MOLECULAR ANALYSES OF NONRIBOSOMAL PEPTIDE SYNTHETASE IN *Ganoderma boninense*

By

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

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May 2015

Chair : Assoc. Prof. Wong Mui Yun, PhD
Institute : Institute of Tropical Agriculture and Food Security

Oil palm plays a significant role in Malaysia's economy as Malaysia is the second-largest palm oil producer after Indonesia. However, the basal stem rot disease (BSR) caused by *G. boninense*, a basidiomycete fungus has affected oil palm yields in most production areas. Many methods to control the disease have been developed but to date, there is no control of BSR disease in the field. Fungi were reported to produce variety of secondary metabolites. Some secondary metabolites are toxic to host plant and may act as virulence factors. Secondary metabolite especially produced by nonribosomal peptide synthetase (NRPS) has recently gained interest due to its wide array of biological activities and virulence factors. Hence, the understanding of fungal secondary metabolism of NRPS at molecular level is essential. In this work, detection of NRPS in *G. boninense* was achieved using PCR-based and biochemical methods. Primers targeted to NRPS conserved region of adenylation (A) domain were used to molecular characterize NRPS. To correlate NRPS and disease incidence during *Ganoderma*-oil palm interaction, RT-PCR was done conducted using samples obtained from glasshouse trial. Chrome Azurol S (CAS) agar plate assay with incorporation of CAS-blue dye was used for siderophore detection. PCR products were sequenced, translated and nucleotide sequences were searched against NCBI database using BLAST tool. The PCR fragments showed similarity to the conserved region of adenylation domain: A2 (LKAGxAYL(VL)P(LI)D, A3 (TSG(TS)TGxPKxV) and A5 (NxYGPxE). A-domain is important as it is the core element of NRPS modules. A-domain acts as the selector and the activator of the cognate substrate. Colour-change reaction in the CAS-blue agar showed the production of siderophore by *G. boninense*. The colour change occurred as a result of ferric iron transfer from the reagent complex to siderophore present in the fungus. In other reports, NRPS genes are found to be involved in the biosynthesis

of siderophores, toxins involved in pathogenesis. RT-PCR of the oil palm samples showed the same fragment size of NRPS as detected in *Ganoderma* fungus. Both progenies of susceptible and tolerant oil palm seedlings showed visible disease symptoms at 4 months after infection. However, RT-PCR of NRPS expressed as early as 1 month after infection and consistent expression at 4 months after infection onwards for both susceptible and tolerant progeny. Information related to NRPS of *G. boninense* obtained in this study will facilitate the understanding of the potential evolution of *Ganoderma* NRPS. Further studies and analysis of these genes and their peptide products may identify important roles of secondary metabolites produced by NRPS in *Ganoderma* physiology, ecology or fungal pathogenicity. This study is the first evidence for the present of NRPS in the genome of *G. boninense* and also its involvement in BSR disease of oil palm.

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

ANALISA MOLEKUL SINTETASE PEPTIDA TANPA RIBOSOM PADA *Ganoderma boninense*

Oleh

JACKIE CHUA

Mei 2015

Pengerusi : Profesor Madya Wong Mui Yun, PhD
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Industri kelapa sawit memainkan peranan penting dalam ekonomi Malaysia yang merupakan pengeluar minyak sawit kedua terbesar selepas Indonesia. Walau bagaimanapun, penyakit reput pangkal batang (RPB) yang disebabkan oleh kulat *Ganoderma boninense* telah menjejaskan hasil pengeluaran kelapa sawit di kebanyakan kawasan. Pelbagai kaedah untuk mengawal penyakit ini telah dilakukan tetapi setakat ini, tidak ada cara untuk mengawal penyakit RPB dengan sepenuhnya. Patogen kulat dilaporkan menghasilkan pelbagai metabolit sekunder. Beberapa metabolit sekunder terutamanya yang dihasilkan oleh sintetase peptida tanpa ribosom (NRPS) telah mendapat perhatian disebabkan kepelbagaiannya dalam aktiviti-aktiviti biologi dan faktor virulen. Oleh itu, pemahaman metabolit sekunder NRPS di peringkat molekular adalah penting. Dalam kajian ini, pengenalpastian dan pencirian NRPS di *G. boninense* dilakukan dengan menggunakan kaedah PCR dan cerakinan kimia. Bahagian NRPS yang merangkumi domain adenilasi (A) digunakan sebagai primer untuk pencarian NRPS dalam kulat *G. boninense.* Untuk melihat hubungan antara NRPS dan kejadian penyakit BSR, amplifikasi PCR transkripsi berbalik (RT-PCR) telah dijalankan menggunakan sampel yang diambil daripada kajian di rumah kaca. Selain daripada itu, ujian piring agar Chrome Azurol S (CAS) dengan CAS-biru digunakan untuk mengesan siderofor. Data jujukan hasil daripada PCR diterjemahkan dan turutan nukleotida dimasukkan dalam pangkalan data NCBI dengan menggunakan sistem BLAST untuk perbandingan. Analisis jujukan menunjukkan persamaan dengan rantau adenilasi iaitu A2 (LKAGxAYL(VL)P(IP)D), A3 (TSG(TS)TGxPKxV) dan A5 (NxYGPxE). A-domain adalah salah satu unsur penting dalam modul NRPS. Ia juga bertindak sebagai pemilih dan pengaktif substrat yang sepadan dalam A-domain. Perubahan warna agar CAS-biru menunjukkan pengeluaran siderofor oleh *G. boninense.* Perubahan warna tersebut berlaku akibat pemindahan ferum dari kompleks reagen untuk siderofor yang dihasilkan oleh kulat tersebut. Dalam laporan lain, gen NRPS didapati terlibat dalam biosintesis siderofor sebagai toksin yang terlibat dalam kepatogenan. Hasil RT-PCR yang dijalankan pada

sampel akar anak sawit yang telah diinokulasi dengan *G. boninense* menunjukkan jalur PCR yang sama dengan jalur PCR untuk NRPS yang dikesan di kulat *Ganoderma*. Anak benih sawit yang toleran dan rentan menunjukkan simptom penyakit yang dilihat pada 4 bulan selepas jangkitan. Walau bagaimanapun, RT-PCR pada sampel akar menunjukkan jalur PCR NRPS seawal 1 bulan selepas jangkitan dan ekspresi yang konsisten pada 4 bulan selepas jangkitan dan seterusnya pada kedua-dua anak benih rentan dan toleran. Maklumat yang berkaitan dengan NRPS *G. boninense* diperolehi dalam kajian ini akan membantu dalam memahami evolusi potensi NRPS dalam *Ganoderma*. Kajian yang lebih lanjut dan analisis gen-gen ini berserta dengan produk peptida NRPS membolehkan kepentingan metabolit sekunder yang dihasilkan oleh NRPS dapat dikenalpasti dalam fisiologi, ekologi atau kepatogenan *Ganoderma*. Kajian ini merupakan laporan pertama mengenai NRPS dalam genom *G. boninense* dan juga penglibatannya dengan penyakit RPB kelapa sawit.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment 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

A	Adenylation
AA	Amino Acid
Acs	Acetyl-coA synthetase
AmAc	Ammonium Acetate
AMP	adenylate
ATP	Adenosine Tri-Phosphate
BAR	Bin-Amphiphysin-Rvs
BLAST	Basic Local Alignment Search Tool
bp	base pair
BSR	Basal Stem Rot
C	Condensation
CAS	Chrome Azurol S
cDNA	complementary DNA
cds	coding sequence
CPO	Crude Palm Oil
CsCl	Cesium Chloride
CTAB	HexadecylTrimethyl-Ammonium Bromide
DHB	2,3-Dihydoxybenzoate
DNA	Deoxyribonucleic Acid
DNAse	Deoxyribonucleic Acidase I
dNTP	Deoxynucleotide TriPhosphate
DSI	Disease Severity Index
EDTA	Ethylenediaminetetraacetic acid
FASs	Fatty Acid Synthetase
FC	Ferricrocin
FsC	Fusarinine C
GADPH	Glyceraldehydes 3”-phosphate Dehydrogenase

HDTMA	Hexadecyltrimethylammonium
HFC	Hydroxyferricrocin
KS	Ketosynthetase
LB	Lysogeny Broth
MEA	Malt Extract Agar
MPOB	Malaysian Palm Oil Board
MPOC	Malaysian Palm Oil Council
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NaOH	Sodium Hydroxide
NRPS	Nonribosomal Peptide Synthetases
ORF	Open Reading Frame
PCP	Peptidyl Carrier Protein
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PheA	Phenylalanine activating A-domain
PKS	Polyketide Synthetase
Ppant	Phosphopantetheinyl prosthetic group
PPi	Pyrophosphate
R	Reductase
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
RWB	Rubber Wood Block
SD-CASA	Simple Double-layered CAS-agar
SOC	Super Optimal Broth
T	Thiolation
TAE	Tris-Acetate-EDTA
TAFC	Triacetylfusarinine C
TE	Thioesterase
tRNA	transfer RNA

CHAPTER 1

INTRODUCTION

Malaysian palm oil industry is one of the major contributors to the economic growth of the country and also to the development of the world palm oil market. Malaysia and Indonesia are producing 85% of the world's palm oil production (Sime Darby Plantation, 2014). The deadly disease for oil palm which is basal stem rot (BSR) caused by the pathogenic fungus, *Ganoderma boninense*, had reduced the yield of oil palm and affected the country's economic performance (Hushiarian *et al.,* 2013). Numerous efforts have been done to ensure the maximum yield of oil palm from practising good agriculture practices including effective formulation of pesticides, biological control and cultural control (include references). Nonetheless, these are only able to minimize the problem in oil palm plantations. To date, there is no cure for BSR disease and this has raised concerns among planters, researchers and those working closely in oil palm sector. Molecular approach has been actively uitilized to explore the fungal biological cycle, virulence factors and to study the interaction with its host. Hence, in-depth study of host plant-pathogenic fungi interactions at molecular level is important. One of the interesting scopes is the discovery of secondary metabolites produce by ~~of~~ fungal pathogens. A class of secondary metabolites of fungal origin that has gained much interest is the nonribosomal peptide synthetases (NRPSs). Several products of NRPSs that have been proven as virulence factors are enniatin which is required for the virulence of *Fusarium avenaceum* on potatoes, HC-toxin for *Cochliobolus carbonum* race 1 on corn and AM-toxin for *Alternaria alternata* on apple (Yoder and Turgeon, 2001). Some fungal NRPS products play crucial roles in plant-microbe relationship too (Oide *et al*., 2006). NRPS biosynthesize small peptides via ribosome independent machinery pathway and responsible for the biosynthesis of bioactive metabolites in bacteria and fungi (Stack *et al*., 2007). They were built up from several domains [adenylation (A), thiolation (T) or peptidyl carrier protein (PCP) and condensation (C) domains] which when grouped together are referred to as a single module. To date, the study of NRPSs has been mostly on filamentous ascomycete fungi and bacteria. No reports were found yet on NRPS produced by *G. boninense.*

Therefore, the objectives of this study were as below:

- To detect nonribosomal peptide synthetase (NRPS) of *Ganoderma boninense* using polymerase chain reaction (PCR).
- To clone and molecular characterize NRPS of *G. boninense*.
- To correlate the expression of *G. boninense* NRPS and basal stem rot disease development in oil palm seedlings.

As NRPSs are abundantly found in fungi, we hypothesized that there is at least one copy of NRPS in *G. boninense* with the conserved core motifs in A-domain and there is a relationship between the *G. boninense* NRPS and basal stem rot disease in oil palm seedlings.

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