally die within 1 or 2 years, whilst mature trees could survive for extra 3 or more years (Corley and Tinker, 2003). Studies on early defense response are crucial in providing information on making better decisions in detecting and managing *G. boninense* infection.

BSR spreads gradually by decaying lower stem and root system by disrupting water and nutrients intake to the upper oil palm part. Shortage of water and nutrients reveal critical symptoms at the foliar part of oil palm such as unopened spear leaves, senescence and

yellowing of upper fronds, reduced and "one-sided mottling" canopy and crown become flattened, and blooming of basidiocarps on the bottom stem. (Chung, 2011; Rees et al., 2012). Ultimately, all of these attacks result in the collapse of the stand (Chung, 2011). *G. boninense* can be classified as hemibiotroph with a transitional state of biotrophs and necrotrophs (Bahari et al., 2018). The initial stage of the fungal attack is the biotrophic state where fungal colonization occurs in an intact state on host plant cells and later switching to necrotrophic state causing extensive cell wall degradation (Chong et al., 2017).

Biotrophs sustain themselves via deriving energy from living cells and preserving intact host cells for nutrient uptake, whereby necrotrophs is opportunistic and kill plant cells rapidly to derive energy and live saprotrophically on the dead remains (Bahari et al., 2018). Normally, plants counteract with the biotrophic attack which happens during the initial stage of infection by boosting the production of reactive oxygen species (ROS) via respiratory burst (Morkunas and Ratajczak, 2014). Hence, plant activates programmed cell death (PCD) to regulate pathogen growth. This response is one of hypersensitive response (HR) when rapid cell death occurs in the local region around the infection (Lam et al., 2001).

On the other hand, necrotrophs alter host cell walls by releasing a high level of cell wall degradation enzyme (CWDE) to enhance host cell wall accessibility (Hok et al., 2010). For example, successful infection of necrotrophic pathogen *Botrytis cinerea* on *Solanum lycopersicum* induces expansin and polygalacturonase which promote cell wall loosening (Cantu et al., 2009). Necrotroph also produces expansin-like protein to aid infiltration of hyphae as well as providing security for hemibiotrophs, *Fusarium graminearum* against plant enzymatic breakdown (Pazzagli et al., 2014; Quarantin et al., 2016). Hemibiotrophs have transitional habits thus depend heavily on successful biotrophic occupation within the host cells before the transition to the necrotrophic phase which is a more aggressive mode of infection. (Vargas et al., 2012). The time for transition from the biotrophic phase to the necrotrophic phase differs among the pathogenic species (Kabbage et al., 2015).

Previous research suggested that *G. boninense* probably can be treated using a systemic fungicide called hexaconale when applied using a suitable technique (Idris et al., 2010a). However, fungicidal treatment becomes limited due to prominent symptoms of *G. boninense* infection can only be observed during the later stage, when the infection is already well-established (Turner and Gillbanks, 2003; Najmie et al., 2011). Countless diagnostic and detection methods were suggested including usage of DNA-PCR, volatile organic compound detection, polyclonal antibodies, fungal ergosterol quantification as well as usage of hyperspectral imaging via satellite sensors (Bridge et al., 2000; Idris et al., 2003; Utomo and Niepold, 2000; Lelong et al., 2010) but some are not exclusive towards *Ganoderma* species and some are unable to detect early infected oil palms due to similarity to healthy palms.

In our study, analyses on gene expression profiles of PR genes in oil palm roots inoculated with *G. boninense* generated several outcomes on a phenomenon involving PR genes in oil palm-*G. boninense* interaction. The knowledge gaps have been identified in isolating *G. boninense* from the infected oil palm, identifying genes that were differentially expressed

due to *G. boninense* infection in oil palm and analysing the expression of pathogenesis-related (PR) genes in the oil palm which was infected with *G. boninense*. We hypothesized that *G. boninense* will be detected in the infected oil palm because of the successful *G. boninense* colonization We also hypothesized that the PR genes expressed in *G. boninense*-treated oil palm will be identified and group based on their PR family. Lastly, we hypothesized that by analysing PR genes expression pattern in infected oil palm based on specific time phase will be able to provide data on which PR genes are phase-specific and could suggest a more efficient method in the future on detecting *G. boninense* in oil palm during the early phase of infection. For future research, these PR genes will be analysed for their potential as biomarkers for *G. boninense* during early plant-pathogen interaction. The experiments were done with the following objectives:

(i) To isolate and detect the presence of *G. boninense* within treated oil palm seedlings.

(ii) To identify the differentially expressed genes (DEGs) in oil palm upon treatment with *G. boninense*.

(iii) To identify and analyse pathogenesis-related (PR) genes expression profiles in oil palm roots due to the oil palm-*G. boninense* interaction.

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LIST OF PUBLICATIONS

Journal

Bahari, M. N., Sakeh, N. M., Abdullah, S. N., Ramli, R. R., and Kadkhodaei, S. (2018). Transciptome profiling at early infection of Elaeis guineensis by *Ganoderma boninense* provides novel insights on fungal transition from biotrophic to necrotrophic phase. *BMC Plant Biology, 18*(1). doi:10.1186/s12870-018-1594-9.

Poster paper presentation

Ramli, R. R., Abdullah, S. N., Bahari, M. N. and Sakeh, N. M. (2018). Expression of Selected Groups of PR Proteins at Early Stage of *Ganoderma boninense* Infection of Oil Palm (*Elaeis guineensis* Jacq.) Seedlings. International Conference on Big Data Applications in Agriculture 2017, Universiti Putra Malaysia, Malaysia.

Ramli, R. R., Abdullah, S. N., Bahari, M. N. and Sakeh, N. M. (2018). Expression of Selected Groups of PR Proteins at Early Stage of *Ganoderma boninense* Infection of Oil Palm Seedlings. 2nd International Conference on Crop Improvement 2015, Universiti Putra Malaysia, Malaysia.

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