UNIVERSITI PUTRA MALAYSIA

ISOLATION AND GENE EXPRESSION AT DIFFERENT GROWTH AND INFECTION STAGES OF Ganoderma boninense CYCLOPHILIN ENCODING cDNAS

LIM FOOK HWA

ITA 2013 6

ISOLATION AND GENE EXPRESSION AT DIFFERENT GROWTH AND INFECTION STAGES OF *Ganoderma boninense* CYCLOPHILIN ENCODING cDNAS

By

LIM FOOK HWA

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

November 2013

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

ISOLATION AND GENE EXPRESSION AT DIFFERENT GROWTH AND INFECTION STAGES OF *Ganoderma boninense* CYCLOPHILIN ENCODING cDNAS

By

LIM FOOK HWA

November 2013

Chairman: Associate Professor Ho Chai Ling, PhD

Faculty: Institute of Tropical Agriculture

Oil palm, the major crop planted in Malaysia, is subjected to various diseases such as Basal Stem Rot (BSR) disease. The disease is mainly caused by *Ganoderma boninense.* However, studies of the fungal infection mechanism and the biological processes involved are still very limited. Fungal cyclophilin (CYP) has been reported to be involved in the pathogenicity of fungi. However, the involvement of CYP in the pathogenicity of *G. boninense* has not been reported. The main objective of this study was to isolate cDNAs encoding CYPs and to profile the expression levels of these genes during different growth and infection stages in *G. boninense.* In this study, five full-length cDNAs encoding CYP have been successfully amplified by polymerase chain reaction (PCR) from *G. boninense*. They were classified as different family members of CYP because significant differences could be observed on their coding sequence and 5' or 3' un-translated regions (UTRs). An *in-vitro* infection test has also been developed by infecting six months old oil palm plantlets with clumps of *G. boninense* mycelium in a 250 ml flask incubated at 28 °C for a duration of eight weeks. Control samples were also prepared by growing either the fungus or the oil palm plantlet in a flask. The fungal samples were collected every two weeks. The infected samples were verified by dissecting the basal stem of the infected oil palm plantlets, detection of *Ganoderma* using *Ganoderma* Selective Media (GSM), PCR and confirmation of *Ganoderma* species with Multiplex PCR-DNA Kit. The findings indicated that *G. boninense* was detected in most of the infected plantlets. For real-time quantitative PCR (qPCR) optimization, a total of seven potential fungal reference genes were tested. *α-Tubulin, β–tubulin* and *eEF2* were found to be the most stable reference genes. The expression of five CYP genes in different types of fungal tissues and infecting mycelium tissues were studied using the qPCR approach and normalized with the reference genes above. Based on the expression patterns, the potential functions of the CYP transcripts were predicted to be involved in the development of basidiomata (GBcyp201), normal cell growth (GBcyp202), stress response (GBcyp203) and fungal pathogenicity (GBcyp205). This work provided genetic information on CYPs encoded by *G. boninense* and

predicted the functions of these CYPs especially in fungal pathogenicity which could be further studied and confirmed. This information may be essential in understanding the molecular infection pathway of *G. boninense*. Besides that, qPCR for *G. boninense* gene expression study has been optimized and this method could be used to study the expressions of other genes in *G. boninense*.

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

PEMENCILAN DAN PENGEKSPRESAN GEN UNTUK cDNA YANG MENGEKODKAN CYCLOPHILIN DARIPADA *Ganoderma boninense* PADA PERINGKAT PERTUMBUHAN DAN JANGKITAN YANG BERBEZA

Oleh

LIM FOOK HWA

November 2013

Pengerusi: Profesor Madya Ho Chai Ling, PhD

Fakulti: Institut Pertanian Tropika

Pokok sawit merupakan tanaman komoditi utama di Malaysia dan terdedah kepada pelbagai jenis penyakit seperti Penyakit Reput Pangkal (BSR). Penyakit ini biasanya disebabkan oleh *Ganoderma boninense*. Walau bagaimanapun, kajian mengenai mekanisma jangkitan kulat dan proses biologi yang terlibat adalah sangat terhad. Gen *cyclophilin* (CYP) daripada kulat telah dilaporkan terlibat dalam proses kepatogenan kulat-kulat yang lain. Namun begitu, penglibatan CYP dalam proses kepatogenan *G. boninense* masih belum dilaporkan lagi. Objektif utama kajian ini adalah untuk memencilkan cDNA yang mengekod CYP yang berbeza dan mengkaji corak pengekspresan gen-gen ini pada setiap peringkat pertumbuhan dan jangkitan yang berbeza di dalam *G. boninense*. Dalam kajian ini, lima jujukan penuh cDNA yang mengekod CYP yang berbeza telah berjaya dipencilkan melalui tindak balas berantai polimerase (PCR) daripada *G. boninense*. Jujukan ini telah diklasifikasikan sebagai ahli keluarga CYP yang berbeza kerana terdapat perbezaan yang ketara pada kawasan jujukan pengekodan dan kawasan tidak terjemah (UTR) 5 ' atau 3'. Satu ujian jangkitan *in-vitro* juga telah dibangunkan dengan menjangkiti anak pokok sawit berumur enam bulan dengan miselium *G. boninense*. Jangkitan telah dijalankan di dalam kelalang 250 ml dan dipelihara pada 28 °C untuk tempoh lapan minggu. Sampel kawalan juga disediakan dengan memelihara hanya kulat atau anak pokok sawit sahaja dalam satu kelalang. Sampel-sampel kulat dikumpul daripada kelalang jangkitan dan kawalan untuk setiap dua minggu. Selain itu, beberapa ujian pengesahan jangkitan termasuk pembedahan pangkal batang anak pokok sawit terjangkit, pengesanan *Ganoderma* melalui *Ganoderma Selective Medium* (GSM), kaedah PCR dan pengesahan spesies *Ganoderma* dengan menggunakan *Multiplex PCR-DNA Kit*. Hasil kajian menunjukkan bahawa *G. boninense* telah dikesan dalam kebanyakan anak pokok yang telah dijangkiti. Bagi pengoptimuman PCR kuantitatif masa-nyata (qPCR), sebanyak tujuh gen rujukan yang berpotensi telah diuji. Didapati *α-tubulin*, *β-tubulin* dan *eEF2* adalah gen-gen rujukan yang paling stabil. Kajian pengekspresan lima jujukan cDNA CYP dalam pelbagai jenis tisu dan tisu miselium yang sedang menjangkit telah dijalankan melalui pendekatan qPCR dan dinormalisasikan dengan gen-gen rujukan di atas. Berdasarkan corak pengekspresan,

fungsi-fungsi yang berpotensi untuk jujukan-jujukan CYP telah diramal terlibat dalam proses penghasilan basidiomata (GBcyp201), pertumbuhan sel normal (GBcyp202), tindak balas terhadap tekanan perubahan persekitaran (GBcyp203) dan kepatogenan kulat (GBcyp205). Kajian ini menyediakan maklumat genetik CYP yang dikodkan oleh *G. boninense* serta meramal fungsi CYP ini terutamanya dalam kepatogenan kulat yang mana boleh dikaji dengan lebih mendalam dan dibuktikan lagi. Maklumat ini mungkin penting dalam memahami tapak jalan molekul jangkitan *G. boninense*. Selain itu, qPCR untuk mengkaji pengekspressan gen *G. boninense* telah dioptimumkan dan kaedah ini boleh digunakan untuk mengkaji pengekspressan gen lain dalam *G. boninense*.

ACKNOWLEDGEMENTS

I would like to express my greatest gratitude to my main supervisor, Associate Professor Dr Ho Chai Ling, for her guidance, suggestions, patience, support and encouragement throughout my study in Universiti Putra Malaysia. I would also like to sincerely thank to Dr Omar Abd Rasid for his direct supervision in the lab, advices and suggestion in my research works. My thanks also go to other supervisory committee members, Dr Ahmad Parveez Ghulam Kadir and Dr Noor Azmi Shaharuddin for their advices, comments and suggestion whenever sought.

Besides that, I want to express my appreciation to Malaysian Palm Oil Board (MPOB) for the scholarship and financial support for me to complete my study. Special thanks to Datuk Dr Choo Yuen May (Director-General of MPOB) and Dr Ravigadevi a/p Sambanthamurthi (Director of ABBC) for allowing me to persuade my study in MPOB. I would also like to thank *Ganoderma* and Diseases Research for Oil Palm (GANODROP) Unit, MPOB for providing the *G. boninense* culture and performing the Multiplex PCR-DNA Kit test and Breeding and Tissue Culture Unit for supplying the oil palm plantlets. I would like to thank Pn. Nor Hanin Ayub, for her guidance on real-time qPCR analysis and to all the Transgenic Technology Group members for their kind assistance.

Finally, I would like to express my deepest appreciation to my father, Lim Boon Hooi and my mother, Kee Mooi Kheng for their long distance support and encouragement. To my beloved wife, Lu Hwei Fong, thank you for your encouragement for my study and kind understanding during my thesis preparation.

I certify that a Thesis Examination Committee has met on **19 November 2013** to conduct the final examination of **Lim Fook Hwa** on his thesis entitled "Isolation and gene expression at different growth and infection stages of *Ganoderma boninense* Cyclophilin encoding cDNAs " in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

Members of the Examination Committee were as follows:

Datin Siti Nor Akmar Abdullah, PhD
Professor
Institut Pertanian Tropika
Universiti Putra Malaysia
(Chairman)

Tan Soon Guan, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

Wong Mui Yun, PhD
Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Internal Examiner)

Latiffah Zakaria, PhD
Associate Professor
School of Biological Sciences
Universiti Sains Malaysia
Malaysia
(External Examiner)

NORITAH OMAR, PhD
Associate Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 21 January 2013

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 Committe were as follow:

Ho Chai Ling, PhD
Associate Professor
Institute of Tropical Agriculture
Universiti Putra Malaysia
(Chairman)

Noor Azmi Shaharuddin, PhD
Senior Lecturer
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

Omar Abd Rasid, PhD
Principle Research Officer
Advanced Biotechnology and Breeding Centre (ABBC)
Malaysian Palm Oil Board (MPOB)
(Member)

Ahmad Parveez Ghulam Kadir, PhD
Senior Principle Research Officer
Advanced Biotechnology and Breeding Centre (ABBC)
Malaysian Palm Oil Board (MPOB)
(Member)

_____
BUJANG BIN KIM HUAT, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date :

DECLARATION

I hereby confirm that:

- this thesis is my original work;
- quotations, illustrations and citations have been duly acknowledged;
- ownership of intellectual property from the thesis is as stipulated in the Memorandum of Agreement (MoA), or as according to the Universiti Putra Malaysia (Research) Rules 2012, in the event where the MoA is absent;
- permission from supervisor and the office of Deputy Vice-Chancellor (Research and Innovation) are required prior to publishing it (in the form of written, printed or in electronic form) including books, journals, modules, proceedings, popular writings, seminar papers, manuscripts, posters, reports, lecture notes, learning modules or any other materials as stated in the Universiti Putra Malaysia (Research) Rules 2012;
- there is no plagiarism or data falsification/fabrication in the thesis, and scholarly integrity is upheld as according to the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) and the Universiti Putra Malaysia (Research) Rules 2012. The thesis has undergone plagiarism detection software.

Signature: _____ Date: _____

Name and Matric No: LIM FOOK HWA (GS 28633)

Declaration by Members of Supervisory Committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) are adhered to.

Signature: _____ Signature: _____

Assoc. Prof. Dr. Ho Chai Ling Dr. Omar Abd Rasid

Signature: _____ Signature: _____

Dr. Noor Azmi Shaharuddin Dr. Ahmad Parveez Ghulam Kadir

TABLE OF CONTENTS

		Page
ABSTRACT		iii
ABSTRAK		v
ACKNOWLEDGEMENTS		vii
APPROVAL		viii
DECLARATION		x
LIST OF TABLES		xiv
LIST OF FIGURES		xv
LIST OF ABBREVIATIONS		xviii
CHAPTER		
1	**INTRODUCTION**	1
2	**LITERATURE REVIEW**	
	2.1 Oil Palm	3
	2.1.1 Origin and History	3
	2.1.2 The Biology of Oil Palm	3
	2.1.3 Economic Importance of Oil Palm	4
	2.2 Basal Stem Rot (BSR) Disease	4
	2.2.1 The History	4
	2.2.2 Disease Symptoms	5
	2.2.3 Disease Epidemiology	6
	2.2.4 Causal Agent of BSR Disease	7
	2.2.5 Detection of BSR Disease	7
	2.2.6 Control of BSR Disease	8
	2.3 *Ganoderma boninense*	9
	2.3.1 Ganodermataceae and *Ganoderma*	9
	2.3.2 Morphological Features of *Ganoderma*	10
	2.3.3 Life cycle of *Ganoderma*	14
	2.4 Interaction of Plant-pathogenic Fungi	15
	2.4.1 Pathogenicity Genes	16
	2.5 Cyclophilins (CYP)	17
	2.5.1 CYP in Fungal Pathogenicity	19
3	**MATERIALS AND METHODS**	
	3.1 Preparation of *G. boninense* Culture	21
	3.2 Isolation of cDNAs Encoding CYP from *G. boninense*	21
	3.2.1 Analysis of Sequences Encoding Fungal *cyp* and Design of Degenerate Primers	21
	3.2.2 Total RNA Extraction from *G. boninense* Tissue	22
	3.2.3 Synthesis of First-strand cDNA from *G. boninense* Total RNA	23
	3.2.4 PCR Amplification of Partial-length cDNAs of	23

cyp
3.2.5 Gel Electrophoresis of PCR Products 24
3.2.6 Purification of PCR Products 24
3.2.7 Extraction of Plasmid DNA from Representative Clones 25
3.2.8 Restriction Enzyme Analysis 25
3.2.9 DNA Sequencing and Analysis of Representative Clones 25
3.2.10 Primers Design Based on Partial-length cDNA Sequences Encoding Different Family Members of CYPs 26
3.2.11 PCR Amplification of 5' and 3' end cDNAs Encoding CYPs 26
3.2.12 End-to-end PCR Amplification of Full-length cDNAs Encoding CYPs 27
3.3 Artificial *In-vitro* Infection Test
3.3.1 Preparation of *G. boninense* Culture 28
3.3.2 Preparation of Oil Palm Plantlets 28
3.3.3 Preparation of Infection Media 28
3.3.4 *In-vitro* Infection Test 28
3.3.5 Collection of *G. boninense* Samples 29
3.3.6 Verification of infected oil palm samples 29
3.4 Quantitative Real-time PCR (qPCR) Analysis
3.4.1 Selection of Potential Reference Genes 30
3.4.1.1 Identification of Potential Reference Genes and Design of Degenerate Primers 30
3.4.1.2 PCR Amplification of Partial-length cDNAs Encoding Potential Reference Genes 31
3.4.1.3 Cloning and Sequencing of PCR Fragments 31
3.4.2 Design of qPCR Primers 31
3.4.3 Extraction of Total RNA from Different Types of *G. boninense* Tissues 32
3.4.4 Estimation of Quantity and Integrity of Total RNA 33
3.4.5 Reverse Transcription of RNA into cDNA 34
3.4.6 qPCR Efficiency Test 34
3.4.7 Determination of the Most Suitable Reference Genes 35
3.4.8 qPCR Gene Expression Analysis 36

4 RESULTS
4.1 Cloning of Full-length cDNAs Encoding CYPs 37
4.1.1 PCR Amplification of Partial-length cDNAs Encoding CYPs 37
4.1.2 DNA Sequencing of Representative Clones 38
4.1.3 Isolation of Full-length cDNAs Encoding CYPs 40
4.1.4 DNA Sequencing of Representative Clones 40
4.1.5 End-to-end PCR Amplification of Full-length cDNAs Encoding CYPs 42
4.2 *In-vitro* Artificial Infection of Oil Palm Plantlets 56
4.2.1 Verification of *In-vitro* Infection of Oil Palm 58

Plantlets
4.3 RT-qPCR Expression Analysis 61
4.3.1 Isolation of Partial-length cDNA Encoding Potential Reference Genes 61
4.3.2 Extraction and Purification of Total RNA from Different *G. boninense* Tissues 63
4.3.3 qPCR Efficiency Test 64
4.3.4 Determination of Suitable Reference Genes for qPCR Analysis 65
4.3.5 qPCR Gene Expression Analysis 68

5 DISCUSSION
5.1 Isolation of cDNAs Encoding CYPs from *G. boninense* 73
5.2 Artificial *In-vitro* Infection Test 73
5.3 Gene Expression Analysis with qPCR 75
5.3.1 Selection of Reference Genes for qPCR Analysis 76
5.3.2 Gene Expression Analysis of CYP with qPCR 76

6 CONCLUSION AND RECOMMENDATIONS FOR FUTURE RESEARCH 80

REFERENCE 82
APPENDICES 93
BIODATA OF STUDENT 121
LIST OF PUBLICATIONS 122