UNIVERSITI PUTRA MALAYSIA

RNAi-MEDIATED GENE SILENCING OF LANOSTEROL 14α-DEMETHYLASE (ERG11)-ENCODING GENE IN Ganoderma boninense

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By

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May 2022

Chair : Professor Wong Mui Yun, PhD
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Oil palm basal stem rot (BSR) disease is caused by several species of *Ganoderma* including *Ganoderma boninense*. Available molecular tools for *G. boninense* are essential for providing the knowledge on the fungal infection process in oil palm. The objectives of this study are to isolate the full-length cDNA encoding Lanosterol 14α-demethylase (*GbERG11*) from *G. boninense*, to optimize a polyethylene glycol (PEG)-mediated protoplast transformation protocol for *G. boninense*, to perform the *GbERG11* gene functional study via RNAi-mediated gene silencing approach and to transform the hpRNA-*GbERG11* vectors into oil palm. A full-length 1980 bp cDNA encoding *GbERG11* was successfully isolated and the GbERG11 shared high similarity (91%) to ERG11 from other basidiomycete fungi. Southern blot and genome data analyses indicated that there is only a single copy of *GbERG11* gene in the *G. boninense* genome. An average concentration of 10^7/ml viable protoplasts were successfully isolated from *G. boninense* mycelium. The *G. boninense* PEG-mediated protoplast transformation using 1 µg of transformation vector, 25% of PEG solution, 10 min of pre-transformation incubation and 30 min of post-transformation incubation has improved the transformation efficiency by 33.5 folds on average. Three hpRNA vectors corresponding to different regions of *GbERG11* were prepared using the *in vitro* recombination between the entry vectors (containing different target regions of *GbERG11*) and hpRNA vector, pH7GWIWG2(I). The *G. boninense* transformed with the hpRNA vectors have shown reduced growth, expression of *ERG11* and ergosterol content as much as 57.3%, 32.9% and 42.9%, respectively as compared to the PER71 (wild type). Less severe infection symptoms were observed on oil palm plantlets inoculated with *G. boninense* transformants as compared to the *G. boninense* PER71 (wild type) in the initial stages of *in vitro* inoculation study. Besides that, particle bombardment of oil palm calli with the hpRNA vectors (*GbERG11*) was performed. The selection process has led to regeneration of 8 putative transgenic greenish polyembryoids. As a conclusion, a gene function study for *G. boninense* has been successfully developed by using the PEG-mediated

protoplast transformation and RNAi-mediated gene silencing approaches. Reduced *GbERG11* gene expression followed by reduced ergosterol content was observed in the *G. boninense* transformants indicating the functional of this molecular tool. The *G. boninense* transformants have showed reduced pathogenicity towards oil palm at the initial stages of the *in vitro* inoculation study, which could highlight the *GbERG11* gene role at the initial stage of infection. The developed molecular tools in this study can be applied for studying other genes in *G. boninense*, as well as other *Ganoderma* species or basidiomycete fungi. The putative transgenic oil palm plantlets can be further evaluated especially on the resistance against *G. boninense*, which could indicate the potential application of RNAi-mediated gene silencing in protecting oil palm against the *Ganoderma* infection.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENYENYAPAN GEN BERPERANTARAAN-RNAi UNTUK GEN YANG MENGEKOD LANOSTEROL 14α-DEMETHYLASE (*ERG11*) DI DALAM *Ganoderma boninense*

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Penyakit reput pangkal pokok sawit (BSR) disebabkan oleh beberapa spesies *Ganoderma* termasuk *Ganoderma boninense*. Penyediaan kaedah molekul untuk *G. boninense* adalah penting untuk menjana pengetahuan mengenai proses jangkitan kulat di dalam pokok sawit. Objektif-objektif kajian ini adalah untuk mengasingkan jujukan penuh cDNA yang mengekod Lanosterol 14α-demethylase (*GbERG11*) daripada *G. boninense,* untuk mengoptimumkan protokol transformasi protoplas berperantaraan polietilena glikol (PEG) untuk *G. boninense*, untuk menjalankan kajian fungsi gen *GbERG11* melalui kaedah penyenyapan gen berperantaraan RNAi dan untuk mentransformasikan vector-vektor hpRNA-*GbERG11* ke dalam pokok sawit.Satu jujukan-penuh 1980 pb cDNA yang mengekod *GbERG11* telah berjaya dipencilkan dan GbERG11 mempunyai kesamaan jujukan yang tinggi (91%) berbanding dengan ERG11 daripada kulat basidiomycete lain. Analisis pemblotan Southern dan data genom menunjukkan hanya terdapat satu salinan gen *GbERG11* di dalam genom *G. boninense*. Protoplas yang subur pada purata kepekatan 10^7 /ml telah berjaya diasingkan daripada miselium *G. boninense*. Transformasi protoplas *G. boninense* berperantaraan PEG dengan menggunakan 1 µg vektor transformasi, 25% larutan PEG, 10 minit inkubasi pra-transformasi dan 30 minit inkubasi pasca-transformasi telah berjaya meningkatkan kecekapan transformasi sebanyak 33.5 kali ganda secara purata. Tiga vektor hpRNA yang mensasarkan kawasan jujukan *GbERG11* yang berlainan telah disediakan dengan menggunakan kaedah rekombinasi *in vitro* antara vektor *Entry* (yang mengandungi kawasan sasaran *GbERG11* yang berbeza) dan vektor hpRNA, pH7GWIWG2 (I). *G. boninense* yang terubahsuai dengan vector-vektor hpRNA telah menunjukkan penurunan kadar pertumbuhan, pengekspresan gen *ERG11* dan kandungan ergosterol sebanyak 57.3%, 32.9% dan 42.9%, masing-masing

berbanding dengan *G. boninense* PER71 (jenis liar). Gejala jangkitan yang kurang serious telah diperhatikan pada anak pokok sawit yang diinokulasi dengan transforman *G. boninense* berbanding dengan *G. boninense* PER71 (jenis liar) pada peringkat awal kajian inokulasi *in vitro.* Selain itu, pengeboman zarah kalus sawit dengan vektor hpRNA (*GbERG11*) telah dilakukan. Proses pemilihan telah membawa kepada regenerasi 8 poliembrioid hijau transgenik putatif. Sebagai kesimpulan, satu kajian fungsi gen untuk *G. boninense* telah berjaya dibangunkan dengan menggunakan kaedah transformasi protoplas berperantaraan polietilena glikol (PEG) dan penyenyapan gen berperantaraan RNAi. Pengurangan pengekspresan gen *GbERG11* disusuli dengan pengurangan kandungan ergosterol telah diperhatikan di kalangan transforman *G. boninense* menunjukkan kefungsian alat molekul ini. Transforman *G. boninense* telah menunjukkan pathogenesis yang kurang terhadap pokok sawit pada peringkat awal kajian inokulasi *in vitro,* di mana mungkin menunjukkan peranan gen GbERG11 pada peringkat awal jangkitan. Alat molekul yang terbangun dalam kajian ini boleh digunapakai untuk mengkaji gen-gen yang lain di dalam *G. boninense*, termasuk spesis *Ganoderma* ataupun kulat basidiomycete yang lain. Anak pokok sawit transgenik putatif boleh dinilai lebih lanjut terutamanya atas kerintangan terhadap *G. boninense*, di mana boleh menunjukkan potensi aplikasi penyenyapan gen berperantaraan RNAi dalam melindungi pokok sawit daripada jangkitan *Ganoderma*.

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LIST OF ABBREVIATIONS

3-MCPDE	Monochloropropane-1, 2-diol esters
5-CFDA	5-Carboxyfluorescein Diacetate
ABBC	Advanced Biotechnology and Breeding Centre
ACTT2	ACT-toxin
AGO	Argonaute
ATMT	*Agrobacterium tumefaciens*-mediated Transformation
ATP	Adenosine triphosphate
bar	bialaphos
BLAST	Basic Local Alignment Search Tool
BLASTN	Basic Local Alignment Search Tool (nucleotide-nucleotide)
BLASTP	Basic Local Alignment Search Tool (protein-protein)
BLASTX	Basic Local Alignment Search Tool (nucleotide-protein)
bp	base pair
BSR	basal stem rot
$CaCl_2$	Calcium cloride
CaMV	Cauliflower mosaic virus
cDNA	complementary DNA
CHS	chalcone synthase
CNB	calcineurin B
CRISPR/Cas9	Clustered regularly interspaced short palindromic repeats*/* CRISPR-associated protein 9

CSPD	Disodium 3-(4-methoxyspiro {l,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.13,7]decan}-4-yl) phenyl phosphate
CTAB	cetyl trimethylammonium bromide
CWDEs	cell wall degrading enzymes
CYM	Complete Yeast Medium
CYP	Cytochromes P450
CYP51	cytochrome P450 lanosterol C14α-demethylase
DIECA	Sodium diethyldithiocarbamate
DMIs	Demethylase inhibitors
DMSO	Dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
dpi	days post inoculation
DSB	double-strand breaks
DSI	disease severity index
dsRBP	dsRNA-binding protein
dsRNA	double stranded RNA
DTT	Dithiothreitol
EDR1	enhanced disease resistance1
EDTA	Ethylenediaminetetraacetic acid
EgBGIA	protease inhibitor
EgCht	chitinase
EgEXPB18	expansin
eGFP	enhanced green fluorescent protein
EgLYK3	PRR protein

EgPR-1	PR-protein
EEO	electroendoosmosis
ERG11	Lanosterol 14α-Demethylase
EST	expressed sequence tag
EU	European Union
ftf1	*Fusarium* transcription factor 1
GanoID	*Ganoderma* Collection and Information Database
GbERG11	*Ganoderma boninense* Lanosterol 14α-demethylase
GE	Glycidyl esters
GM	genetically modified
GHG	greenhouse gases
gpd	glyceraldehyde-3-phosphate dehydrogenase
gusA	beta-glucuronidase
HIGS	Host Induced Gene Silencing
H_2O_2	Hydrogen peroxide
HPLC	High Performance Liquid Chromatography
hpRNA	hairpin RNA
hpt	hygromycin B phosphotransferase
HR	homologous recombination
inHg	Inch of mercury
IPTG	Isopropyl β-D-1-thiogalactopyranoside
ID	identification
JA	oil palm jasmonate
Jacq.	Jacquin
JGI	Joint Genome Institute

kb	kilo base
LB	Luria-Bertani
MAPK	MAP kinase
MgSO4	Magnesium sulfate
MIC	Minimum inhibitory concentration
miRNAs	microRNAs
Mlo	MILDEW RESISTANT LOCUS O
MM	Mannitol and Na malate
MMC	Mannitol, Na malate and $CaCl_2$
MPOB	Malaysian Palm Oil Board
MPK	mitogen activated protein kinase
mRNA	Messanger RNA
MS	Murashige and Skoog
NAA	α-Naphtaleneacetic acid
NaFeEDTA	Ferric sodium EDTA
NCBI	National Centre for Biotechnology Information
NEP	necrosis- and ethylene inducing protein
NHEJ	non-homologous end joining
NH_4Ac	Ammonium acetate
NTC	Non template control
OD	Optical density
ORF	Open reading frame
OsERF922	rice transcription factor encoding gene
PAMPs	Pathogen-associated molecular patterns
PCR	Polymerase chain reaction

PDA	Potato dextrose agar
PDB	Potato dextrose broth
PDR	parasite-derived resistance
PEG	polyethylene glycol
PIRG	percentage inhibition of radial growth
piRNAs	PIWI-interacting RNAs
PRRs	Pattern recognition receptors
PSB	Plant Systems Biology
psi	Pounds per square inch
PTGS	post-transcriptional gene silencing
PTI	PAMP-triggered immunity
PVP	Polyvinylpyrrolidone
qPCR	Real-time quantitative PCR
RACE	Rapid Amplification of cDNA Ends
RdRP	RNA-dependent RNA polymerase
REMI	restriction enzyme-mediated integration
RFP	red fluorescent protein
RIN	RNA integrity number
RISC	RNA-induced silencing complex
RLC	RISC-loading complex
RM	Ringgit Malaysia
RNA	Ribosomal ribonucleic acid
RNAi	RNA interference
RNAP	RNA Polymerase
ROS	reactive oxygen species

SA	oil palm salicylate
SDS	Sodium dodecyl sulfate
siRNAs	small interfering RNAs
SPAD	Soil Plant Analysis Development
sRNA	small RNAs
SRS	Substrate Recognition Site
SSC	saline-sodium citrate
ssRNA	single-stranded RNA
TAE	Tris-acetate-EDTA
TALENS	Transcription Activator Like Effector Nucleases
TE	Tris EDTA
T-DNA	transfer-DNA
TGS	Transcriptional gene silencing
Ti	Tumour-inducing
Tris-HCL	Tris-Hydrochloride
U	enzyme unit
UPM	universal primer mix
URA3	orotidine 5'-monophosphate decarboxylase
USR	upper stem rot
UTR	untranslated region
UV	ultraviolet
xg	Times gravity
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
VIB	Vlaams Instituut voor Biotechnologie

vir	virulence
WAK	cell wall-associated kinase
wpi	weeks post inoculation
w/v	Weight/volume
ZFNs	Zinc Finger Nucleases

CHAPTER 1

INTRODUCTION

Oil palm (*Elaeis guineensis* Jacq.) is an important oil-bearing crop which has contributed to about 40% of global vegetable oil output by only utilizing 0.4% of the worldwide agricultural land (Jackson *et al*., 2019). Palm oil has been mainly used as edible product and important raw material in non-food industries ranging from high value oleo chemicals to production of biodiesel (Parveez *et al*., 2021). Oil palm can also supply basic biomass-derived material for production of paper and plywood (Sulaiman *et al*., 2012).

The Malaysian oil palm industry currently faces several challenges including limitation of land for oil palm cultivation area expansion, stagnant of national oil palm average yield production, climate change, shortage of labour and pest and diseases (Fry, 2009; Murphy, 2014; Alam *et al*., 2015; Kushairi *et al*., 2019). Some of the challenges could be overcame through implementation of new government policy, modification of the palm oil processing procedure and improve good agricultural practice. However, for the challenges such as pest and diseases, comprehensive study on the pest or pathogen is necessary to ensure effective control measures could be implemented.

Oil palm Basal stem rot (BSR) disease is caused by a few species of *Ganoderma* and has obtained alarming attention in Southeast Asia especially Malaysia and Indonesia. In addition, the BSR is also becoming an emerging disease in Colombia, which is another palm oil producing country in South America (Castillo *et al*., 2022). The disease was initially reported in Malaya in 1931 (Thompson, 1931). The first oil palm plantation was setup in Malaya in 1917 at the Tenammaran Estate, Kuala Selangor and oil palm cultivation area has increased significantly in the 1960s due to the Malaysian government crop diversification programme. The oil palm BSR was only found on the older palms initially, which are least economic important. However, during the last 30 years, the BSR started to be observed on younger palms, as young as 1-2 years after planting (Singh, 1991). As a result of the disease incidence, as much as 80% of palms could be killed when they are only half way of their economic lifecycle. The 10.3% of BSR infection rate could cause an estimated loss of RM25.0 billion to the Malaysian oil palm industry by 2045 (Olaniyi and Szulczyk, 2020). A recent BSR survey in Malaysian oil palm estates showed increased disease incidence from 1.5% (1995) to 7.4% (2017) (Idris *et al*., 2019).

Besides BSR, oil palm upper stem rot (USR) incidences were also reported in Malaysia, Indonesia and Papua New Guinea (Rakib *et al*., 2014; Rees *et al*., 2012; Pilotti, 2005). Unlike BSR, the *Ganoderma* infection was occurred on the upper part of the oil palm stem or trunk and the airborne-basidiospores or the *Ganoderma* inoculum that grew on dead material (produced during the

harvesting process) could be the causal agents of the USR (Hasan *et al*., 2005; Nur-Rashyeda *et al*., 2021). *G. zonatum* has been identified as the primary pathogen for oil palm USR, follow by *G. boninense* and *G. miniatocinctum* (Rakib *et al*., 2015).

Various efforts have been initiated for detection and control of the oil palm BSR. The disease control principles aimed to minimize the disease incidence in the replanting area, prolong the productive life of the disease palms and delay the progress of *Ganoderma* infection (Sariah *et al.*, 2011). Besides that, several approaches have been initiated with the aim at understanding the disease and pathogen at the molecular level. The works include *Ganoderma* genome sequencing (Utomo *et al*., 2018), transcriptomic and metabolomic data analyses during the oil palm-*Ganoderma* interaction (Zain *et al.*, 2013; Ho *et al.*, 2016) and isolation of potential pathogenicity genes from *G. boninense* (Rasid *et al.*, 2014; Lim *et al.*, 2017; Teh *et al*., 2019).

However, there is still lacking of molecular methods for gene functional study for *Ganoderma* to complement the current available molecular studies. The gene function protocol is important to validate the vast molecular data generated from the transcriptomic and metabolomic analyses especially those that are involved in the *Ganoderma* pathogenicity and interaction with oil palm (Ho *et al*., 2016). Gene function study provides in depth understanding of the candidate gene/metabolome which could then highlight the interaction and infection pathway of pathogen in the host (Zhang *et al*., 2018). The generated knowledge is important to understand the pathogen and design effective BSR control measures for oil palm in future (Wouw and Howlett, 2011; Ho *et al*., 2016).

Lanosterol 14α-Demethylase (ERG11) is an abundant hemease superfamily and involves in the ergosterol biosynthetic pathway in fungi (Zhang *et al*., 2019). Ergosterol is an important component of the fungal cell membrane (Barrett-Bee and Dixon, 1995; Veen and Lang, 2005). For decades, fungal ERG11 has been targeted for disease control purpose by using Azole-group fungicides (Hof, 2001; Sheng *et al.*, 2009). For oil palm BSR, the Azole group fungicides were found effective to inhibit the *Ganoderma* growth in oil palm and prolong the diseased palm lifespan (Idris *et al*., 2002; 2004). *Ganoderma* ergosterol was used to quantify the progression of the oil palm BSR disease in a number of studies (As'wad *et al*., 2011; Chong, 2012; Muniroh *et al*., 2014). In a transcriptomic study, *Ganoderma ERG11* transcript was found in the oil palm inoculated with *G. boninense* (Ho *et al*., 2016). It is hypothesized that the *G. boninense ERG11* and ergosterol could play essential role during the oil palm infection process and downregulation or gene silencing of the gene will reduce the ability of the fungus to colonize oil palm. However, the molecular information of *G. boninense ERG11* (*GbERG11*) and its gene function study are still unavailable until now.

This study was aimed at establishing a molecular tool via RNAi-mediated gene silencing approach for gene function study in *G. boninense*. The *ERG11* from *G. boninense* was used as a model gene in this study. Gene function study of

ERG11 in *G. boninense* required a reliable *G. boninense* genetic transformation protocol to ensure successful transformation and integration of the vector construct into the *G. boninense* genome. An RNAi-mediated gene silencing approach was used to downregulate the expression of *ERG11* and the consequence in *G. boninense* in term of growth appearance, expression level and pathogenicity towards oil palm was studied. The potential hairpin RNA (hpRNA) vectors were then transformed into oil palm in the effort to generate resistant oil palm towards *Ganoderma* and BSR. The main objectives of this study are:

a. To isolate and characterize the cDNA encoding *ERG11* from *G. boninense.*
b. To develop and optimize a polyethylene glycol (PEG)-mediated protoplast transformation system for *G. boninense.*
c. To determine the efficacy of RNAi-mediated gene silencing in *G. boninense* transformants.
d. To determine the capability of the hpRNA vector(s) to transform into oil palm.

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