MOLECULAR CLONING AND CHARACTERIZATION OF PUTATIVE VIRULENCE FACTORS OF *Ganoderma boninense*

By

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

December 2023

FBSB 2023 8

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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Chairman : Professor Ho Chai Ling, PhD
Faculty : Biotechnology and Biomolecular Sciences

Ganoderma boninense is a white rot fungus which causes basal stem rot (BSR) disease in oil palm. BSR is responsible for yield loss to the oil palm industry, which is the major economic growth driver of the country. To date, the existing solutions for the disease are not effective and the virulence factors responsible for the disease occurrence are understudied. The objectives of this study are to isolate the transcripts for putative virulence factors from *G. boninense*; to profile their gene expressions during fungal growth and plant-pathogen interactions; and to predict the transcription factor binding sites (TFBS) at the promoter sequence of selected putative virulence factors across different *Ganoderma* species. Four transcripts were selected from a previous study with the following criteria: i. present in the *G. boninense*-treated oil palm root transcriptomes (absent in control), ii. present in whole genome sequence of *G. boninense*, iii. encodes for secreted protein, and iv. has homologs involved in plant pathogenesis. This is the first report on the characterisation of metalloproteinase (*U14090*), aspartic protease (*U42611*), polysaccharide deacetylase (*U128397*), and lipase (*U56931*) as candidate virulence factors from *G. boninense*. The full-length cDNAs of the candidates were isolated from *G. boninense* BLSM5B, cloned, and verified by sequencing. Expression of these genes in mycelial cultures harvested at different time-points (7-, 14-, 21-, and 28-days after inoculation (dai) in potato dextrose broth), upon direct contact with host, and treatment with elicitors (i.e., salicylic acid, jasmonic acid, and hydrogen peroxide), were analysed by real-time PCR (qPCR). The gene expression of *U56931* was up-regulated in mycelial cultures at 21- and 28-dai in comparison to that at 7-dai, whereas the expression of *U42611* was down-regulated in mycelial cultures at 28-dai in comparison to that at 7-dai. Salicylic acid which is involved in plant biotic stress response was shown to be able to down-regulate the gene expression of *U56931*. The expression of these candidate genes was not responsive to elicitation of jasmonic acid, and hydrogen peroxide although jasmonic acid, and hydrogen peroxide also mediate plant defence signalling and

respiratory burst oxidation, respectively. On the other hand, the gene expression of *U14090* was up-regulated in *G. boninense* in contact with oil palm roots for 24- and 48-hours compared to the uninoculated control. In addition, common and unique TFBS in the promoter regions of candidate genes in selected *Ganoderma* species were predicted and compared. The findings provided an insight into the expression of putative virulence factors during plant-pathogen interactions. This information is important for gene-targeted study in the development of effective cure against *G. boninense* to minimize yield loss of the oil palm industry.

Keywords: *Ganoderma*; plant pathogen interactions; basal stem rot; basidiomycete; oil palm

SDG: GOAL 15: Life on Land

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

PENGKLONAN DAN PENCIRIAN MOLEKUL FAKTOR VIRULEN PUTATIF DARIPADA *Ganoderma boninense*

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Ganoderma boninense merupakan kulat pereput putih yang menyebabkan penyakit reput pangkal batang (BSR) pokok kelapa sawit. BSR mengakibatkan kerugian kepada industri kelapa sawit yang merupakan pemacu utama pertumbuhan ekonomi negara. Setakat ini, penyelesaian yang sedia ada untuk penyakit tersebut adalah kurang berkesan dan faktor virulen yang menjadi punca penyakit tersebut masih kurang difahami. Objektif kajian ini adalah untuk memencilkan transkrip untuk faktor virulen putatif daripada *G. boninense*; memprofil pengekspresan gen tersebut semasa pertumbuhan kulat serta interaksi patogen dengan tumbuhan; dan meramal tapak pengikatan faktor transkripsi (TFBS) yang terletak di promoter faktor virulen putatif yang terpilih daripada spesies *Ganoderma* yang berbeza. Empat transkrip telah dipilih daripada kajian terdahulu berdasarkan kriteria berikut: i. wujud dalam transkriptom akar kelapa sawit yang dirawat dengan *G. boninense* (tidak wujud dalam kawalan), ii. wujud di jujukan genom keseluruhan *G. boninense*, iii. mengekod protein rembesan, dan iv. mempunyai homolog yang terlibat dalam patogenesis tumbuhan. Kajian ini merupakan laporan pertama pencirian metaloproteinase putatif (*U14090*), protease aspartat putatif (*U42611*), deasetilase polisakarida putatif (*U128397*), dan lipase putatif (*U56931*) sebagai calon faktor virulen daripada *G. boninense*. Jujukan lengkap cDNA bagi calon telah dipencilkan daripada *G. boninense* BLSM5B, diklonkan, dan disahkan melalui penjujukan. Pengekspresan gen tersebut dalam kultur miselia pada titik masa penuaian yang berbeza (7-, 14-, 21-, dan 28-hari selepas inkubasi (dai) dalam kaldu dekstrosa kentang), selepas pendedahan kontak terus dengan perumah, dan rawatan dengan elisitor (asid salisilik, asid jasmonik, dan hidrogen peroksida), telah dianalisis dengan tindakbalas berantai polimerase-masa nyata (qPCR). Pengekspresan gen *U56931* telah dipertingkatkan pada 21- dan 28-dai berbanding dengan 7-dai, manakala pengekspresan *U42611* telah dikurangkan berbanding dengan 7-dai. Asid salisilik yang terlibat dalam tindak balas tekanan biotik tumbuhan telah mengurangkan pengekspresan gen *U56931*.

Pengekspresan calon gen tidak menunjuk tindak balas terhadap asid jasmonik dan hidrogen peroksida walaupun asid jasmonik dan hidrogen peroksida memperantarakan signal pertahanan tumbuhan dan ledakan oksidatif respiratori masing-masing. Selain itu, pengekspresan gen *U14090* telah dipertingkatkan dalam miselia *G. boninense* yang berkontak terus dengan akar kelapa sawit pada 24- dan 48-jam selepas inokulasi berbanding dengan kawalan yang tidak diinokulasi. Di samping itu, TFBS umum dan unik di kawasan promoter calon gen daripada spesies *Ganoderma* yang berbeza telah diramal dan dibandingkan. Hasil kajian ini telah memberi permahaman terhadap pengekspresan faktor virulen putatif daripada *G. boninense* semasa interaksi patogen dengan tumbuhan. Maklumat ini adalah penting untuk kajian bersasarkan gen bagi pembangunan rawatan yang berkesan terhadap *G. boninense* agar dapat mengurangkan kerugian hasil industri kelapa sawit.

Kata Kunci: *Ganoderma*; interaksi patogen dengan tumbuhan; reput pangkal batang; basidiomiset; kelapa sawit

SDG: MATLAMAT 15: Kehidupan Darat

ACKNOWLEDGEMENTS

I am deeply indebted to Prof. Dr. Ho Chai Ling for accepting me into the programme. It is an honour to be supervised by Prof. Ho for her dedication and expertise, in guiding me through this study to get the work done in meticulous detail. I would also like to thank Assoc. Prof. Dr. Mohd Termizi Yusof for his constructive kind advice during the project initiation. Sincere thanks go to Datu Dr. Lulie Melling FASc., for her full support throughout this study. This study wouldn't have been possible without her approval and encouragement.

I would like to acknowledge the Sarawak Government for the research funding and Ta Ann Holdings for the education sponsorship. Also, I would like to thank members of the Sarawak TROPI Molecular and Microbiology Research Division for their guidance, advice, and understanding. Their dedication and perseverance have laid the foundation for current research facilities. Thank you, Simon, for picking up oil palm seedlings for me. My sincere gratitude to the interns: Jee Jing and Lai Hui Ting for wrapping up my work in 2019; Matthew Lim Juan Hock and Au Wen Shen for helping in RNA sample extraction and quantification in 2021.

I would like to extend my appreciation to members of UPM FBSB Biotech 3 Molecular Biology Lab, Mu'azr Amer Hamzah, Chan Kok Yan, and Nur Aini Mohd Kassim, for their guidance and friendship. Especially Mu'azr for his kind help in facilitating communication throughout the study. To all the kind people who have accompanied me on this journey, I can't thank you enough for your kindness and bringing sunshine into this journey.

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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X-Gal	5-bromo-4-chloro-3-indoly1-β-D-galacto-pyranoside
A230	absorbance at 230 nm
A260	absorbance at 260 nm
A280	absorbance at 280 nm
T_a	annealing temperature
BSR	basal stem rot
bp	base pair
BLAST	Basic Local Alignment Search Tool
$CaCl_2$	calcium chloride
CE	carbohydrate esterase family
CWDE	cell wall degrading enzyme
CABI	Centre for Agriculture and Biosciences International
CTAB	cetyltrimethylammonium bromide
CoPN	Code of Good Nursery Practice for Oil Palm Nurseries
CDS	coding sequence
cDNA	complementary DNA
CDD	Conserved Domain Database
COX	cytochrome c oxidase
dai	day after inoculation
°C	degree celsius
dATP	deoxyadenosine triphosphate
dNTP	deoxynucleotide triphosphate
DNase	deoxyribonuclease
DNA	deoxyribonucleic acid
ETI	effector-triggered immunity

eef2	elongation factor 2
ET	ethylene
EDTA	ethylenediaminetetraacetic acid
EU	European Union
FFB	fresh fruit bunch
hpi	hour post inoculation
H_2O_2	hydrogen peroxide
HR	hypersensitive response
ISR	induced systemic resistance
IQ	integrity and quality
IPTG	isopropyl β-D-1-thiogalactopyranoside
JA	jasmonic acid
LB	Luria-Bertani
$MgCl_2$	magnesium chloride
MSPO	Malaysian Sustainable Palm Oil Certification Scheme
MEA	malt extract agar
MEB	malt extract broth
T_m	melting temperature
MAMPs	microbial-associated molecular patterns
NCBI	National Center for Biotechnology Information
NEP	necrosis and ethylene inducing protein
NTC	no template control
nr	non-redundant
NB-LRR	nucleotide-binding and leucine-rich repeat
PTI	PAMP-triggered immunity
PAMPs	pathogen-associated molecular patterns
PHI-base	pathogen-host interactions database

PRR	pattern recognition receptor
PCI	phenol: chloroform: isoamyl alcohol
PCR	polymerase chain reaction
PVPP	polyvinylpolypyrrolidone
PDB	potato dextrose broth
Cq	quantification cycle
RAMS	randomly amplified microsatellites
ROS	reactive oxygen species
qPCR	real-time PCR
R	resistance
RT-PCR	reverse transcription polymerase chain reaction
RNase	ribonuclease
RNA	ribonucleic acid
rpm	rotations per minute
SA	salicylic acid
TROPI	Sarawak Tropical Peat Research Institute
sRNA	small RNA molecule
NaCl	sodium chloride
SAR	systemic acquired resistance
RQ	the calculated relative gene expression level
TFBS	transcription factor binding site
Tris-HCl	Tris(hydroxymethyl)aminomethane hydrochloride
T2SS	type II secretion system
T3SS	type III secretion system
UPW	ultrapure water
U-	Unigene
UPM	Universiti Putra Malaysia

USR	upper stem rot
wgs	whole genome sequence

CHAPTER 1

INTRODUCTION

The African oil palm (*Elaeis guineensis* Jacq.) is an effective oil crop which plays an important role in fulfilling demands of global oil consumption and usage. To date, Malaysia is the second largest exporter of palm oil (Malaysian Palm Oil Board, 2022a). An increasing annual revenue was obtained from the palm oil industry, growing from RM 3,632.6 million in 1960 to RM 130.24 billion in 2022 (Lim, 1987; Malaysian Palm Oil Board, 2022a). It is a pillar of the Malaysian economy driving the nation's economic prosperity and development. Much efforts have been invested on crop improvements to increase oil yield.

Ganoderma boninense is a hemibiotrophic fungal pathogen which causes the basal stem rot (BSR) and upper stem rot (USR) in oil palm tree. The BSR disease has led to 50–80% reduction in oil yield, and ultimately causes plant death (Corley & Tinker, 2016). The disease control methods rely largely on the application of chemical fungicides (Idris *et al.*, 2004; Nur-Rashyeda *et al.*, 2021) and field sanitisation (Virdiana *et al.*, 2010), while integrated approaches such as *Bacillus subtilis* bio-fungicide tablets were only tested at nursery scale (Puspita *et al.*, 2019), BSR prevention-formulated new fertilizer technology GanoCare® was tested at both nursery and field trials (Rebitanim *et al.*, 2020), and chitosan-dazomet nanoparticles were still under lab-scale development and testing (Maluin *et al.*, 2019). There is still no effective method for *G. boninense* disease control. One of the reasons underlying this problem is the late onset of disease symptoms that complicates disease control efforts (Siddiqui *et al.*, 2021).

An in-depth understanding of the molecular events occurring during the plant-pathogen interaction before the emergence of the symptoms is of great importance. During a plant-pathogen interaction, pathogens secrete an array of virulence factors contributing to the occurrence of disease (Kamoun, 2006). Previous studies reported on how established model plants (e.g. *Arabidopsis thaliana* and *Oryza sativa*) develop plant immunity to counter pathogenic attack, especially induced defence which promotes resistance towards secondary infection, and how this could eventually lead to marker development for the molecular breeding of resistant cultivars (He *et al.*, 2022). These findings could be applied to *E. guineensis-G. boninense* pathosystem.

With recent advancements in molecular technologies, the understanding of *G. boninense* has been further improved at the biology level, especially in population studies (Midot *et al.*, 2019; Wong *et al.*, 2021; Wong *et al.*, 2022). In addition, an increasing number of next-generation sequencing technologies were applied to decipher the *E. guineensis-G. boninense* interaction (Bahari *et al.*, 2018; Ho *et al.*, 2016). However, information on the virulence factors contributing to the BSR is limited. The fungal transcripts in *Ganoderma*-treated oil palm seedlings could be potential candidates of virulence factors contributing

to BSR (Ho *et al.*, 2016). The colonization of oil palm roots by *G. boninense* is hypothesized to be facilitated by secreted virulence proteins in infected oil palm roots that breached the host defence through suppression or avoidance of chitin-triggered immunity.

In this study, four transcripts were selected from a previous study with the following criteria: i. present in the *G. boninense*-treated oil palm root transcriptomes (absent in control), ii. present in whole genome sequence of *G. boninense*, iii. encodes for secreted protein, and iv. has homologs involved in plant pathogenesis. The candidates are putative metalloproteinase (*U14090*), aspartic protease (*U42611*), polysaccharide deacetylase (*U128397*), and lipase (*U56931*).

The objectives of this study are:

1. To isolate the transcripts of selective putative virulence factors from *G. boninense*;
2. To profile the gene expressions of selective putative factors during fungal growth and plant-pathogen interactions; and
3. To predict the transcription factor binding sites (TFBS) at the promoter sequence of selected putative virulence factors in pathogenic *G. boninense* and non-pathogenic *Ganoderma* species (*G. sinense* and *G. lucidum)*.

CHAPTER 2

LITERATURE REVIEW

2.1 Oil palm

The oil palms are under the *Elaeis* genus, which has derived from the Greek word '*elaion*', meaning oil. They are monocots in the family Arecaceae of the order Arecales, that have broad leaf-blade with a well-developed vascular system resembling those of functional woody dicots despite the lack of secondary growth, thus attributing to their narrow ecological amplitude in the tropical regions (Cronquist, 1981). There are two species under the *Elaeis* genus, which are *E. guineensis* Jacq, the African oil palm and *E. oleifera* (Kunth) Cortes, the American oil palm. The African oil palm *E. guineensis* being a commercially cultivated crop, has drawn more attention due to its economic importance, whereas *E. oleifera* which produces a lower yield is used for oil palm breeding for certain features (Corley & Tinker, 2016).

Oil palm fruits produce two types of oil, i.e., crude palm oil and palm kernel oil which are extracted from the mesocarp and seed, respectively (Kelanaputra *et al.*, 2018). The oil yield is different in the three variants of *E. guineensis* having different shell thickness and ratios of mesocarp to kernel, i.e., *dura* (thick shell), *pisifera* (no shell), and *tenera* (thin shell) (Beirnaert & Vanderweyen, 1941). In the early 20th century, *dura* was the main cultivated variant. The crossing of *dura* and *pisifera* has produced *tenera*, which has a thinner shell and produces a higher oil yield than its female parent *dura*, while overcoming sterility in its male parent *pisifera* (Kelanaputra *et al.*, 2018; Singh *et al.*, 2013). Since then, *tenera* has become the dominant cultivated variant in the oil palm industry.

2.1.1 Economic importance of oil palm

Elaeis guineensis Jacq. is a highly efficient oil-bearing crop plant producing 3.3 tonnes of oils per hectare, accounting for >30% of the global demand for oils and fats (World Wide Fund for Nature, 2023). It produces a high-quality edible vegetable oil for daily cooking and manufacture of palm oil products, including beverages, food, personal care, cleaning, cosmetics, and biofuel (Malaysian Palm Oil Council, 2023). Oil palm is therefore essential for food security of the growing world population and national prosperity.

The first commercial oil palm cultivation in the Malaysia/Malaya started in 1917 (Basiron, 2007). The expansion of oil palm cultivation was accelerated only in the 1960s upon the implementation of the agricultural diversification programme under The Plan of Development for Malaya, which aimed to improve the farmers' livelihood and reduce rubber dependency (Courtenay, 1984). Since then, oil palm planted area has increased from 54,000 hectares in 1960 to 5.6 million

hectares in 2022 (Basiron, 2007; Malaysian Palm Oil Board, 2022b). Oil palm has become the main commodity crop in Malaysia with the highest production, contributing to 35.2 per cent of the gross value of the agriculture sector in 2021 (Department of Statistics Malaysia, 2022).

Malaysia is the world's second-largest producer and exporter of palm oil. In 2022, Malaysia produced 18.45 million tonnes of palm oil and exported 15.71 million tonnes of palm oil, which has constituted 31.08% of the global palm oil export (Malaysian Palm Oil Board, 2022a, 2022c; Oil World as cited by Ministry of Plantation and Commodities, 2023). The export of all palm products recorded for the period of January to December 2022 amounted to RM 130.24 billion, a major source of export revenue (Malaysian Palm Oil Board, 2022a). The industry has also created job opportunities for more than 0.5 million people, supporting the livelihood of the employees and their families (Ministry of Plantation Industries and Commodities, 2018). Thus, the oil palm industry is important for the economic growth of the nation.

2.1.2 Challenges in oil palm cultivation

The oil palm cultivation has encountered a few major challenges in recent years, including labour shortages, environmental concerns, pests, and diseases. Oil palm cultivation is labour intensive, and the Malaysian palm oil industry relies heavily on foreign workforce for plantation maintenance and fresh fruit bunch (FFB) harvesting. In 2020, Malaysia imposed border restrictions to fight the COVID-19 pandemic, and this has caused a severe plantation worker shortage, causing the ripen FFBs unharvested. This has led to lower FFB yield, reduced crude palm oil production, and delay in tree replanting (Parveez *et al.*, 2022). Consequently, the total oil palm planted area in Malaysia in December 2022 was reduced to 5.67 million hectares, from 5.73 million hectares in 2021, 5.87 million hectares in 2020, and 5.90 million hectares in 2019, respectively (Malaysian Palm Oil Board, 2021, 2022b; Parveez, 2020). As the border reopened and the industry is recovering, the prospective for the palm oil industry remains optimistic for 2023 (Ministry of Plantation and Commodities, 2023).

On the other hand, the expansion of oil palm cultivation areas over the last few decades had raised concerns regarding environmental impacts. In 2018, European Union (EU) voted to ban palm oil which was derived from deforestation. This has caused a major impact towards Malaysian palm oil industry as EU was the second largest importer in 2017, while the cost to the reputation of the industry was tremendous (Malaysian Palm Oil Council, 2018). To counter this issue, the Ministry of Plantation Industries and Commodities had made Malaysian Sustainable Palm Oil Certification Scheme (MSPO) mandatory at the end of 2019 (Ministry of Plantation Industries and Commodities, 2017). Also, the Ministry of Primary Industries proposed to cap oil palm plantations at 6.5 million hectares by 2023 as an effort towards sustainable oil palm cultivation while focusing more on yield and seedling improvement (Ministry of Primary

Industry, 2019). As suitable land for oil palm cultivation is limited, enhancing crop yield while reducing crop loss is important.

Ganoderma disease is one of the most threatening diseases to oil palm cultivation (Nur-Rashyeda *et al.*, 2021). It is caused by the fungal pathogen *G. boninense*, which often occurs as BSR or USR. In Malaysia, BSR is predominant, whereas USR is less common (Midot *et al.*, 2019; Rakib *et al.*, 2017; Wong *et al.*, 2022). BSR was first reported in 1931 in 25-year old oil palms (Thompson, 1931). BSR incidence was reported as <1% within the 10–15th year of planting and increased up to >25% at 22 years, whereas the FFB yield in the infected area declined after the 10th year of planting and reached 24.1% at the 22nd year (Roslan & Idris, 2012). To date, efficient preventive or control method for this disease is still unavailable (Darlis *et al.*, 2023). The disease incidence is especially alarming among smallholders who lack the knowledge and skills on *Ganoderma* disease management (Kamu *et al.*, 2016). The *Ganoderma* BSR disease yield loss suffered by the planters was estimated up to 68.73%, based on observations from 461 infected palms over 12 months in three estates (same plantation company) located in Sabah (Assis *et al.*, 2020).

2.2 White rot fungus *Ganoderma boninense*

2.2.1 Biology and epidemiology

Ganoderma boninense is a white fungus of the Polyporoid clade under the Basidiomycota division. Basidiomycete fungi have a fruiting body structure called basidiocarp (also known as basidiome or basidioma), which produces basidiospores externally from the club-shaped basidia. The basidiocarps of *G boninense* are usually glossy, have yellowish-brown to reddish-brown dorsal surface, in stalked or sessile form, while the basidiospores are yellow to yellowish brown, in ellipsoid shape, having dimensions varied from 8.5–11.5 x 4.2–6.5 µm (Ho & Nawawi 1985; Latiffah & Ho, 2005; Pilotti *et al.*, 2004; Rakib *et al.*, 2014a).

Basidiomycetes including *G. boninense* alternate between self-sterile haploid and dikaryotic mycelia (dominant) throughout their life cycle (Pilotti *et al.*, 2002; Raper, 1978). During sexual reproduction, karyogamy of nuclei from two different mating types produces a diploid zygote (2n), which undergoes meiosis to produce four haploid basidiospores (n). As the basidiospores germinate to monokaryotic hyphae and form primary mycelia, the compatible hyphae of different mating type fuse and give rise to dikaryotic mycelium (n+n) which contains two haploid nuclei within a single cell, distinguished with clamp connections on the hyphae (Pilotti *et al.*, 2002). Then, the secondary mycelia can grow into a basidiocarps, having the dikaryon cells in the form of basidia which will fuse during karyogamy to allow genetic reassortment (Figure 2.1) (Clark *et al.*, 2020).

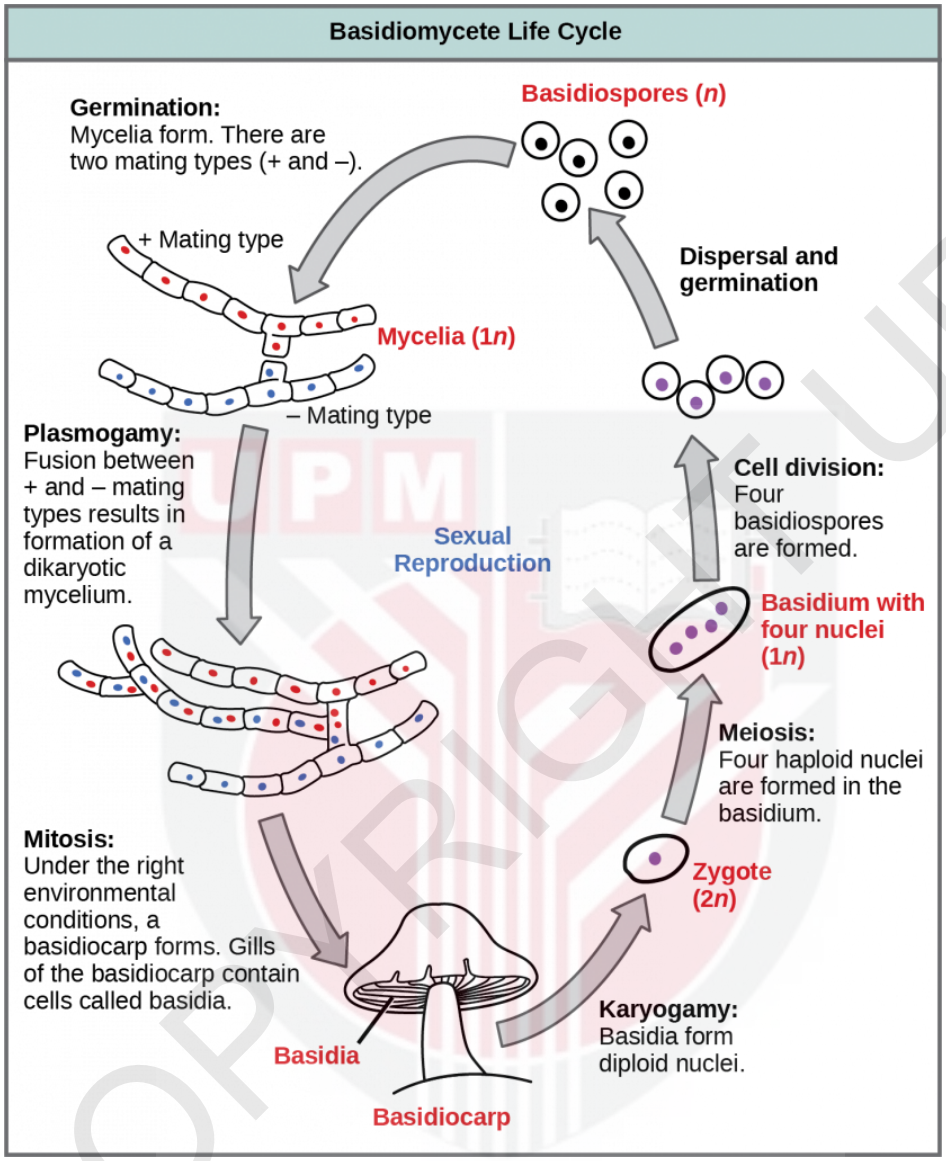


Figure 2.1 : Basidiomycete life cycle. Basidiomycetes alternate between monokaryotic haploid hyphae and the dominant dikaryotic mycelia, which may grow into the mushroom structure known as basidiocarp (Clark *et al*., 2020). Creative Commons Attribution 4.0 International Public License (CC BY 4.0)

Ganoderma boninense is different from brown rot fungus which only degrades the cellulose of the cell wall, it is able to degrade lignin and cellulose in the plant cell wall, giving a white residual appearance (Paterson, 2007). Owing to its ability to degrade lignin, *G. boninense* could cause BSR and USR in oil palm. Besides oil palm, *G. boninense* was also reported in other palm species (*Cocos nucifera*, *Livistona subglobosa, Wodyetia bifurcate, and Areca* spp.), forest oak

(*Casuarina torulosa*), and *Albizia* spp. (Centre for Agriculture and Biosciences International & Steyaert, 1975; Mohd Farid *et al.*, 2018; Peries, 1974).

According to the record as of October 2022 in the Centre for Agriculture and Biosciences International (2022), BSR incidence caused by *G. boninense* was reported in 24 countries in Africa, Asia, North America, Oceania, and South America (Table 2.1). It is widespread in Malaysia and Indonesia, the main palm oil exporting countries (Centre for Agriculture and Biosciences International, 2022). Despite of geographical barriers, the presence of high gene flow was demonstrated in the *G. boninense* populations collected from different regions as reported in four separate studies: i. Sumatra and Peninsular Malaysia (Mercière *et al.*, 2017); ii. Solomon Islands, Papua New Guinea, Indonesia, and Malaysia (Pilotti *et al.*, 2021); iii. Sumatra, Sarawak, and Peninsular Malaysia (Wong *et al.*, 2021); and iv. Sumatra, Sabah, Sarawak and Peninsular Malaysia (Wong *et al.*, 2022).

Owing to sexual reproduction of this fungus, a high genetic diversity was observed in each population (Mercière *et al.*, 2017; Pilotti *et al.*, 2018; Pilotti *et al.*, 2021; Wong *et al.*, 2021; Wong *et al.*, 2022). A lower genetic heterozygosity within the populations has occurred possibly due to inbreeding for better adaptation and survivability (Pilotti *et al.*, 2003; Wong *et al.*, 2021; Wong *et al.*, 2022). Fungal biology and genetic evolution are important for a better understanding of the adaption of *G. boninense* to environmental stress and host co-evolution.

Table 2.1 : Distribution of *G. boninense* in various continents by countries

Present and widespread	
Asia	Indonesia*, Malaysia* (Peninsular Malaysia, Sabah, Sarawak)
Oceania	Australia (New South Wales, Queensland, Victoria)
Present but extent is unrecorded	
Africa	Angola*, Cameroon*, Democratic Republic of the Congo*, Ghana*, Nigeria*, São Tomé and Príncipe*, Tanzania*, Zimbabwe*
Asia	China (Hainan), India (Maharashtra), Japan (Kyushu), Philippines, Singapore*, Sri Lanka, Taiwan, Thailand*
North America	Honduras*
Oceania	Australia (Tasmania), Papua New Guinea*, Samoa, Solomon Islands*
South America	Colombia*

Note: * represents incidence reported in oil palm, otherwise was in other hosts. (Centre for Agriculture and Biosciences International, 2022), Attribution-NonCommercial-ShareAlike 3.0 Unported (CC BY-NC-SA 3.0).

2.2.2 Pathogenesis

Pathogenesis is defined as the sequential events during an infection that gives rise to a disease, which starts from the adhesion of a pathogen to the host surface where early recognition is initiated, followed by replication, and disease spread (Baron *et al.*, 1996). There are two modes of infections for *G. boninense*, which are by direct root or inoculum contact and by basidiospores. According to Singh (1991), *G. boninense* invades oil palm roots before spreading to the stem causing palm death. Microscopic examination has confirmed the infection route. The *G. boninense* first colonised the root surface, penetrated through the root hair base and crossed the epidermal and cortical layers, then degraded plant tissues possibly through the action of cell wall degrading enzymes (Alexander, 2017; Nusaibah *et al.*, 2016; Rees *et al.*, 2009). *G. boninense*-infected roots or -colonised debris left in the plantation could serve as an inoculum that infect nearby healthy trees or replanted new trees. It was depicted as a weak pathogen which is, unable to grow in non-sterile soil or organic debris, and has an optimal growth at a temperature of 25–30 °C (Flood *et al.*, 2000; Rees *et al.*, 2007).

The findings of mating compatibility analysis and randomly amplified microsatellites (RAMS) fingerprinting analysis showed a high genetic diversity of *G. boninense* within a plantation, between neighbouring infected palms, and within an individual infected palm (Pilotti, 2005; Pilotti *et al.*, 2018; Pilotti *et al.*, 2003; Rees *et al.*, 2007). The high genetic diversity among *Ganoderma* isolates, implied spores as a mode of infection rather than clonal mycelia contact from infected roots and residual debris (Ariffin, 1996; Miller *et al.*, 1995; Sanderson, 2005). As a tetrapolar heterothallic basidiomycete, *G. boninense* undergoes sexual reproduction which favours out-crossing that allow genetic recombination and produces offsprings with diverse genotypes (Pilotti *et al*., 2002). A diseased oil palm host could be infested by multiple isolates of *G. boninense* with different genotypes, implying spores as a mode of infection (Miller *et al*., 1999; Pilotti, 2005). Besides, the infection mode by clonal mycelia contact was also reported where a host was infected by several identical isolates (Pilotti, 2005).

Despite that, there was no direct evidence which showed basidiospores as a possible infection mode, since attempts at artificial infection using basidiospores were unsuccessful (Hasan & Flood, 2003). Besides, the monokaryotic mycelium from basidiospores was found to be non-infective, and *G. boninense* is only pathogenic in dikaryotic form after a compatible mating between two monokaryons (Pilotti *et al.*, 2002; Rees *et al.*, 2007). Rees *et al.* (2012) have shown the germination of basidiospores on cut surfaces (fronds, peduncles, and stems) of oil palm in plantation. Then, Pilotti *et al.* (2018) have observed the presence of both dikaryons and monokaryons from seedlings grew in BSR-infected field. This has confirmed the viability of basidiospore-derived mycelium within the rhizosphere.

G. boninense is a hemibiotroph which could switch from biotroph to a destructive necrotroph (Rees *et al.*, 2009). *G. boninense* can infect oil palms of any stage,

including both young and mature palms. Infected young palms usually exhibit one-sided yellowing, mottling and necrosis of lower fronds, shorter and chlorotic unfolded leaves, pale and retarded palm growth as well as unopened spear leaves; whereas diseased mature palms also show similar symptoms with drooping dead fronds which form a skirting appearance (Ariffin *et al.*, 2000; Soepena *et al.*, 2000).

Once infected palms showed foliar symptoms, ~50% of the stem could have rotten before its spread to the vascular tissues. This lead to disrupted water and nutrient supply to the aerial part of the palm, displaying symptoms resembling wilting and malnutrition (Turner & Gillbanks, 2003). The palms could die within 6–24 months and 2–3 years after the first sign of symptoms in young palms and mature palms, respectively (Ariffin *et al.*, 2000; Turner & Gillbanks, 2003). The basidiocarp of *G. boninense* may grow at the basal stem in BSR or one metre above the ground in USR (Rakib *et al.*, 2014b; Sariah *et al.*, 1994). Different strains of *G. boninense* tend to manifest in the host at different rates, affecting vegetative growth and survivability of the host differently (Goh *et al.*, 2014; Lo *et al.*, 2023). The late onset of disease symptoms has posed a great challenge to disease detection and control, calling for an urgent need for integrated disease detection, control, and management strategies.

2.3 Plant-pathogen interactions

A host plant refers to a plant species of which a pathogen could cause disease, whereas a non-host plant is a plant species which is not responsive to a pathogen (Freeman & Beattie, 2008). When a disease develops, the host-pathogen interaction is referred to as compatible; and if little or no disease develops, it is known as an incompatible response. An immune cultivar is protected by nonhost resistance and does not display any disease symptoms, a highly resistant cultivar may show little symptoms, whereas a highly susceptible cultivar exhibits severe symptoms (Freeman & Beattie, 2008; Niks, 2014). A successful disease infection requires the pathogen to evade host detection and/or suppress host defences. Hosts deployed a multilayer-defence to protect themselves, first with existing constitutive preformed defence and later with inducible defences triggered by biotic and abiotic stimuli (Mauch-Mani *et al.*, 2017).

On the other hand, pathogens exhibit diverse nutritional requirements, lifestyles and infect their hosts differently. Biotrophs colonise and feed on the host while bypassing the hypersensitive response (HR) in the host; necrotrophs secrete toxin to kill the host before colonisation; and hemibiotroph has a lifestyle switching from asymptomatic biotrophy to destructive necrotrophy (Doehlemann *et al.*, 2017; McCombe *et al.*, 2022). Pathogens produce virulence factors to counteract the host defence responses, facilitate host cell wall invasion, nutrient acquisition, and host defence avoidance or suppression (Doehlemann *et al.*, 2017). However, host-pathogen interactions may result in a gradual plant immunity, which produces partial host resistance or susceptibility, and ultimately

results in host range expansion or shrinkage, affected by exogenous factors such as circadian clock, light quality, photoperiod, humidity, temperature, developmental stage, tissue contact, and host-pathogen co-evolution (Niks & Marcel, 2009; Panstruga & Moscou, 2020).

2.3.1 Constitutive resistance

Constitutive resistance consists of non-specific preformed barriers in the plant that act as the first line of defence, either in a structural or chemical form, to counter pathogenic penetration and colonization. Preformed structural barriers in the plant include surface wax, cuticle and bark, cell wall, and casparian strips (Moore *et al.*, 2011). Surface wax covering epidermal layer serves as the interface protecting plant surface from external threats such as disease-causing pathogen. Cuticle, bark, and cell wall are the outer intact layer that pathogen has to penetrate before successful entry to the plant. Cell wall usually possesses various receptors for pathogen recognition facilitating downstream defence signalling. Casparian strip at the root endodermis layer form a seal between plant cells, restricting nutrient access and growth rate of pathogen through the down-regulation of genes involved in casparian strip formation (*MYB36*) and suberin biosynthesis (*HORST*) upon pathogenic attack (Fröschel *et al.*, 2021; Holbein *et al.*, 2019).

Plants produce diverse secondary metabolites (antifungal, phenolic compounds), during the course of development and to counter biotic stress, even in the absence of pathogen (Lattanzio *et al.*, 2006; Mamarabadi *et al.*, 2018). Phenolic compounds consist of more than 8000 reported molecules, which are differentiated by their chemical structures e.g., phenolic acids, flavonoids, tannins, phenolic lignans, and phenolic stilbenes. Gallic acid is a phenolic acid suggested to inhibit ergosterol biosynthesis, hence has antifungal activity (Li *et al.*, 2017). Flavonoids inhibit fungal activity by disrupting cell wall, causing mitochondrial dysfunction, inhibiting cell wall formation, cell division, synthesis of RNA/DNA/protein, and efflux pump (Aboody & Mickymaray, 2020). Tannins were reported as antifungal agents against *Penicillium digitatum*, the citrus pathogen by causing fungal cell wall disruption and leakage of intracellular contents (Zhu *et al.*, 2019).

2.3.2 Induced resistance

Induced resistance is defined as a state of plant having enhanced defence upon stimulation, where the stimuli can be from beneficial microbial interactions, pest and pathogens attack, or abiotic stresses (De Kesel *et al.*, 2021; Mauch-Mani *et al.*, 2017). The plant perceives signals from those stimuli through pattern recognition receptors (PRRs) at the cell surface which recognise microbial/pathogen-associated molecular patterns (MAMPs/PAMPs) and results in PAMP-triggered immunity (PTI). The pathogens may deploy effectors to overcome PTI. This may involve recognition by intracellularly localised resistance (R) proteins which have a signature intracellularly localised

nucleotide-binding and leucine-rich repeat (NB-LRR) architecture, activation of an amplified defence response which is known as effector-triggered immunity (ETI), and a hypersensitive response (HR) at the infection site (Jones & Dangl, 2006) (Figure 2.2).

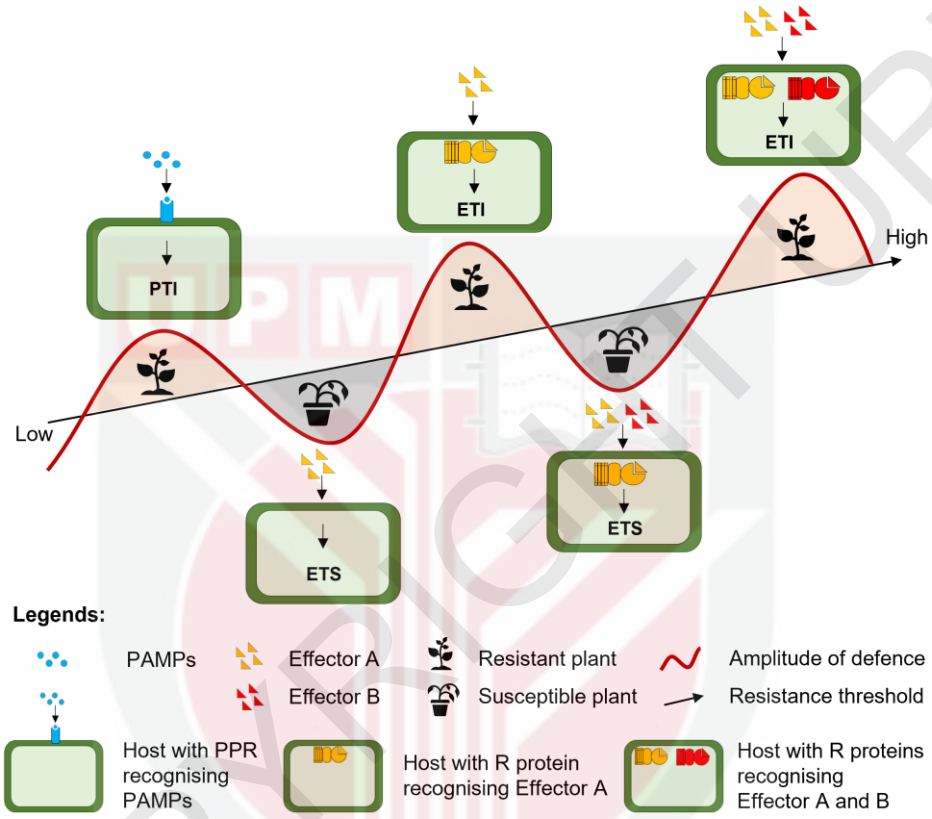


Figure 2.2 : Induced defence in plant. Pathogen-associated molecular patterns as PAMPs; pattern recognition receptor as PPR; resistance protein recognising effector as R protein; PAMP-triggered immunity as PTI; effector-triggered immunity as ETI; effector-triggered susceptibility as ETS

However, natural selection may drive the co-evolutionary arms race between the host and pathogen to increase species fitness, where the host may acquire more R genes to recognize more pathogenic races and activate ETI, while the pathogen may gain or lose effectors to avoid ETI (Jones & Dangl, 2006). Effector proteins recognised by the R proteins are known as avirulence effectors. On the other hand, the absence or rapid evolution of avirulence genes caused the breakdown in plant resistance and may result in virulent phenotype, as demonstrated in the rice blast and wheat–powdery mildew pathosystems (Damchuay *et al.*, 2020; Hu *et al.*, 2022; Müller *et al.*, 2022).

A plant hinders disease progression by activating its defence machinery (e.g., phytohormones, antioxidants, secondary metabolites, pathogenesis-related proteins, phytoalexin, melatonin, carotenoid, and cell wall modification) (Kaur *et al.*, 2022b; Vlot *et al.*, 2021). A plant escalates its defence mainly by modulating its signalling molecules including reactive oxygen species (ROS, such as hydrogen peroxide (H_2O_2)) and phytohormones (such as salicylic acid (SA), jasmonic acid (JA), or ethylene (ET)) (Tian *et al.*, 2016; Yang *et al.*, 2020; Zeng *et al.*, 2023). SA provides resistance against biotrophs and hemibiotrophs through systemic acquired resistance (SAR). JA and ET provide resistance against necrotrophs through induced systemic resistance (ISR), and H_2O_2 plays an important role in plant resistance through the modulation of both SAR and ISR (Yu *et al.*, 2022). Upon defence induction at the site of infection, systemic signals may be transmitted throughout the plant, providing the uninfected tissues with faster and/or elevated resistance against secondary infection (Vlot *et al.*, 2021). Hence, induced defence is also known as systemic resistance. Induced resistance could be a potential alternative towards integrated plant disease management. The application of environmentally friendly plant defence inducer could replace chemical fungicides which raise environmental concerns.

2.4 Current understanding on oil palm-*G. boninense* interactions

Oil palm and *G. boninense* interact when the spores or mycelia of *G. boninense* are in contact with the oil palm, breaching the cell surface to ease nutrient acquisition, and later multiply before dispersal. A cascade of cellular and molecular events occur during this process. With recent advancements in molecular technologies, the understanding of *G. boninense* has been further improved. A growing number of sequencing projects have been conducted on *G. boninense*, which has facilitated comparative genomic analyses to better understand the underlying mechanisms contributing to the virulence of *G. boninense* (Dhillon *et al.*, 2021; Ho, 2023; Ho *et al.*, 2016; Ho *et al.*, 2018, 2019; Isaac *et al.*, 2018; Khairi *et al.*, 2022; Ramzi *et al.*, 2019; Sulaiman *et al.*, 2018; Wong *et al.*, 2019). These studies have contributed to candidate genes for further experimental validation.

2.4.1 Response of oil palm to *G. boninense* infection

G. boninense-infected oil palm roots may employ chitinases for fungal cell wall degradation, releasing chitin elicitors (a structural component in fungal cell wall), hence triggering PTI through up-regulation of gene(s) encoding chitinases (Bahari *et al.*, 2018; Ho *et al.*, 2016). Ho *et al.* (2016) first suggested SA-mediated defence in oil palm upon *G. boninense* infection, where accumulation of transcripts related to SA methylation to its active form was found in *G. boninense*-treated oil palm. SA interacts antagonistically with JA. This may down-regulate JA-inducible phenylalanine ammonia lyase (*PAL*) genes in oil palm (Ho *et al.*, 2016; Tan *et al.*, 2016; Tee *et al.*, 2013). SA-mediated defence in oil palm during biotrophic infection was supported by the up-regulation of *EgNPR1* at 3 wpi, a SA receptor which is important for the positive regulation of

SA biosynthesis and homeostasis regulation (Liu *et al.*, 2020b; Mohan *et al.*, 2022). Positive correlation between SA accumulation and lignification was observed to confer oil palm resistance against *G. boninense* infection (Faizah *et al.*, 2022).

Lipidomic analysis revealed a distinct lipid profile between *G. boninense*-infected and non-infected root cell walls, with a higher sterol level in the infected oil palm roots as compared to the uninfected host. The sterol level may vary according to the disease tolerance of the host towards *G. boninense* (Alexander *et al.*, 2017; Nurazah *et al.*, 2013; Nusaibah *et al.*, 2011; Nusaibah *et al.*, 2016). The host reprogrammed its lipid sterol metabolism to counter pathogenic attack at different infection stages (Alexander *et al.*, 2019). It also altered its pyruvate metabolism, glycolysis or gluconeogenesis pathways, as an energy saving strategy for defence response against *G. boninense* infection (Alexander *et al.*, 2022). Similarly, it may modulate its resources or suppress other biological processes, to prioritise defence response against *G. boninense* (Dhillon *et al.*, 2021; Ho *et al.*, 2019). Induced defence in oil palm has been demonstrated to be transmitted systemically, where the defence response of infected roots can be detected in the leaves at transcript level (Ho *et al.*, 2019) and enzymatic level (Naher *et al.*, 2012).

2.4.2 Countermeasures of *G. boninense* during pathogenesis

G. boninense interacting with the host was found to have an increased abundance of transcripts encoding for enzymes responsible for lignin degradation and lignin modification, and a reduced abundance of transcripts responsible for fungal respiration, chitin degradation, and pathogenesis (Dhillon *et al.*, 2021). The ability of fungal pathogen to develop countermeasures in avoiding plant defence signal perception and elicitation encompass its ability to modify fungal chitin surface to avoid recognition and degradation by plant chitinase (Gong *et al.*, 2020). *G. boninense* infection has also caused the down-regulation of a gene encoding for mediator responsible for R protein recognition (Ho *et al.*, 2016). However, information on interacting proteins from host and pathogen is lacking. Candidate resistance genes from oil palm have been annotated (Ab-Halim *et al.*, 2022; Chan *et al.*, 2017), however, effector prediction from *G. boninense* was only published recently (Ho, 2023; Khairi *et al.*, 2022). A parallel research on both *R-Avr* genes is necessary to understand interacting proteins from both host and pathogen.

Several candidate proteins from *G. boninense* including cyclophilins (Lim *et al.*, 2017), hydrophobin (Sulaiman *et al.*, 2018), pheromone receptor (Madihah *et al.*, 2019), necrosis and ethylene inducing protein (NEP)-like proteins (Teh *et al.*, 2019), laccases and manganese peroxidases (Ho *et al.*, 2020) have been characterised. Of which, recombinant GbNEP was shown to be able to induce necrosis in non-host plants (tobacco and tomato), while its necrosis-inducing activity in oil palm host has yet to be proven (Teh *et al.*, 2019). These studies

presented the current progress and knowledge gap in the virulence factors. More research has to be conducted to identify the functional virulence factors.

2.5 Selected putative virulence factors for this study

Doehlemann *et al.* (2017) described virulence factor as a disease-causing agent that counteracts the host defence responses, facilitates host cell wall invasion, nutrient acquisition, and host defence avoidance or suppression. Virulence factors could occur as effectors, cell wall degrading enzymes (CWDEs), hydrolytic enzymes, organic acids, secondary metabolites, or small RNA molecules (sRNAs) (Doehlemann *et al.*, 2017; Han *et al.*, 2021; Inoue *et al.*, 2023; Jiao *et al.*, 2022; Mapuranga *et al.*, 2023; Zhang *et al.*, 2021; Zhang *et al.*, 2020).

Effectors caused host susceptibility in R proteins-lacking hosts but activated ETI if the host has the corresponding R proteins which are capable to recognise the effectors (Han *et al.*, 2021; Jia *et al.*, 2000; Jones & Dangl, 2006). CWDEs and hydrolytic enzymes are important for cell wall degradation and host invasion (Rafiei *et al.*, 2021; Rauwane *et al.*, 2020). Organic acids acidify the host surface for successful infection (Wang & Wang, 2020). Fungal secondary metabolites cause host toxicity and improve the fungal fitness (Pusztahelyi *et al.*, 2015), whereas pathogenic sRNAs may mimic the host sRNAs to avoid host immune response (Weiberg & Jin, 2015).

In this study, four cDNA transcripts were selected based on previous findings by Ho *et al.* (2016). They encode for metalloproteinase, aspartic protease, lipase, and polysaccharide deacetylase, respectively. These transcripts were only found in the transcriptome of *G. boninense*-treated oil palm roots, and not in the transcriptome of untreated oil palm roots. Virulence factors are often secreted by plant pathogens into their host, to hijack host defence (Kunkel & Chen, 2006), hence these candidates may play a role in the pathogenesis of *G. boninense*.

2.5.1 Metalloproteinase

Metalloproteinase is a hydrolase classified as a metalloendopeptidases (EC 3.4.24), which catalyses the addition of water to a peptide bond. Its activity is dependent on the presence of a protein-bound metal ion(s) (Auld, 2013). Pathogenic fungi are known to secrete zinc-ion-dependent metalloproteinases such as M43 metalloproteinase (Pan *et al.*, 2020a), M35 deuterolysin (Han *et al.*, 2021), and M36 fungalysin (Wang *et al.*, 2022). M43 pappalysin-like metalloproteinase is characterised by a HEVGHWLGLYH motif (Pan *et al.*, 2020a), whereas M35 and M36 metalloproteinases are known by the signature HEXXH motif with histidine (H) residues involve in zinc-binding. However, there is an additional GTXDXXYG motif in M35 deuterolysin for the third zinc ligand in asparagine residue (D) (Hori *et al.*, 2001). The roles of fungal metalloproteinases in plant-pathogen interaction are shown in Table 2.2. Metalloproteinases involve

in fungal virulence with the following roles: suppress host immune system (Han *et al.*, 2021; Hu *et al.*, 2022; Sanz-Martín *et al.*, 2016), cleave/inhibit host chitinase (Jashni *et al.*, 2015; Ökmen *et al.*, 2018; Pan *et al.*, 2020a), initiate ETI (Jia *et al.*, 2000), elicit cell death and ROS accumulation (Pan *et al.*, 2020a). A lower conidia production and inhibited spore germination were observed in the metalloproteinase gene mutant in *F. graminearum* (Wang *et al*., 2022), suggesting that metalloproteinase is essential for the fungal survival and dispersal.

2.5.2 Aspartic protease

Aspartic protease is a hydrolase (EC 3.4.23 aspartic endopeptidases). It has two aspartic acid residues vital for their catalytic activity (Haertlé, 2015). Aspartic protease was suggested to be involved in pathogen nutrition (Hislop *et al.*, 1982). It was found to be expressed in appressorium but not in conidia (Plummer *et al.*, 2004). Studies on the role of fungal aspartic protease in host-pathogen interaction were tabulated in Table 2.2. Aspartic proteases were found to be dispensable in *Botrytis cinerea* and *Glomerella cingulate*, suggested for alternative or multiple peptidase action during host-pathogen interaction (Plummer *et al.*, 2004; Ten Have *et al.*, 2010). Aspartic protease may play a role during the early infection stage to evade and degrade host structural and defence components (Bueno *et al.*, 2012; Poussereau *et al.*, 2001b). Aspartic protease was also found to degrade grape chitinase in a winemaking study (Theron *et al.*, 2017, 2018; Van Sluyter *et al.*, 2013). From the industrial application perspective, aspartic protease is a potential enzyme to improve the quality of wine. At the same time, aspartic protease may have a potential role to break down chitinase, a host pathogenesis-related protein (Kaur *et al.*, 2022a). On the other hand, bacterial aspartic protease was found to determine the host specificity in different strains of *Ralstonia solanacearum* (Jeong *et al.*, 2011).

2.5.3 Lipase

Lipase is a hydrolase categorised under EC 3.1.1.3 triacylglycerol hydrolases, which releases free fatty acid and glycerol during the hydrolytic activity on a water insoluble triacylglycerol (Hou & Shimada, 2009). Lipase is a known virulence factor in many pathosystems (Gaillardin, 2010). Lipase could contribute to fungal virulence by promoting fungal adhesion, growth, development, reproduction, stress response, mycotoxin reproduction, lipase activity, lipid turnover, and suppressing plant immune response (Table 2.2) (Blümke *et al.*, 2014; Feng *et al.*, 2011; Feng *et al.*, 2009; Kim *et al.*, 2023; Nguyen *et al.*, 2011a; Voigt *et al.*, 2005). Lipid metabolism in peroxisomes was suggested to be critical in *B. cinerea* disease establishment, following an observation where ~52% genes in the peroxisome pathway were up-regulated (>2-fold) upon host exposure (Han *et al.*, 2022). However, genome-wide single gene deletion mutants of 86 genes encoding for lipase in *F. graminearum* showed that, only phospholipase D mutant displayed a reduced virulence while other mutants were having normal virulence level (Kim *et al.*, 2023). Functional

redundancy of lipase gene could compensate for possible loss of function in other single gene mutant and retain the virulent phenotype (Bravo-Ruiz *et al.*, 2013; Kim *et al.*, 2023).

2.5.4 Polysaccharide deacetylase

Polysaccharide deacetylase is a member of the carbohydrate esterase family 4 which catalyses metal-dependent de-acylation of acetylated polysaccharides (acetyl xylan, chitin, and peptidoglycan). Polysaccharide deacetylase includes acetyl xylan esterase (EC 3.1.1.72), chitin deacetylase (EC 3.5.1.41), chitooligosaccharide deacetylase (EC 3.5.1.-), peptidoglycan deacetylase (EC 3.5.1) (Armendáriz-Ruiz *et al.*, 2018). Peptidoglycan is a component of bacterial cell wall, chitin is a component of the fungal cell wall, whereas xylan is a component of hardwood cell wall. In the context of microbial interaction, peptidoglycan deacetylase and chitin deacetylase are virulence factors in bacteria (Boamah *et al.*, 2023) and fungi, respectively (Liu *et al.*, 2023). The roles of polysaccharide deacetylases in fungal virulence include suppress Bax-induced cell death (Xu *et al.*, 2020), suppress chitin-induced plant defence (Cord-Landwehr *et al.*, 2016; Gao *et al.*, 2019; Martínez-Cruz *et al.*, 2021; Xu *et al.*, 2020), promote fungal invasion (Yang *et al.*, 2022), and growth (Martínez-Cruz *et al.*, 2021), inhibit plant cell death (Dai *et al.*, 2021), and involve in cell wall integrity (Rizzi *et al.*, 2021) (Table 2.2).

Based on other pathosystems (Table 2.2), pathogens secrete an array of proteins to interfere host immune to cause successful plant infection. As the current information on *G. boninense* virulence factor is still lacking, with a few candidates with homologs involved in fungal virulence were selected for further characterisation. The candidates selected in this study will provide an insight on *G. boninense* response to host exposure.

Table 2.2 : Reported roles of metalloproteinase, aspartic protease, lipase, and polysaccharide deacetylase in host-pathogen interaction

Pathogen	Host	Gene product	Role	Reference
Colletotrichum graminicola	maize	M36 metalloprotease	• involves in virulence • expressed during biotrophic stage to suppress host immune system	Sanz-Martín *et al.* (2016)
Colletotrichum graminicola	maize	M36 metalloprotease	• transcript expression peaked during lifestyle switching into necrotrophy	Vargas *et al.* (2012)
Fusarium graminearum	wheat	M36 metalloprotease	• involves in virulence • involves in conidia production and spore germination • suppresses host resistance through interaction with host CAMTA protein	Wang *et al.* (2022)
Fusarium oxysporum	tomato	M36 metalloprotease	• involves in virulence • works in synergistic action with serine protease to cleave host chitinase	Jashni *et al.* (2015)
Fusarium oxysporum; Colletotrichum higginsianum; Alternaria brassicicola; Verticillium longisporum; Pyrenopeziza brassicae	mustard plant	M36 metalloprotease	• cleaves host chitinase	Naumann and Wicklow (2013)

Table 2.2 : Continued

Pathogen	Host	Gene product	Role	Reference
Fusarium verticillioides	maize	M36 metalloprotease	• cleaves host chitinase	Naumann *et al.* (2011)
Ustilago maydis	maize	M36 metalloprotease	• involves in virulence • cleaves and deactivates host chitinase	Ökmen *et al.* (2018)
Magnaporthe oryzae	rice	M35 metalloproteinase	• direct binding to R protein to initiate ETI in resistant host	Jia *et al.* (2000)
Magnaporthe oryzae	rice	M35 metalloproteinase	• direct binding to R protein to initiate ETI in resistant host	Jia *et al.* (2000)
Magnaporthe oryzae	rice	M35 metalloproteinase	• involves in virulence • supresses PTI in susceptible host by increases cytochrome c oxidase (COX) activity and decreases the ROS accumulation	Han *et al.* (2021)
Rhizoctonia cerealis	wheat	M43 metalloproteinase	• involves in virulence • expressed during infection • elicits cell death and ROS accumulation • inhibits host chitinase	Pan *et al.* (2020a)
Botrytis cinerea	grape, strawberry, and raspberry	aspartic proteinase	• non-essential for virulence	Ten Have *et al.* (2010)

Table 2.2 : Continued

Pathogen	Host	Gene product	Role	Reference
Glomerella cingulata	apple	aspartic peptidase	• transcript induced during appressorium formation • non-essential for virulence	Plummer *et al.* (2004)
Sclerotinia sclerotiorum	sunflower	non-aspartyl acid protease	• transcript induced during infection and peaked at necrotrophic stage	Poussereau *et al.* (2001a)
Sclerotinia sclerotiorum	sunflower	aspartyl protease	• expressed during infection	Poussereau *et al.* (2001b)
Sclerotinia sclerotiorum	common bean	aspartyl protease	• involves in pathogenic invasion	Bueno *et al.* (2012)
Blumeria graminis	wheat	lipase	• promotes fungal adhesion and development	Feng *et al.* (2009)
Fusarium graminearum	maize, wheat, and barley	lipase	• involves in virulence	Voigt *et al.* (2005)
Fusarium graminearum	maize, wheat, and barley	lipase	• releases fatty acids • suppresses callose biosynthesis and plant immune response	Blümke *et al.* (2014)
Fusarium graminearum	maize, wheat, and barley	lipase	• involves in fungal growth, reproduction, stress responses, pathogenicity, mycotoxin production, and lipase activity	Kim *et al.* (2023)

Table 2.2 : Continued

Pathogen	Host	Gene product	Role	Reference
Fusarium graminearum	maize, wheat, and barley	autophagy-like lipase	• involves in fungal growth, lipid turnover, and pathogenesis	Nguyen *et al.* (2011a)
Stagonospora nodorum	wheat	lipase	• involves in fungal adhesion	Feng *et al.* (2011)
Puccinia striiformis f. sp. *tritici*	wheat	polysaccharide deacetylase	• suppresses Bax-induced cell death • suppresses chitin-induced plant defence	Xu *et al.* (2020)
Verticillium dahliae	cotton, tomato, and sunflower	polysaccharide deacetylase	• converts chitin to ligand-inactive chitosan • avoids chitin-triggered host immunity	Gao *et al.* (2019)
Blumeria gramini	wheat	chitin deacetylase	• promotes fungal invasion by interfering PTI	Yang *et al.* (2022)
Pestalotiopsis sp.	tropical tree	chitin deacetylase	• inactivates chitin elicitor • avoids plant immune system elicitation	Cord-Landwehr *et al.* (2016)
Podosphaera xanthii	cucurbits	chitin deacetylase	• involves in fungal growth • suppresses chitin-triggered immunity	Martínez-Cruz *et al.* (2021)
Pyricularia oryzae	rice	chitin deacetylase	• inhibits plant cell death	Dai *et al.* (2021)
Ustilago maydis	maize	chitin deacetylase	• involves in fungal virulence and cell wall integrity	Rizzi *et al.* (2021)

CHAPTER 3

MATERIALS AND METHODS

3.1 Fungal culture

Ganoderma boninense BLSM5B was provided by Sarawak Tropical Peat Research Institute (TROPI), Kota Samarahan, Sarawak. It was collected from Balingian, Sarawak (GPS coordinate: N02°57'51.7", E112°30'28.8") (Midot *et al.*, 2019), and maintained in tap water to preserve its pathogenicity (Tung *et al.*, 2018), which was confirmed through reinoculation of oil palm seedlings (Lo *et al.*, 2023). The revived culture was maintained on malt extract agar [1.275 % (w/v) maltose, 0.275 % (w/v) dextrin, 0.235 % (w/v) glycerol, 0.078 % (w/v) peptone, 1.5 % (w/v) agar] (MEA) (Difco, US) at 30 °C in dark condition. A mycelial plug (with a diameter of 6 mm) was taken from the actively growing outer zone of a 14-day-old culture and inoculated in 30 mL malt extract broth [0.6 % (w/v) malt extract, 0.18 % (w/v) maltose, 0.6 % (w/v) dextrose, 0.12 % (w/v) yeast extract] (MEB) (Difco, US). The fungal culture was incubated at 30 °C in the dark at static condition for 14 days before the mycelia were harvested and immediately frozen in liquid nitrogen before kept at -80 °C until use.

3.2 Isolation of transcripts encoding putative virulence factors from *G. boninense* BLSM5B

3.2.1 Selection of candidate transcripts and primer design for the isolation of full-length coding sequence (CDS)

Four partial transcripts that were present only in *G. boninense*-treated oil palm root transcriptome were mined from the transcriptome reported by Ho *et al.* (2016). By using Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/), highly similar sequences from white-rot fungi were retrieved from GenBank (e-value < 1e-5; percentage identity > 40%). Gene-specific primers were designed for the amplification of the full-length CDS. The primer sequences were examined for specificity using Primer3 version 0.4.0 (http://bioinfo.ut.ee/primer3-0.4.0/primer3/) (Untergasser *et al.*, 2012), PCR Primer Stats (https://www.bioinformatics.org/sms2/pcr_primer_stats.html) (Stothard, 2000), and Oligonucleotide Properties Calculator version 3.27 (Kibbe, 2007) (http://biotools.nubic.northwestern.edu/OligoCalc.html) (primer design parameters: length: 17–23 nucleotides; GC content: 40–60%; melting temperature, T_m: 48–58 °C; maximum difference in primer pair T_m: 5 °C; absence of hairpin formation). Details of the primers are tabulated in Table 3.1.

Table 3.1 : Details of primers used in this study

Primer	Sequence (5'–3')	GC content (%)	T_m (°C)	T_a (°C)	Expected size of amplicon (bp)
Putative metalloproteinase (ON922904)					
U14090F	ATGCGTCGTTTGTTCGTCG	52.6	56.2	62.7	2481
U14090R	AAATCAGAACGGCCACAGC	52.6	56.1		
U14090qF	GGGTGACTTCTTGGCTACG	57.9	55.5	50.9	93
U14090qR	CGGATACCCTTACCGATGTTT	47.6	54.4		
Putative aspartic protease (ON922905)					
U42611F	ATGTCTCGCGGCCTATTCTC	55.0	56.9	63.7	1251
U42611R	TCAGTTCGTGGTTGCGGT	55.6	57.2		
U42611qF	GTCATCAACGTTGAGGTTGG	50.0	53.9	56.3	110
U42611qR	GAGCTCTGCACGAACGA	58.8	55.0		
Putative lipase (ON922906)					
U56931F	ATGCTTCCTGGACTGTC	52.9	51.5	58.8	939
U56931R	CTTCATTGCTGCAGTTC	47.1	48.6		
U56931qF	ACAGATAATGGGAACGGAGC	50.0	54.6	50.9	113
U56931qR	CCCGCTACATACTCAATCCATAC	47.8	54.9		
Putative polysaccharide deacetylase (ON922907)					
U128397F	ATGAAGCTCGCGCTTGTC	55.6	56.0	62.7	1413
U128397R	CTACAAGAAGAGAGCAAAAGCG	45.5	53.9		
U128397qF	GACGCGCTTGCACTGAAT	55.6	56.5	60.0	114
U128397qR	GATGGTAACACCGGACGAAG	55.0	55.5		
***G. boninense* endogenous control: elongation factor 2 (eef2)**					
GER 1qF	TGGTCAAGAACATCCGTAT	42.1	50.8	54.0	173
GER 2qR	CGCTAACAAAGACAAGGG	50.0	50.9		
***G. boninense* endogenous control: α-tubulin**					
GTR 7qF	GCACCGACTCTGGTGATGCT	60.0	59.9	60.0	100
GTR 8qR	GATAGGCTATGGTCGCGAAG	55.0	55.1		
Sequencing primers					
M13F	GTTTTCCCAGTCACGAC				
M13R	AGTGTGTCCTTTGTCGATACTG				

For CDS isolation, forward primers were suffixed with -F, while reverse primers were suffixed with -R. For qPCR, forward primers were suffixed with -qF, while reverse primers were suffixed with -qR. Primers for *G. boninense* endogenous control were adapted from Lim *et al.* (2014). M13F and M13R are universal primers for sequencing (Messing & Vieira, 1982). Abbreviations: T_a for annealing temperature, T_m for melting temperature, eef2 for elongation factor 2.

3.2.2 Total RNA extraction

The extraction protocol was adapted from Wang *et al.* (2005) with slight modifications. Fungal mycelia (~10 g) retrieved from -80 °C freezer were ground into fine powder in liquid nitrogen using a pestle and mortar. The powder was transferred to a 50 mL Nalgene tube containing 10 mL modified cetyltrimethylammonium bromide (CTAB) buffer [2% (w/v) CTAB, 100 mM Tris-HCl (pH 8.0), 2 M sodium chloride (NaCl), 25 mM ethylenediaminetetraacetic acid (EDTA) (pH 8.0), 2% (w/v) polyvinylpolypyrrolidone (PVPP), 2% (w/v) β-mercaptoethanol]. The sample was incubated in a water bath at 65 °C for 30 min and mixed at every 10 min interval by inverting the tubes. Then, an equal volume of phenol: chloroform: isoamyl alcohol (PCI) (25: 24: 1; saturated phenol at pH 5: chloroform: isoamyl alcohol) was added to the sample, mixed gently, and centrifuged at 4 °C, 12,000 x g for 20 min, before the aqueous layer was collected. Subsequently, the PCI extraction was repeated on the aqueous layer, the collected clear aqueous layer was mixed with lithium chloride to a final concentration of 4.3 M, aliquoted into five 2.0 mL microcentrifuge tubes, and kept at -80 °C overnight. Next, the tubes were centrifuged at 4 °C, 20,000 x g for 20 min. The pellet was dissolved in 300 µL of sterilised ultrapure water (UPW), followed by the addition of 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volume of absolute ethanol, and incubated at -80 °C overnight. The tubes were centrifuged at 4 °C, 20,000 x g for 20 min. The pellet was resuspended in 500 µL of 70 % (v/v) ethanol and centrifuged again. Lastly, the supernatant was discarded, and the pellet was vacuum dried before it was resuspended in 50 µL of ice-cold UPW and stored at -80 °C until use. NanoPhotometer® P-Class (Implen GmbH, Germany) was used to examine the concentration and purity of the RNA samples at wavelengths 230 nm, 260 nm, and 280 nm. RNA samples with A_{260}/A_{280} and A_{260}/A_{230} = ~2.0 were considered to be free of protein and phenolic contaminants, respectively. Then, the RNA sample (~500 µg) was heat-denatured in 1.5X RNA gel loading buffer [95 % (v/v) ultrapure formamide, 0.025 % (w/v) bromophenol blue, 0.025 % (w/v) xylene cyanol FF, and 5 mM EDTA (pH 8.0)] at 70 °C for 10 min. The samples were examined by 1% (w/v) agarose gel electrophoresis, stained by SYBR Safe (Thermo Fisher Scientific, US) and viewed using Amersham™ Typhoon™ Biomolecular Imager (GE Healthcare, US) to check for RNA integrity. In addition, RNA IQ (integrity and quality) assay was carried out to confirm the RNA integrity. RNA IQ number ranges from 1–10, with a higher number (>8) indicating better sample quality (Thermo Fisher Scientific Inc, 2023a). Samples having intact and distinct bands were used for further experiments.

3.2.3 DNase-treatment

DNase-treatment was carried out using DNase I (RNase-free) (NEB, UK) according to the manufacturer's protocol. A volume of 100 µL reaction containing ~10 µg RNA, 1X DNase I Reaction Buffer, and 2 U DNase I was set up on ice. The reaction mixture was incubated at 37 °C for 15 min, followed by the addition of 0.5 M EDTA (1 µL) to a final concentration of 5 mM. Then, the sample was extracted with PCI. Autoclaved UPW (200 µL) and an equal volume of PCI (300

µL) were added to the reaction, mixed well, and centrifuged at 4 °C, 20,000 x g for 20 min. The aqueous layer was transferred to a new 2 mL tube followed by adding 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volume of absolute ethanol. The sample was kept at -80 °C overnight. Then, the tubes were centrifuged at 4 °C, 12,000 x g for 20 min. The pellet was resuspended in 500 µL of 70 % (v/v) ethanol and centrifuged again. Lastly, the supernatant was discarded, and the pellet was vacuum dried before being resuspended in 20 µL of ice-cold UPW and stored at -80 °C until use. The RNA concentration, purity, and integrity were examined as described in subsection 3.2.2.

3.2.4 cDNA synthesis

The first-strand cDNA was synthesised using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) according to the manufacturer's protocol. The reaction mix contained 3 µg total RNA, 8.33 µM oligo $(dT)_{18}$ primer, and nuclease-free water to a total volume of 12 µL. The reaction was mixed, centrifuged, and incubated at 65 °C for 5 min. Then, the reaction was put on ice and added with other components to a final volume of 20 µL containing 1X Reaction Buffer, 1 U RiboLock RNase Inhibitor, 1 mM dNTP mix, and 10 U RevertAid M-MuLV Reverse Transcriptase. After a brief mixing and centrifugation, the reaction was incubated at 42 °C for 60 min and terminated at 70 °C for 5 min. Then, the reaction was kept at -20 °C until use.

3.2.5 Cloning of candidate transcripts

3.2.5.1 Gradient PCR

Each PCR mix (10 µL) contained 1X KAPA HiFi Buffer (Fidelity Buffer for *U42611*, *U56931*, and *U128397*, GC buffer for *U14090*), 0.3 mM KAPA dNTP mix, 0.3 µM forward primer, 0.3 µM reverse primer, and 0.3 U KAPA HiFi DNA polymerase (KAPA Biosystems, US). The primer sequences are listed in Table 3.1. The thermal cycling program was as follows: 95 °C for 3 min, followed by 30 cycles of 98 °C for 20 s, 58.8–67.7 °C for 15 s (T_a range is different for each target, optimum T_a is shown in Table 3.1), 72 °C for 90 s; and a final extension at 72 °C for 90 s. Upon completion of thermal cycling, the amplified PCR products were analysed by 1% (w/v) agarose gel electrophoresis at 100 V for 45 min.

3.2.5.2 Purification of PCR products

QIAquick Gel Extraction Kit (QIAGEN, Germany) was used for PCR product purification. An agarose gel slice containing the expected amplicons was excised and put into a pre-weighed 2.0 mL microcentrifuge tube. The weight of the agarose gel was measured and added with 3 volumes of Buffer QG followed by incubation at 50 °C until the gel slice has fully dissolved. Then, 1 volume of

isopropanol was added to the mixture and mixed gently. The mixture was transferred to the QIAquick column and centrifuged at 17,900 x g for 1 min. After discarding the flow-through, 500 µL of Buffer QG was added to the column and centrifuged again. The flow-through was discarded and 750 µL of PE Buffer was added to the column and incubated for 7 min before centrifugation. Then, the column was transferred to a clean 1.5 mL microcentrifuge tube and eluted with 20 µL of Buffer EB added to the centre of the QIAquick membrane. The column was incubated for 10 min before centrifugation to collect the purified PCR products. Purified PCR product was analysed by 1% (w/v) agarose gel electrophoresis, and quantified by NanoPhotometer® P-Class (Implen GmbH, Germany).

3.2.5.3 Adenylation of purified PCR products

A-tailing reaction mixture (50 µL) was set up for purified PCR products. Each reaction contained 1X Colorless GoTaq Flexi Buffer, 0.2 mM dATP (NEB, UK), 1.5 mM magnesium chloride ($MgCl_2$), 500 ng purified PCR product, and 1 U GoTaq DNA polymerase (Promega, US). After gently mixing and a brief centrifugation (30 s), the tube containing the reaction mixture was incubated at 72 °C for 30 min. Then, the reaction mixture was purified using QIAquick PCR Purification Kit (QIAGEN, Germany) according to the manufacturer's protocol. Purified A-tailed PCR product was quantified by NanoPhotometer® P-Class (Implen GmbH, Germany).

3.2.5.4 Ligation

The ligation reaction (10 µL) contained 1X Ligation Buffer A, 1X Ligation Buffer B, 50 ng T&A cloning vector (Yeastern Biotech, Taiwan), 2 U yT_4 DNA ligase, and purified A-tailed PCR products (1:3 molar ratio of vector to insert). The reaction mixture was gently mixed and briefly centrifuged (30 s) before incubation at 4 °C overnight.

3.2.5.5 Preparation of competent cells

Escherichia coli One Shot TOP10/P3 (genotypes: F^- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L *end*A1 *nup*G / P3: Kan^R Amp^R (am) Tet^R (am)) (Invitrogen, US) was used as cloning host. A single colony of the *E. coli* was picked from a plate culture and inoculated into 10 mL of LB medium [1% (w/v) tryptone, 1% (w/v) sodium chloride, and 0.5% (w/v) yeast extract] and cultured at 37 °C for overnight at 160 rpm. An aliquot of the overnight culture was added at a 1:10 ratio to fresh LB medium and cultured until the absorbance reading at 600 nm reached 0.4–0.6. Then, the bacterial cells were kept on ice for 10 min before being harvested at 4,500 x g, 4 °C for 5 min. The supernatant was discarded, and the cell pellet was gently resuspended in 0.1 volume (of the original culture) of 0.1 M cold $CaCl_2$ (1 mL). The cell suspension was incubated

on ice for 20 min and centrifuged at 4, 500 x g, 4 °C for 5 min. The supernatant was discarded. The cells were resuspended in 0.05 volume of storage media (0.5 mL) [0.1 M cold $CaCl_2$ and 15% (v/v) glycerol]. Lastly, the cell suspension was aliquoted into prechilled 1.5 mL microcentrifuge tubes (300 µL per tube) for storage at -80 °C.

3.2.5.6 Transformation

The ligation mixture was aliquoted into a tube containing 300 µL of *E. coli* One Shot TOP10/P3 competent cells and incubated on ice for 30 min. Then, the mixture was incubated at 42 °C for 45 s before being transferred onto the ice for another 5 min. LB medium was added to make a final volume of 1 mL and incubated at 37 °C, 160 rpm for 1 h. Subsequently, the bacterial culture was plated on LB plate with 100 µg/mL ampicillin, agar surface supplemented with 4 µM of isopropyl β-D-1-thiogalactopyranoside (IPTG) and 1.2 mg of 5-bromo-4-chloro-3-indoly1-β-D-galacto-pyranoside (X-Gal). The plate was incubated at 37 °C overnight.

3.2.5.7 Colony PCR

A total of six single white colonies were picked randomly from the LB plate. Each colony was resuspended in 5 µL of autoclaved UPW and used as a template for colony PCR. The PCR mixture (10 µL) contained 1X Green GoTaq Flexi Buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP mixture, 0.3 mM M13F primer, 0.3 mM M13R primer (as shown in Table 3.1), 1 µL of cell suspension and 0.5 U GoTaq DNA polymerase (Promega, US). The thermal cycling program was as follows: 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 90 s; and a final extension at 72 °C for 7 min. The amplified PCR products were examined by 1% (w/v) agarose gel electrophoresis.

3.2.5.8 Plasmid extraction

The putative positive transformants screened by PCR were inoculated into 10 mL of LB broth supplemented with 100 µg/mL ampicillin and each incubated at 37 °C, 160 rpm for overnight. The bacterial cells were harvested by centrifugation at 8,000 x g, 4 °C for 5 min. The cell pellet was resuspended in 200 µL of ice-cold resuspension buffer or Solution I [50 mM glucose, 25 mM Tris-HCl (pH 8.0), and 10 mM EDTA (pH 8.0)], followed by the addition of 300 µL of lysis buffer or Solution II (0.2 N sodium hydroxide, and 1% sodium dodecyl sulfate, SDS), and 300 µL of ice-cold neutralisation buffer or Solution III (3 M potassium acetate and 2 M glacial acetic acid). The tubes were inverted gently and incubated on ice for 10 min. Then, the tube was centrifuged at 10,000 x g for 10 min. An equal volume of PCI (25:24:1) was then added to the supernatant and centrifuged at 10,000 x g for 10 min. The aqueous phase of the sample was transferred to a new tube added with 0.6 volume of isopropanol and 0.1 volume of 3 M sodium acetate. The mixture was mixed gently and kept at -20 °C for 2 h

to allow nucleic acid precipitation. Then, the tube was centrifuged at 10,000 x g for 5 min, the pellet collected was washed with 600 µL of ice-cold absolute ethanol, centrifuged at 10,000 x g for 5 min before the pellet was being air-dried in a laminar flow cabinet for 15 min. Lastly, the sample was eluted in 30 µL of autoclaved UPW. The sample was checked by 1% (w/v) agarose gel electrophoresis and quantified by NanoPhotometer® P-Class (Implen GmbH, Germany).

3.2.5.9 Restriction enzyme analysis

A reaction mixture (25 µL) containing 1X CutSmart Buffer (50 mM potassium acetate, 20 mM tris-acetate, 10 mM magnesium acetate, 100 µg/mL bovine serum albumin, pH 7.9), 30 U *Hind*III (NEB, UK), and 3 µg of plasmid DNA was prepared. The reaction mixture was incubated at 37 °C for 1 h followed by inactivation at 80 °C for 20 min. Then, 1.0% (w/v) agarose gel electrophoresis was performed. Plasmids containing the insert of the expected size were sent for Sanger sequencing.

3.2.6 Sequence analysis

The chromatograms were examined, and the raw sequences were trimmed using BioEdit Sequence Alignment Editor version 7.2.5 (Hall, 1999). The identity of the sequences was confirmed using BLASTx (https://blast.ncbi.nlm.nih.gov/) (Altschul *et al.*, 1990; Boratyn *et al.*, 2012) against the non-redundant protein sequences (nr) database (default setting). The presence of the sequences in other *G. boninense* strains was also confirmed using BLASTn, searched against the whole genome sequence (wgs) database (default setting). Then, the nucleotide sequences were translated to amino acid sequences using ExPASy translate tool (https://web.expasy.org/translate/) (Artimo *et al.*, 2012), followed by signal peptide identification using SignalP Version 5.0 (https://services.healthtech.dtu.dk/service.php?SignalP-5.0) (Almagro Armenteros *et al.*, 2019), subcellular localisation prediction using WoLF PSORT (https://wolfpsort.hgc.jp/) (Horton *et al.*, 2007), and transmembrane topology prediction using DeepTMHMM (https://dtu.biolib.com/DeepTMHMM) (Hallgren *et al.*, 2022). Transcripts were searched against pathogen-host interactions database (PHI-base) version 4.14 using PHIB-BLAST for their potential pathogenic phenotype or interactions (http://phi-blast.phi-base.org/) (Urban *et al.*, 2021). The top five sequence homologs were aligned to the transcript using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) (Sievers *et al.*, 2011), and motif annotations were derived from the Conserved Domain Database (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) (Lu *et al.*, 2019) on default setting.

Phylogeny construction was carried out using Geneious Prime® version 2022.1.1 (https://www.geneious.com) (Kearse *et al.*, 2012). Sequence homologs sharing sequence similarity >60% were retrieved from NCBI nr database, aligned with Clustal Omega version 1.2.2 (Sievers *et al.*, 2011), and the tree was

built using Geneious tree builder (parameters: Jukes-Cantor genetic distance model; neighbour-joining method; an outgroup from Ascomycota division; 1000 bootstrap resampling; 50% support threshold).

3.3 Gene expression profiling of selected putative virulence factors during fungal growth and plant-pathogen interaction

3.3.1 Primer design for quantitative real-time PCR (qPCR) analysis

All primers used for qPCR were designed using PrimerQuest™ Tool (https://www.idtdna.com) (Integrated DNA Technologies, Coralville, Iowa, USA) (primer design parameters: length: 17–30 nucleotides; GC content: 35–65%; melting temperature, T_m: 59–65 °C; maximum difference in primer pair T_m: 5 °C; absence of hairpin formation; amplicon size: 100–200 bp; forward primer spans the intron/exon boundary), confirmed by PCR Primer Stats from Sequence Manipulation Suite (Stothard, 2000) (https://www.bioinformatics.org/sms2/pcr_primer_stats.html) and synthesised by Integrated DNA Technologies P/L (Singapore). The details of the primers are included in Table 3.1. The primers were used for gradient PCR to check for their optimum annealing temperature, T_a. The primer specificity was confirmed by the standard curve and melting curve. Standard curves were generated spanning 5 orders of magnitude (cDNA at 400 ng, 80 ng, 16 ng, 3.2 ng, and 0.64 ng, respectively), in triplicates. Details of reaction mixture components and thermal cycling parameters are described in sub-section 3.3.3.

3.3.2 Preparation of fungal materials

3.3.2.1 Fungal samples harvested at different time-points

G. boninense BLSM5B was cultured in 30 mL potato dextrose broth (PDB) (20% (w/v) potato infusion and 2% (w/v) dextrose) (HiMedia, India) and incubated at 30 °C in the dark at static condition. The fungal mycelia were harvested at Day-7, Day-14, Day-21, and Day-28 by centrifugation at 4,137 x g for 5 min. Day-7 and Day-14 represent exponential growth stage, whereas Day-21 and Day-28 represent decline stage (Nawawi & Ho, 1990). The collected samples were frozen in liquid nitrogen and kept at -80 °C until use.

3.3.2.2 Phytohormone and elicitor treatments

Seven-day-old *G. boninense* BLSM5B was treated with salicylic acid (SA) (Sigma-Aldrich, US), jasmonic acid (JA) (Duchefa Biochemie, Netherlands), or hydrogen peroxide (H_2O_2) (Sigma-Aldrich, US) in a final concentration of 1 mM in 30 mL PDB according to Ho *et al.* (2020). Untreated fungal culture was used as control. All flasks were incubated for one day at 30 °C in the dark and static

condition. The fungal mycelia were harvested by centrifugation at 4,137 x g for 5 min. The collected samples were stored at -80 °C before RNA extraction.

3.3.2.3 Oil palm *in-planta* experiment

Five-month-old oil palm seedlings of FGV Yangambi ML161 (Deli x Yangambi ML161) were purchased from Sarjoh Plantation Nursery Sdn. Bhd., a CoPN (Code of Good Nursery Practice for Oil Palm Nurseries) certified nursery premise. The seedlings were then uprooted and washed in running tap water until the soil residues were removed. The roots were rinsed in 0.5% (v/v) sodium hypochlorite for 10 min twice and subsequently in 70% (v/v) ethanol for 5 min. The rinsing was repeated twice. Then the seedlings were left overnight in sterilised UPW before final rinsing using sterilised UPW on the day of the experiment. The roots of each seedling were put in contact with a seven-day-old *G. boninense* BLSM5B liquid culture in a 150 mL conical flask containing 30 mL of PDB. Untreated *G. boninense* culture grown under the same condition was used as control. Three *G. boninense* samples in contact with the oil palm seedlings and one untreated *G. boninense* sample were collected at 12-, 24-, and 48-hours post inoculation, respectively. The collected samples kept as described in the previous section. The experiment was repeated. The first experiment and the repeated experiment were designated as *in-planta* 1 and *in-planta* 2, respectively.

3.3.3 Gene expression analysis by qPCR

Total RNA extraction and cDNA synthesis were as described in sub-sections 3.2.2–3.2.4. Real-time PCR was performed using QuantStudio™ 6 Pro Real-Time PCR System (Applied Biosystems, US). Each qPCR reaction (10 µL) was prepared in triplicates containing 20 ng cDNA, 1X PowerUp™ SYBR™ Green Master Mix (Applied Biosystems, US), 0.5 mM forward primer, 0.5 mM reverse primer (refer to Table 3.1), and RT-PCR Grade Water (Invitrogen, US). A no template control (NTC) was also prepared in parallel for each primer pair. The thermal cycling program was set at 50 °C for 2 min, 95 °C for 2 min; 40 cycles of 95 °C for 15 s, 60 °C for 1 min; followed by a melt curve analysis from 60 °C to 95 °C. Data analysis was performed using Design & Analysis Software version 2.4.3 (Thermo Fisher Scientific, 2020). Reference genes were adapted from Lim *et al.* (2014) with elongation factor 2 (eef) and α-tubulin genes for normalization based on the $2^{-\Delta\Delta C}{}_{T}$ method (Livak & Schmittgen, 2001). For gene expression analysis, the transcript abundance of *G. boninense* harvested on Day-14, Day-21, and Day-28 was compared to that of Day-7; the transcript abundance of *G. boninense* treated with SA, JA, or H_2O_2 was compared to that of untreated *G. boninense*; the transcript abundance of *G. boninense* in contact with oil palm seedlings was compared to that of untreated *G. boninense*. T-test was conducted on the Cq (quantification cycle) values at p-value<0.05. Transcript abundance was expressed as RQ (the calculated relative gene expression level). The gene was considered to be differentially expressed when the expression fulfilled both t-test p-value<0.05 and $|\log_2 RQ| \geq 1$.

3.4 Prediction of transcription factor binding sites (TFBS) of selected putative virulence factors in selected *Ganoderma* species

3.4.1 Promoter sequence mining

The 1000 bp upstream sequence from the translational start site was retrieved from NCBI GenBank whole genome sequence (wgs) database for each of the candidate sequences. Details of the sequence were tabulated in Table 3.2.

Table 3.2 : Accession number for the sequences retrieved from GenBank

Organism/ Strain/ BioProject Accession	Nucleotide accession/ Contig number			
	U14090	***U42611***	***U56931***	***U128397***
G. boninense/ G3/ PRJNA421251	PJEW 03000011.1/ contig 11	PJEW 03000008.1/ contig 8	PJEW 03000012.1/ contig 12	PJEW 03000002.1/ contig 2
G. sinense/ ZZ0214-1/ PRJNA42807	AYKW 01000010.1/ contig 18	AYKW 01000056.1/ contig 6	AYKW 01000068.1/ contig 8	AYKW 01000020.1/ contig 27
G. lucidum/ BCRC 36111/ PRJNA564693	JAAIFM 010000066.1/ contig 66	JAAIFM 010000016.1/ contig 16	JAAIFM 010000008.1/ contig 8	JAAIFM 010000001.1 contig 1

3.4.2 TFBS prediction

RStudio Version 2021.9.0.351 (RStudio Team, 2021) was used for a refined TFBS analysis with R package TFBSTools version 1.31.2 (Tan & Lenhard, 2016). The promoter gene sequences were matched against the fungal transcription factor binding profiles in JASPAR2022 (https://jaspar2022.genereg.net/) (Fornes *et al.*, 2020) public database with a cut-off value of 90% using the R package mentioned earlier. Then, the outputs were analysed to identify the profiles unique only to *G. boninense* and illustrated using VennDiagram version 1.7.3 (Chen, 2018) (R scripts available in Appendix H).

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Selection of candidate putative virulence factors for cloning

Pathogens are known to secrete an array of virulence factors during host infection for penetration, immunity evasion, and nutrient acquisition (Doehlemann *et al.*, 2017). The previous finding of Ho *et al.* (2016) provided the first overview of the molecular interactions between oil palm and its fungal pathogen, *G. boninense* and biocontrol agent, *Trichoderma harzianum*. While closing the knowledge gap, it also opened more research questions to be answered. Fungal transcripts found in the *G. boninense*-treated roots provided insight into the *G. boninense* gene expression during its pathogenesis and allowed molecular characterisation of specific genes of interest.

Out of the 3041 fungal transcripts identified, 1395 transcripts were present only in the *G. boninense*-treated oil palm roots but were absent in both untreated and *Trichoderma*-treated oil palm roots (Ho *et al.*, 2016). However, there are uncertainties about the origin of the transcripts, whether they are from the *G. boninense* secretome expressed during pathogenesis or from the residual inoculum. Selection of the candidate genes was therefore rather challenging. Literature search on the possible mechanisms of which the transcripts present only in the *G. boninense*-treatment are responsible, followed by a search against the *G. boninense* whole genome shotgun (wgs) database deposited in NCBI helped to confirm the presence of the candidates in the *G. boninense* genome and select putative virulence factors for the current study.

Four transcripts were selected from a previous study with the following criteria: i. present in the *G. boninense*-treated oil palm root transcriptomes (absent in control), ii. present in whole genome sequence of *G. boninense*, iii. encodes for secreted protein, and iv. has homologs involved in plant pathogenesis. The selected candidates were *Unigene-14090* encoding for putative extracellular elastinolytic metalloproteinase, *Unigene-42611* encoding for putative aspartic protease, *Unigene-56931* encoding for GDSL-like lipase/acylhydrolase, and *Unigene-128397* encoding for polysaccharide deacetylase from carbohydrate esterase family CE4 (Table 4.1).

Table 4.1 : Selected fungal transcripts from *G. boninense*-treated oil palm root transcriptome

Unigene ID	Number of read counts* in			Sequence description
	G	T	UT	
Unigene-14090	832	0	0	extracellular elastinolytic metalloproteinase
Unigene-42611	1208	0	0	aspartic protease
Unigene-56931	52	0	0	GDSL-like lipase/acylhydrolase
Unigene-128397	3171	0	0	polysaccharide deacetylase from carbohydrate esterase family CE4

*Read counts denote the number of gene fragments mapped in the sequencing analysis where samples were treated in G: artificial inoculation with *G. boninense*; T: artificial inoculation with *T. harzianum*; and UT: untreated oil palm seedlings (Adapted from Ho *et al.* (2016)). Unigene is abbreviated as "U-" in the remaining text.

4.2 RNA templates for cloning

The total RNA extracted from *G. boninense* BLSM5B was DNase-treated and checked for integrity and purity before use. Genomic DNA was removed successfully and was not observed in the agarose gel image. Two distinct bands were observed for each RNA sample indicating that the RNA samples were intact (Figure 4.1). The intact RNA was also confirmed by RNA IQ assay with an overall reading >8.7, indicating >87% intact RNA (Thermo Fisher Scientific Inc, 2023a). RNA IQ assay utilized selective dye binding method which is highly specific to RNA thus reducing interference from contaminants. Nanophotometer reading showed that the samples have an A_{260}/A_{280} of 2.0–2.2 and A_{260}/A_{230} of 1.8–2.3, having minimum contamination from proteins and phenolic compounds (Table 4.2). cDNA was synthesized from this RNA sample for gene isolation.

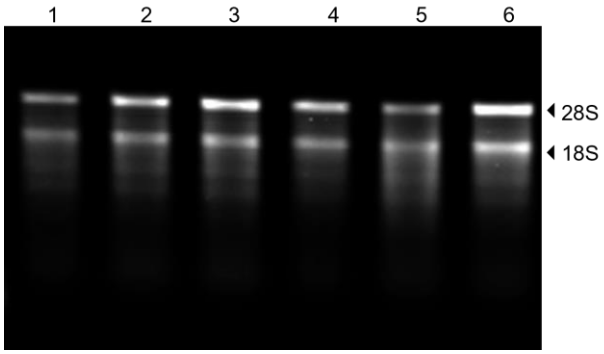


Figure 4.1 : RNA quality of DNase-treated RNA samples. Lanes 1–6: Samples 1–6. The 18S and 28S ribosomal RNA bands are indicated by arrows

Table 4.2 : RNA quality of DNase-treated RNA samples

Sample	RNA IQ	A_{260}/A_{280}	A_{260}/A_{230}
1	8.7	2.2	2.1
2	8.9	2.0	2.4
3	9.1	2.2	2.4
4	9.2	2.2	2.0
5	9.4	2.2	1.8
6	8.9	2.2	2.3

4.3 PCR cloning of putative virulence factors

At the initial stage of this research project, only the genomic data of *G. boninense* from strains NJ3 and G3, of which both were collected from infected oil palm in Indonesia, were available at NCBI. The full-length candidate genes were found in the genomic data of strain G3. Thus, the sequences from strain G3 were chosen as a reference for primer design and transcription factor binding site analysis.

4.3.1 Putative metalloproteinase (*Unigene 14090*)

Initially, the PCR for the cloning of candidate transcript *U14090* encoding for metalloproteinase was carried out using KAPA HiFi Fidelity Buffer at an annealing temperature (T_a) between 62–69 °C. However, the PCR product was in low concentration with primer dimer. When the KAPA HiFi GC Buffer was used, a high concentration of specific PCR product was obtained at T_a of 64.8 °C (Figure 4.2). The expected size of PCR product was ~2,500 bp.

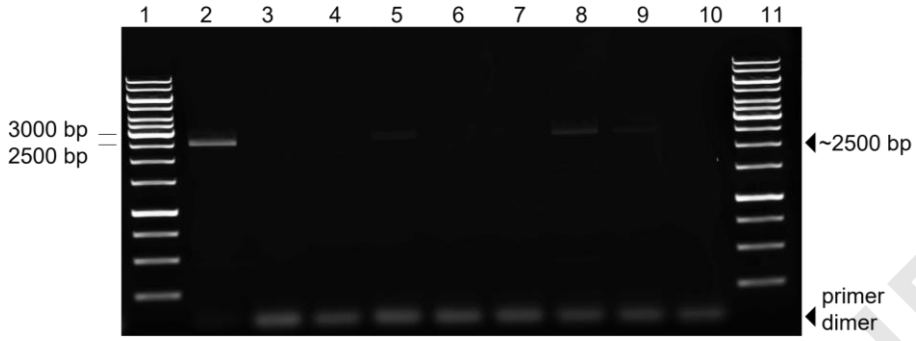


Figure 4.2 : Gel image of PCR products encoding *U14090* amplified *from G. boninense* using different buffers. Lanes 1 and 11: 1kb DNA Ladder, Lane 2: 1X GC buffer, Lane 3: 0.75X GC buffer/0.25X Fid buffer, Lane 4: 0.65X GC buffer/0.35X Fid buffer, Lane 5: 0.5X GC buffer/0.5X Fid buffer, Lane 6: 0.35X GC buffer/0.65X Fid buffer, Lane 7: 0.25X GC buffer/0.75X Fid buffer, Lane 8: 1X Fid buffer, Lane 9: 1X Fid buffer+0.25 mM $MgCl_2$, and Lane 10: negative control. (KAPA HiFi GC Buffer is abbreviated as GC buffer and KAPA HiFi Fidelity Buffer is abbreviated as Fid buffer)

Subsequently, the T_a was optimized at 62.7–67.7 °C using KAPA HiFi GC Buffer, a single specific band was observed at 62.7–66.4 °C, with the brightest band intensity at 62.7 °C (Figure 4.3). Large-scale amplification of *U14090* amplicon was then conducted at 62.7 °C and purified (Figure 4.4). The purified PCR product was ligated to pTA plasmid (pTA/*U14090*), transformed to *E. coli,* and confirmed by colony PCR (Figure 4.5). Five out of six of the selected bacterial colonies contained *U14090* insert of the expected size. The correct gene insertion into the multiple cloning sites region of the plasmid was confirmed by restriction enzyme digestion analysis. The insert was released from the cloning vector upon digestion by restriction enzyme (Hind*III*) flanking the cloning site (Figure 4.6). The pTA/*U14090* plasmid was confirmed by sequencing.

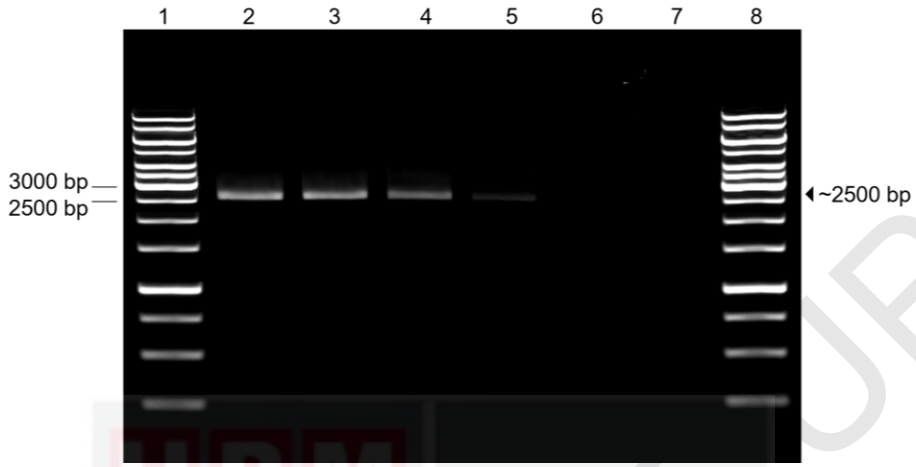


Figure 4.3 : Gel image of PCR products encoding *U14090* amplified from *G. boninense* BLSM5B at different annealing temperatures. Lanes 1 and 8: 1 kb DNA Ladder, Lanes 2–6: PCR products amplified at T_a of 62.7 °C, 63.7 °C, 64.8 °C, 66.4 °C, and 67.7 °C respectively, and Lane 7: negative control

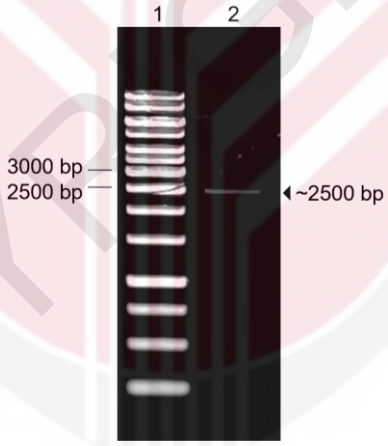


Figure 4.4 : Gel image of *U14090* purified PCR products. Lane 1: 1 kb DNA Ladder, Lane 2: purified PCR product

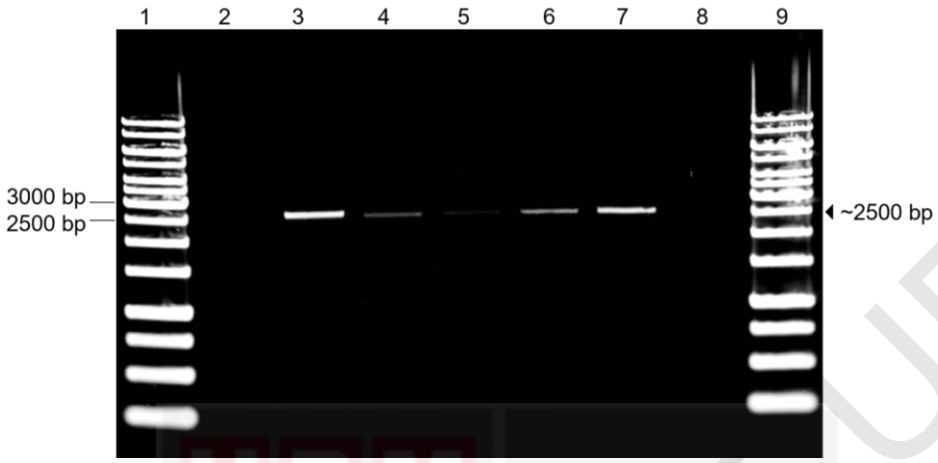


Figure 4.5 : Gel image of *U14090* colony PCR products. Lanes 1 and 9: 1 kb DNA Ladder, Lanes 2–7: colonies 1–6, and Lane 8: negative control

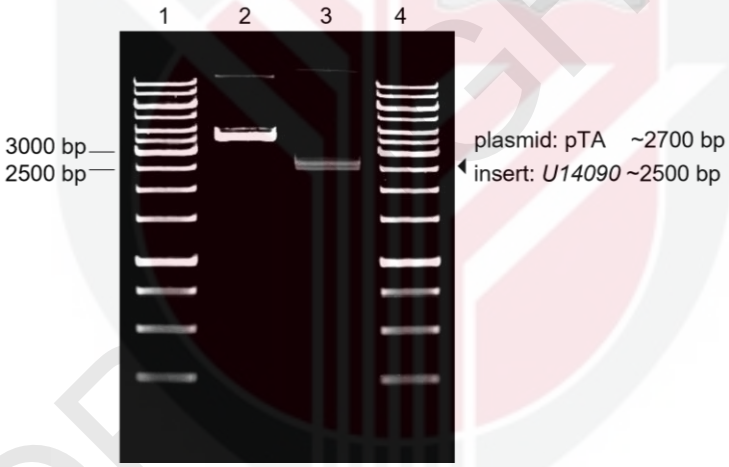


Figure 4.6 : Gel image of *U14090* purified plasmid DNA and products of *Hind*III restriction enzyme analysis. Lanes 1 and 4: 1 kb DNA Ladder, Lane 2: Undigested pTA/*U14090* plasmid DNA, and Lane 3: *Hind*III-treated plasmid DNA

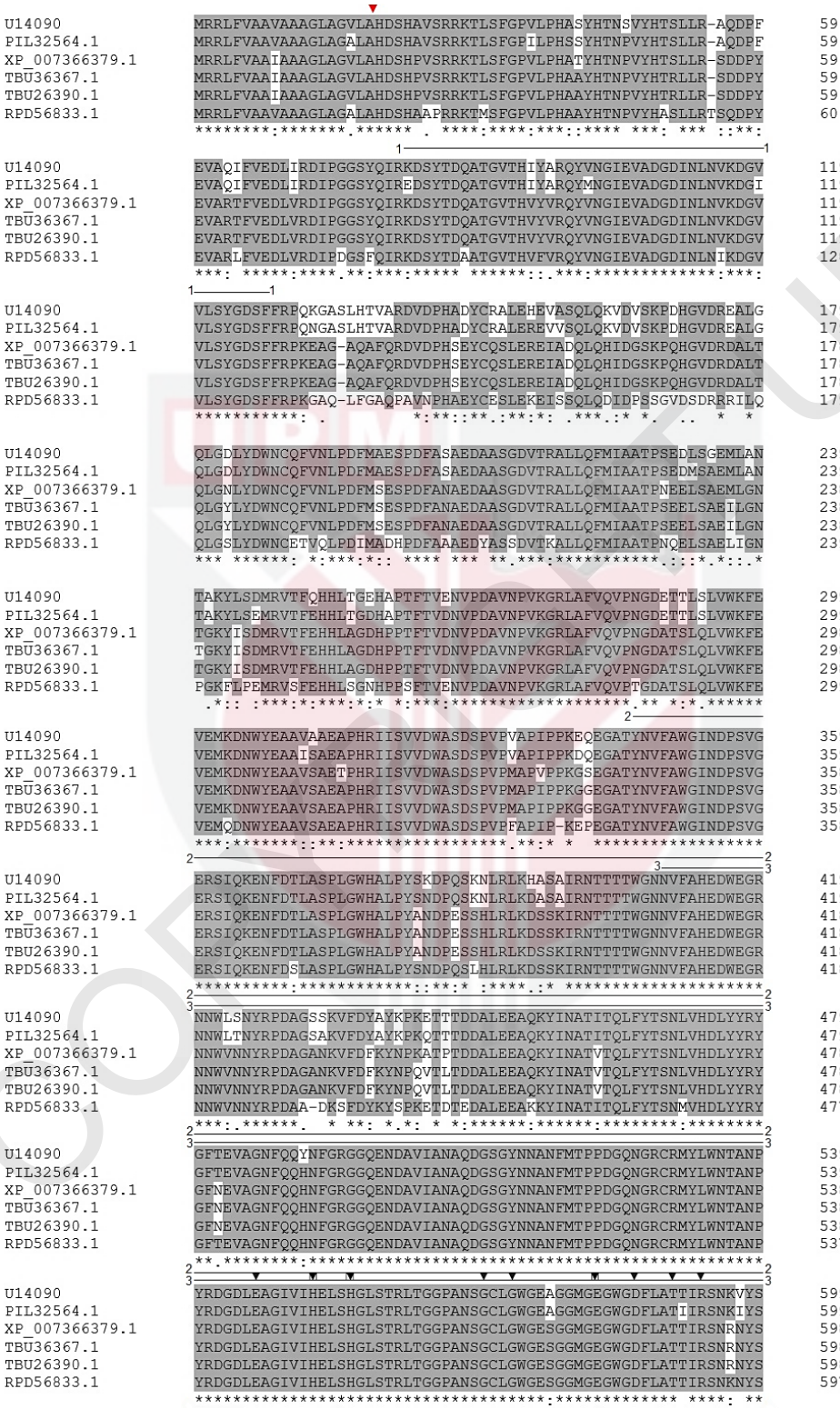
From the sequencing result, the full-length CDS was found to be 2,481 bp encoding 826 amino acids. BLASTx analysis showed that the cloned *U14090* shares 94% sequence similarities to a sequence from *G. sinense*, and up to 87% to sequences from other white-rot fungi such as *Dichomitus squalens* and *Lentinus tigrinus* (Table 4.3).

Table 4.3 : BLASTx results for *U14090* encoding metalloproteinase isolated from *G. boninense* BLSM5B

Description	E-value	Identity	Accession
Ganoderma sinense ZZ0214-1	0.0	94%	PIL32564.1
Dichomitus squalens LYAD-421 SS1	0.0	87%	XP_007366379.1
Dichomitus squalens	0.0	87%	TBU36367.1
Dichomitus squalens	0.0	87%	TBU26390.1
Lentinus tigrinus ALCF2SS1-6	0.0	82%	RPD56833.1

The translated amino acid sequence of *U14090* was predicted to contain a signal peptide cleavage site at position 19–20, with no transmembrane domains. It was predicted to be localised extracellularly. Three conserved domains were predicted by Conserved Domain Database (CDD), which included pfam07504: fungalysin/thermolysin propeptide (FTP) motif (amino acids: 82–127), pfam02128: fungalysin metallopeptidase (M36) (amino acids: 346–762), and cd09596: peptidase M36 family (amino acids: 409–757) which contains HExxH (active site) motif (amino acids: 552–556) (Lu *et al.*, 2019) (Figure 4.7).

According to the Fungal Secretome KnowledgeBase (FunSecKB; http://proteomics.ysu.edu/secretomes/fungi.php) which contains >478 k fungal protein sequences from 118 fungi, peptidase M36 family contains many fungal-secreted proteins (Lum & Min, 2011). FTP which was suggested to prevent premature enzyme activation or work as a chaperone for proper enzyme folding, is a conserved domain found in the propeptide of M4 and M36 peptidases (Markaryan *et al.*, 1996; Tang *et al.*, 2003). The presence of a HExxH motif in *U14090* could imply the functional conservation of M36 metalloproteinase having a chaperone prodomain. M36 metalloproteinase with a mutated prodomain containing FTP motif has non-functional proteolytic activity and was incapable of penetrating the host barrier (Na Pombejra *et al.*, 2018).


U14090 MRRLFVAAVAAAGLAGVLAHDSHAVSRRKTLSFGPVLPHASYHTNSVYHTSLLR-AQDPF 59
PIL32564.1 MRRLFVAAVAAAGLAGALAHDSHAVSRRKTLSFGPILPHSSYHTNPVYHTSLLR-AQDPF 59
XP_007366379.1 MRRLFVAAIAAAGLAGVLAHDSHPVSRRKTLSFGPVLPHATYHTNPVYHTSLLR-SDDPY 59
TBU36367.1 MRRLFVAAIAAAGLAGVLAHDSHPVSRRKTLSFGPVLPHAAYHTNPVYHTRLLR-SDDPY 59
TBU26390.1 MRRLFVAAIAAAGLAGVLAHDSHPVSRRKTLSFGPVLPHAAYHTNPVYHTRLLR-SDDPY 59
RPD56833.1 MRRLFVAAVAAAGLAGALAHDSHAAPRRKTMSFGPVLPHAAYHTNPVYHASLLRTSQDPY 60
U14090 EVAQIFVEDLIRDIPGGSYQIRKDSYTDQATGVTHIVARQYVNGIEVADGDINLNVKDGV 119
PIL32564.1 EVAQIFVEDLIRDIPGGSYQIREDSYTDQATGVTHIVARQYMNGIEVADGDINLNVKDGI 119
XP_007366379.1 EVARTFVEDLVRDIPGGSYQIRKDSYTDQATGVTHVYVRQYVNGIEVADGDINLNVKDGV 119
TBU36367.1 EVARTFVEDLVRDIPGGSYQIRKDSYTDQATGVTHVYVRQYVNGIEVADGDINLNVKDGV 119
TBU26390.1 EVARTFVEDLVRDIPGGSYQIRKDSYTDQATGVTHVYVRQYVNGIEVADGDINLNVKDGV 119
RPD56833.1 EVARLFVEDLVRDIPDGSFQIRKDSYTDAATGVTHVFVRQYVNGIEVADGDINLNIKDGV 120
U14090 VLSYGDSFFRPQKGASLHTVARDVDPHADYCRALEHFVASQLQKVDVSKPDHGVDREALG 179
PIL32564.1 VLSYGDSFFRPQNGASLHTVARDVDPHADYCRALEREVVSQLQKVDVSKPDHGVDREALG 179
XP_007366379.1 VLSYGDSFFRPKEAG-AQAFQRDVDPHSEYCQSLEREIADQLQHIDGSKPQHGVDRDALT 178
TBU36367.1 VLSYGDSFFRPKEAG-AQAFQRDVDPHSEYCQSLEREIADQLQHIDGSKPQHGVDRDALT 178
TBU26390.1 VLSYGDSFFRPKEAG-AQAFQRDVDPHSEYCQSLEREIADQLQHIDGSKPQHGVDRDALT 178
RPD56833.1 VLSYGDSFFRPKGAQ-LFGAQPAVNPHAEYCESLEKEISSQLQDIDPSSGVDSDRRRILQ 179
U14090 QLGDLYDWNCQFVNLPDFMAESPDFASAEDAASGDVTRALLQFMIAATPSEDLSGEMLAN 239
PIL32564.1 QLGDLYDWNCQFVNLPDFMAESPDFASAEDAASGDVTRALLQFMIAATPSEDMSAEMLAN 239
XP_007366379.1 QLGNLYDWNCQFVNLPDFMSESPDFANAEDAASGDVTRALLQFMIAATPNEELSAEMLGN 238
TBU36367.1 QLGYLYDWNCQFVNLPDFMSESPDFANAEDAASGDVTRALLQFMIAATPSEELSAEILGN 238
TBU26390.1 QLGYLYDWNCQFVNLPDFMSESPDFANAEDAASGDVTRALLQFMIAATPSEELSAEILGN 238
RPD56833.1 QLGSLYDWNCETVQLPDIMADHPDFAAAEDYASSDVTKALLQFMIAATPNQELSAELIGN 239
U14090 TAKYLSDMRVTFQHHLTGEHAPTFTVENVPDAVNPVKGRLAFVQVPNGDETTLSLVWKFE 299
PIL32564.1 TAKYLSEMRVTFEHHLTGDHAPTFTVDNVPDAVNPVKGRLAFVQVPNGDETTLSLVWKFE 299
XP_007366379.1 TGKYISDMRVTFEHHLAGDHPPTFTVDNVPDAVNPVKGRLAFVQVPNGDATSLQLVWKFE 298
TBU36367.1 TGKYISDMRVTFEHHLAGDHPPTFTVDNVPDAVNPVKGRLAFVQVPNGDATSLQLVWKFE 298
TBU26390.1 TGKYISDMRVTFEHHLAGDHPPTFTVDNVPDAVNPVKGRLAFVQVPNGDATSLQLVWKFE 298
RPD56833.1 PGKFLPEMRVSFEHHLSGNHPPSFTVENVPDAVNPVKGRLAFVQVPTGDATSLQLVWKFE 299
U14090 VEMKDNWYEAAVAAEAPHRIISVVDWASDSPVPVAPIPPKEQEGATYNVFAWGINDPSVG 359
PIL32564.1 VEMKDNWYEAAISAEAPHRIISVVDWASDSPVPVAPIPPKDQEGATYNVFAWGINDPSVG 359
XP_007366379.1 VEMKDNWYEAAVSAETPHRIISVVDWASDSPVPMAPVPPKGSEGATYNVFAWGINDPSVG 358
TBU36367.1 VEMKDNWYEAAVSAEAPHRIISVVDWASDSPVPMAPIPPKGGEGATYNVFAWGINDPSVG 358
TBU26390.1 VEMKDNWYEAAVSAEAPHRIISVVDWASDSPVPMAPIPPKGGEGATYNVFAWGINDPSVG 358
RPD56833.1 VEMQDNWYEAAVSAEAPHRIISVVDWASDSPVPFAPIP-KEPEGATYNVFAWGINDPSVG 358
U14090 ERSIQKENFDTLASPLGWHALPYSKDPQSKNLRLKHASAIRNTTTTWGNNVFAHEDWEGR 419
PIL32564.1 ERSIQKENFDTLASPLGWHALPYSNDPQSKNLRLKDASAIRNTTTTWGNNVFAHEDWEGR 419
XP_007366379.1 ERSIQKENFDTLASPLGWHALPYANDPESSHLRLKDSSKIRNTTTTWGNNVFAHEDWEGR 418
TBU36367.1 ERSIQKENFDTLASPLGWHALPYANDPESSHLRLKDSSKIRNTTTTWGNNVFAHEDWEGR 418
TBU26390.1 ERSIQKENFDTLASPLGWHALPYANDPESSHLRLKDSSKIRNTTTTWGNNVFAHEDWEGR 418
RPD56833.1 ERSIQKENFDSLASPLGWHALPYSNDPQSLHLRLKDSSKIRNTTTTWGNNVFAHEDWEGR 418
U14090 NNWLSNYRPDAGSSKVFDYAYKPKETTTDDALEEAQKYINATITQLFYTSNLVHDLYYRY 479
PIL32564.1 NNWLTNYRPDAGSAKVFDYAYKPKQTTTDDALEEAQKYINATITQLFYTSNLVHDLYYRY 479
XP_007366379.1 NNWVNNYRPDAGANKVFDFKYNPKATPTDDALEEAQKYINATVTQLFYTSNLVHDLYYRY 478
TBU36367.1 NNWVNNYRPDAGANKVFDFKYNPQVTLTDDALEEAQKYINATVTQLFYTSNLVHDLYYRY 478
TBU26390.1 NNWVNNYRPDAGANKVFDFKYNPQVTLTDDALEEAQKYINATVTQLFYTSNLVHDLYYRY 478
RPD56833.1 NNWVNNYRPDAA-DKSFDYKYSPKETDTEDALEEAKKYINATITQLFYTSNMVHDLYYRY 477
U14090 GFTEVAGNFQQYNFGRGGQENDAVIANAQDGSGYNNANFMTPPDGQNGRCRMYLWNTANP 539
PIL32564.1 GFTEVAGNFQQHNFGRGGQENDAVIANAQDGSGYNNANFMTPPDGQNGRCRMYLWNTANP 539
XP_007366379.1 GFNEVAGNFQQHNFGRGGQENDAVIANAQDGSGYNNANFMTPPDGQNGRCRMYLWNTANP 538
TBU36367.1 GFNEVAGNFQQHNFGRGGQENDAVIANAQDGSGYNNANFMTPPDGQNGRCRMYLWNTANP 538
TBU26390.1 GFNEVAGNFQQHNFGRGGQENDAVIANAQDGSGYNNANFMTPPDGQNGRCRMYLWNTANP 538
RPD56833.1 GFTEVAGNFQQHNFGRGGQENDAVIANAQDGSGYNNANFMTPPDGQNGRCRMYLWNTANP 537
U14090 YRDGDLEAGIVIHELSHGLSTRLTGGPANSGCLGWGEAGGMGEGWGDFLATTIRSNKVYS 599
PIL32564.1 YRDGDLEAGIVIHELSHGLSTRLTGGPANSGCLGWGEAGGMGEGWGDFLATTIRSNKIYS 599
XP_007366379.1 YRDGDLEAGIVIHELSHGLSTRLTGGPANSGCLGWGESGGMGEGWGDFLATTIRSNRNYS 598
TBU36367.1 YRDGDLEAGIVIHELSHGLSTRLTGGPANSGCLGWGESGGMGEGWGDFLATTIRSNRNYS 598
TBU26390.1 YRDGDLEAGIVIHELSHGLSTRLTGGPANSGCLGWGESGGMGEGWGDFLATTIRSNRNYS 598
RPD56833.1 YRDGDLEAGIVIHELSHGLSTRLTGGPANSGCLGWGESGGMGEGWGDFLATTIRSNKNYS 597

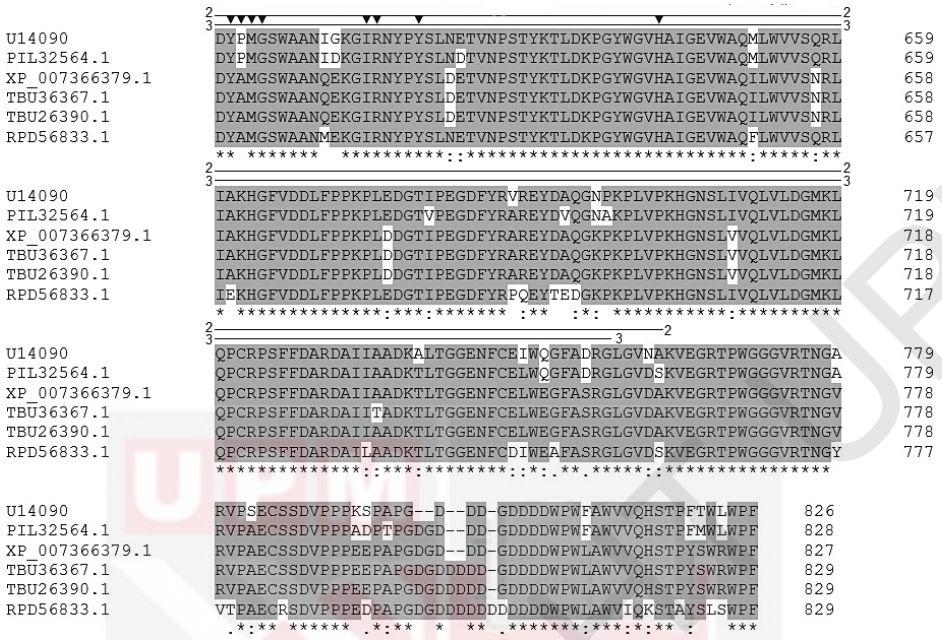


Figure 4.7 : Multiple sequence alignment of the *U14090* metalloproteinase based on BLASTX result. Fully conserved amino acid residues are labelled with '*', strongly similar properties (Gonnet PAM 250 matrix > 0.5) are labelled with ':', weakly similar properties (Gonnet PAM 250 matrix < 0.5 and > 0) are labelled with '.', residues conservation >50% are shaded in grey. The active sites are indicated by '▼', zinc binding sites are indicated by '□', and signal peptide cleavage site is indicated by '▼'. 1: fungalysin/thermolysin propeptide (FTP) motif, 2: fungalysin metallopeptidase (M36), and 3: peptidase M36 family

Phylogeny analysis revealed that *U14090* from *G. boninense* BLSM5B clustered together with the fungal metalloproteinase sequences from the order Polyporales of the division Basidiomycota (Figure 4.8). The most similar sequence of *U14090* is from *G. sinense*, the *Ganoderma* species often used for medicinal purposes, with 94% sequence similarity. Despite the function of metalloproteinase from Polyporales is not known, it may have chitinase cleaving activity which interferes with plant resistance as found in the metalloproteinases from other fungal pathogens (Jashni *et al.*, 2015; Naumann *et al.*, 2011; Ökmen *et al.*, 2018).

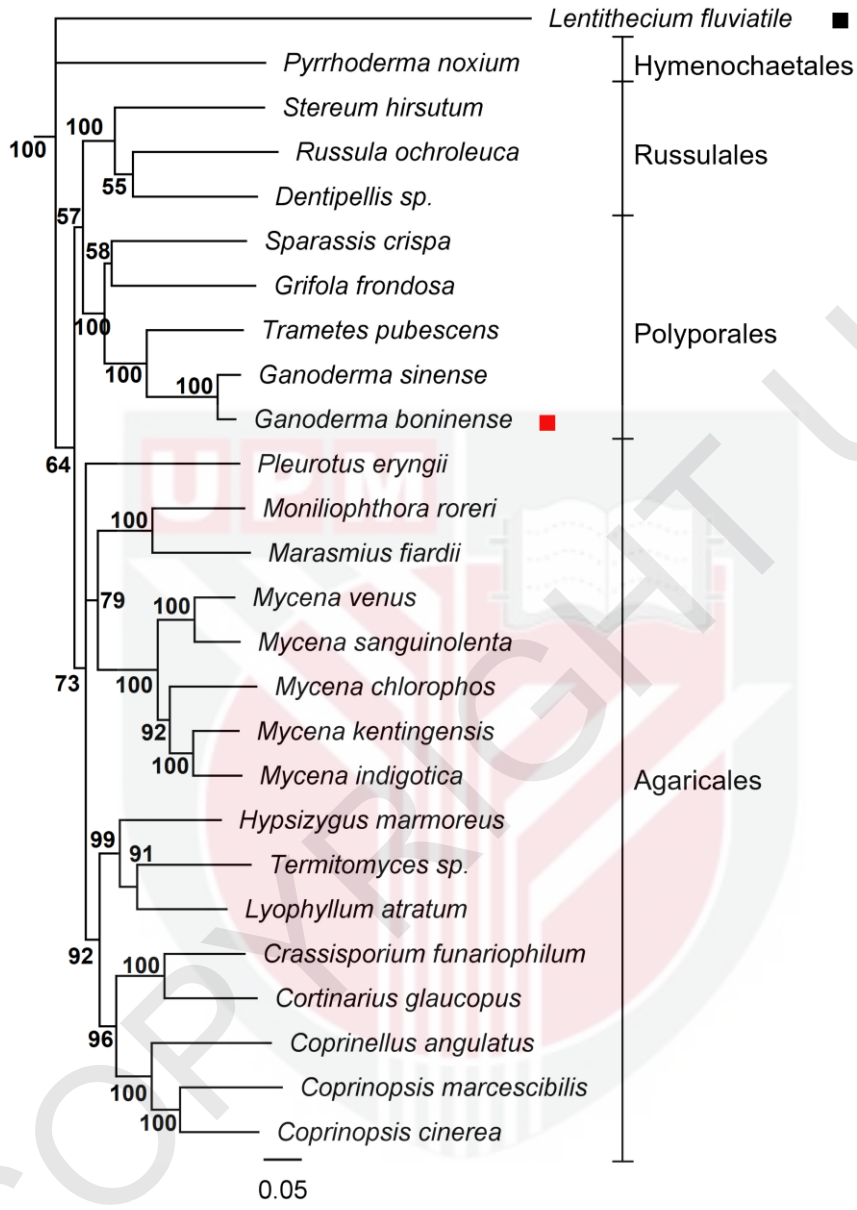


Figure 4.8 : Phylogenetic tree inferred from *U14090* amino acid sequence using the neighbour-joining method. The bootstrap support % based on 1000 replicates is indicated next to the node and the scale bar corresponds to 0.05 substitutions per site. The outgroup from Ascomycota division is labelled with '■'. The sequence obtained from this study is labelled with '■', otherwise were retrieved from GenBank. Details of sequences obtained from GenBank are included in Appendix A

According to the PHI-base (http://www.phi-base.org/), a curated database for experimentally validated pathogen-host interaction, *U14090* is mapped to *Cgfl* from *Colletotrichum graminicola*, which encodes for fungalysin metalloproteinase (Table 4.4). It displayed reduced virulence in gene deletion mutant (Sanz-Martín *et al.*, 2016). Thus, *U14090* might function as a virulence factor in *G. boninense* but this remains to be determined experimentally.

Table 4.4 : Sequence homologs of *U14090* involved in pathogenesis

PHI No.	Gene	Pathogen/ Host	Gene Mutant
PHI:12176	*Cgfl*	*Colletotrichum graminicola/ Zea mays*	effector (plant avirulence determinant)

U14090 may encode for a putative secreted metalloproteinase under the M36 fungalysin family (elastinolytic metalloproteinase) which clustered with shelf fungi often associated with wood rot. Elastinolytic metalloproteinase is one of the important virulence factors for invasive aspergillosis, which was secreted during the host cell penetration (Markaryan *et al.*, 1994). It was first cloned and sequenced from pathogenic *Aspergillus fumigatus* (Sirakova *et al.*, 1994). A few M36 metalloproteinase have been reported in other plant pathogens, which included *Alternaria brassicicola*, *Colletotricum graminicola*, *C. higginsianum*, *Fusarium oxysporum*, *F. graminearum*, *F. verticillioides*, *Puccina helianthi*, *Pyrenopeziza brassicae*, *Ustilago maydis*, and *Verticillium longisporum*, but not in *G. boninense* (Jashni *et al.*, 2015; Lu *et al.*, 2021; Naumann & Wicklow, 2013; Naumann *et al.*, 2011; Ökmen *et al.*, 2018; Sanz-Martín *et al.*, 2016; Vargas *et al.*, 2012). The transcript sequence of *U14090* was deposited in GenBank with the accession number ON922904.

4.3.2 Putative aspartic protease (*Unigene 42611*)

Candidate transcript *U42611* encoding aspartic protease was first amplified by gradient PCR to identify the optimum T_a. The expected size of PCR product was ~1,300 bp. When a range of T_a from 62–66.4 °C were used for gradient PCR, a single, specific band was observed (Figure 4.9). The brightest band was observed at T_a of 63.7 °C. Large-scale amplification of *U42611* amplicon was then conducted at 63.7 °C and purified (Figure 4.10).

The purified PCR product was ligated to pTA plasmid (pTA/*U42611*), transformed to *E. coli*, and confirmed by colony PCR (Figure 4.11). Four out of six of the selected bacterial colonies contained *U42611* insert of the expected size. The correct gene insertion into the multiple cloning sites region of the plasmid was confirmed by restriction enzyme digestion analysis. The insert was released from the cloning vector upon digestion by *Hind*III flanking the cloning site (Figure 4.12). The pTA/*U42611* plasmid was sent for sequencing.

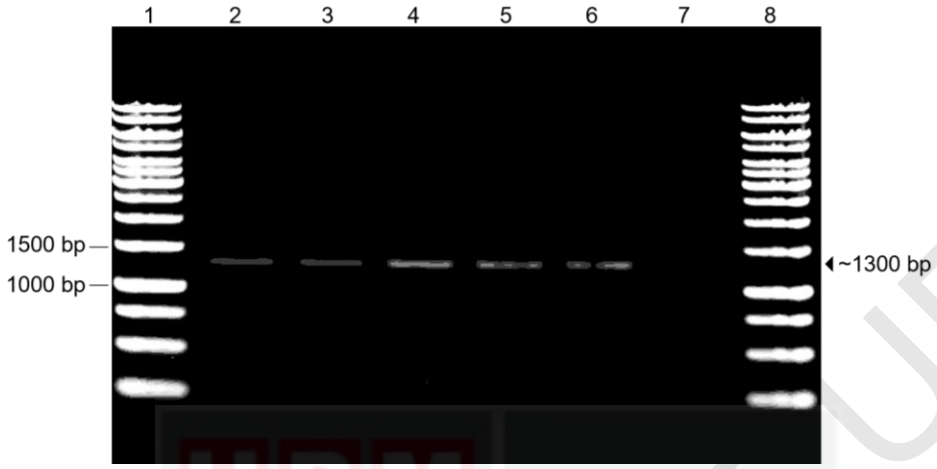


Figure 4.9 : Gel image of PCR products encoding *U42611* amplified from *G. boninense* BLSM5B at different annealing temperatures. Lanes 1 and 8: 1 kb DNA Ladder, Lanes 2–6: PCR products amplified at T_a of 62.0 °C, 62.7 °C, 63.7 °C, 64.8 °C, and 66.4 °C respectively, and Lane 7: negative control (63.7 °C)

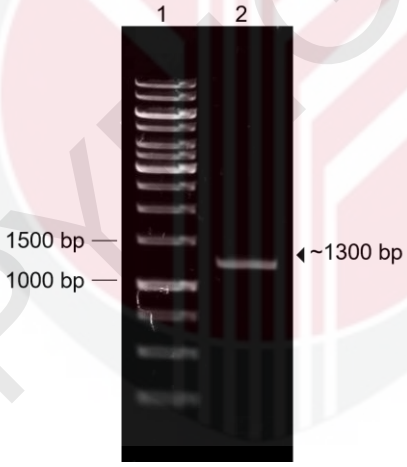


Figure 4.10 : Gel image of *U42611* purified PCR products. Lane 1: 1 kb DNA Ladder, Lane 2: purified PCR product

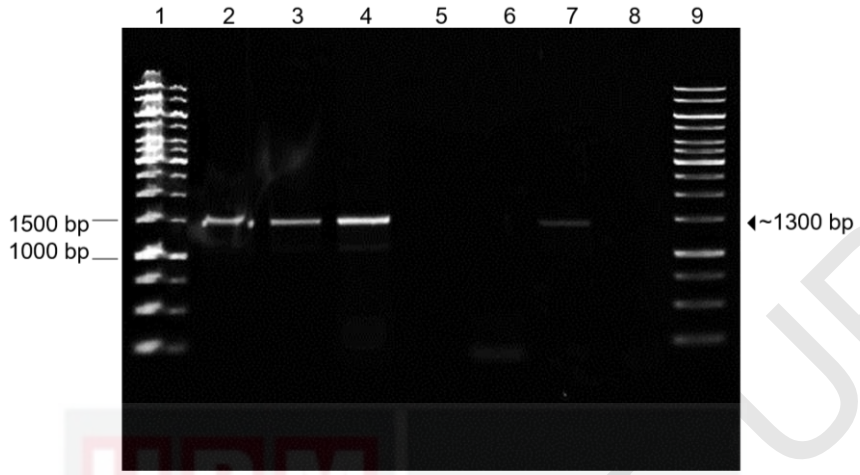


Figure 4.11 : Gel image of *U42611* colony PCR products. Lanes 1 and 9: 1 kb DNA Ladder, Lanes 2–7: colonies 1–6, and Lane 8: negative control

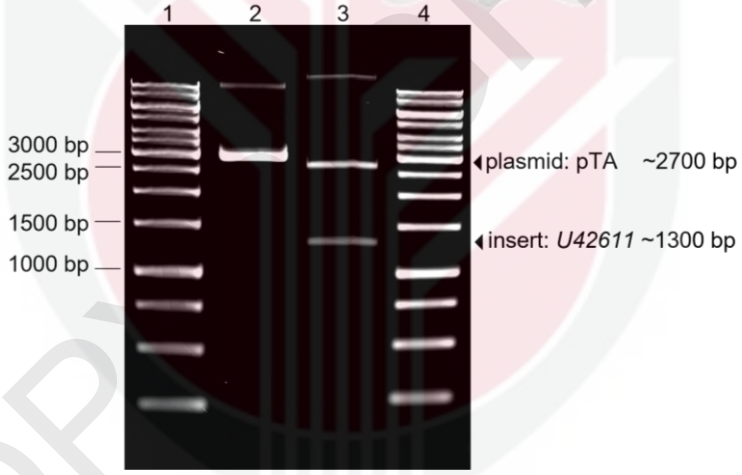


Figure 4.12 : Gel image of *U42611* purified plasmid DNA and products of *Hind*III restriction enzyme analysis. Lanes 1 and 4: 1 kb DNA Ladder; Lane 2: Undigested pTA/*U42611* plasmid DNA; and Lane 3: *Hind*III-treated plasmid DNA

From the sequencing result, the full-length CDS was found to be 1,251 bp encoding 416 amino acids. BLASTx analysis showed that the cloned *U42611* shares 92% sequence similarities with a sequence homolog from *G. sinense*, and up to 81–85% to sequences from other white-rot fungi such as *D. squalens*, *Trametes cinnabarina*, *Polyporus brumalis*, and *T. coccinea* (Table 4.5).

Table 4.5 : BLASTx results for *U42611* encoding aspartic protease isolated from *G. boninense* BLSM5B

Description	E-value	Identity	Accession
Ganoderma sinense ZZ0214-1	0.0	92%	PIL25289.1
Dichomitus squalens	0.0	85%	TBU39746.1
Trametes cinnabarina	0.0	81%	CDO71514.1
Polyporus brumalis	0.0	81%	RDX41559.1
Trametes coccinea BRFM310	0.0	82%	OSD00664.1

The translated amino acid sequence of *U42611* was predicted to contain a signal peptide cleavage site at position 20–21, with no transmembrane domains. It was predicted to be localised extracellularly. Three conserved domains were predicted by CDD, which included cd05471: pepsin-like aspartic protease (amino acids: 88–406), pfam00026: eukaryotic aspartyl protease (amino acids: 88–406), and PTZ00013: plasmepsin 4 (PM4) (amino acids: 93–406) (Lu *et al.*, 2019) (Figure 4.13).

All the three domains could be grouped under the same superfamily (cl11403: pepsin_retropepsin_like superfamily) (Lu *et al.*, 2019). Pepsin-like aspartic protease is categorised as the peptidase family A1 (pepsin A, clan AA) which is usually secreted as a zymogen that undergone autocatalysis at acidic pH (Davies, 1990; Rawlings & Barrett, 1995), whereas plasmepsin 4 is an aspartic proteinase from the malaria parasite, which plays a role in the parasite's virulence and is a potential target for drug development (Dame *et al.*, 2003; Liu, 2017; Spaccapelo *et al.*, 2011). The presence of the domains in *U42611* indicates the gene product might possess similar virulent properties as in plasmepsin 4.

Phylogeny analysis revealed that *U42611* from *G. boninense* BLSM5B clustered together with the fungal aspartic protease amino acid sequences from the order Polyporales of the division Basidiomycota (Figure 4.14). The most similar sequence homolog of *U42611* is from *G. sinense*, of the same genus. Aspartic protease was suggested to help pathogen to obtain nutrients from its host (Hislop *et al.*, 1982). Recently it was found to possess chitinase degrading ability and its actual involvement in plant-pathogen interaction is yet to be demonstrated (Theron *et al.*, 2017, 2018; Van Sluyter *et al.*, 2013).

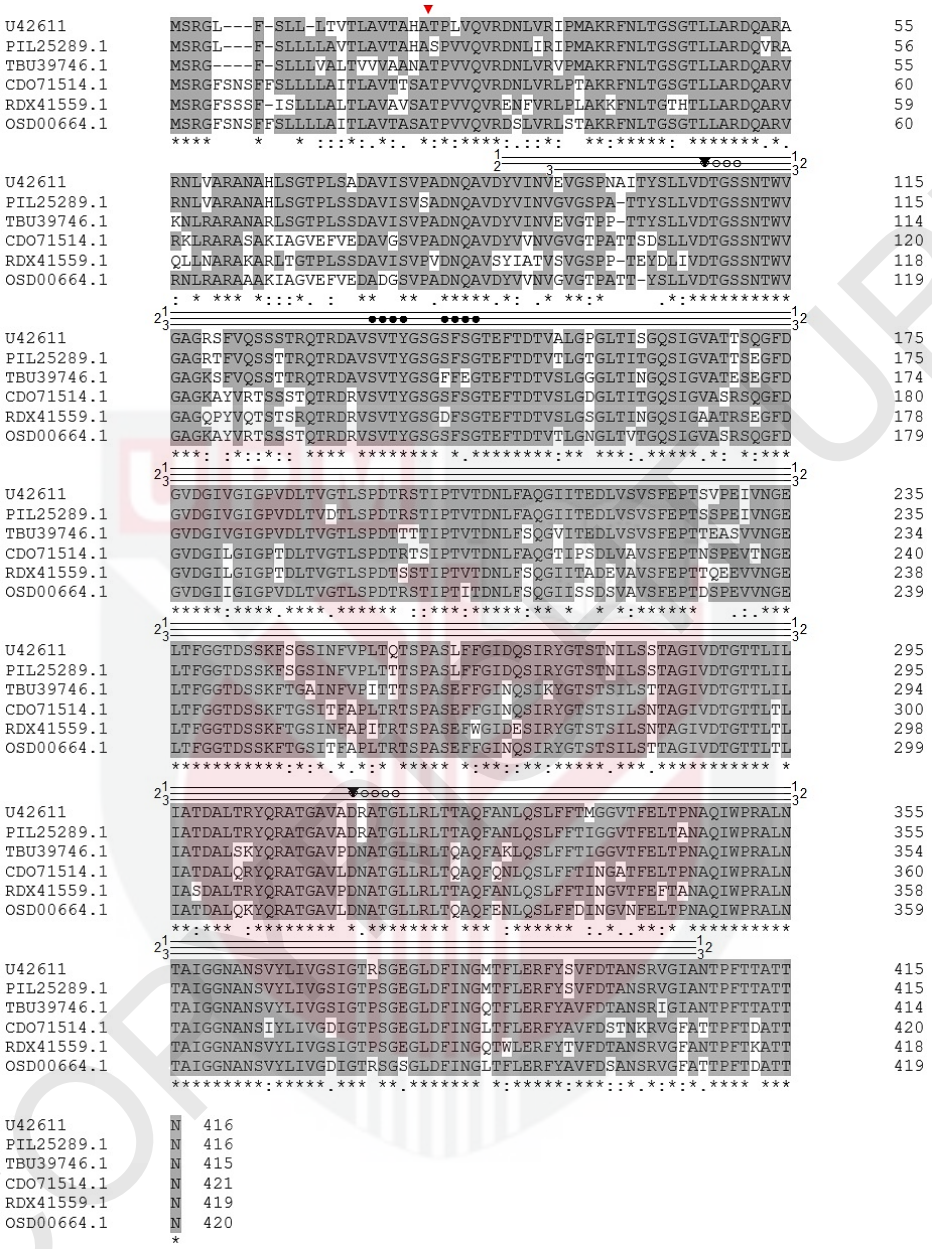


Figure 4.13 : Multiple sequence alignment of the *U42611* aspartic protease based on BLASTX result. Fully conserved amino acid residues are labelled with '*', strongly similar properties (Gonnet PAM 250 matrix > 0.5) are labelled with ':', weakly similar properties (Gonnet PAM 250 matrix < 0.5 and > 0) are labelled with '.', residues conservation >50% are shaded in grey. The catalytic active sites are indicated by '▼', catalytic motif by '○', active site flap by '●', and signal peptide cleavage site is indicated by '▼'. 1: pepsin-like aspartic protease, 2: eukaryotic aspartyl protease, and 3: plasmepsin 4

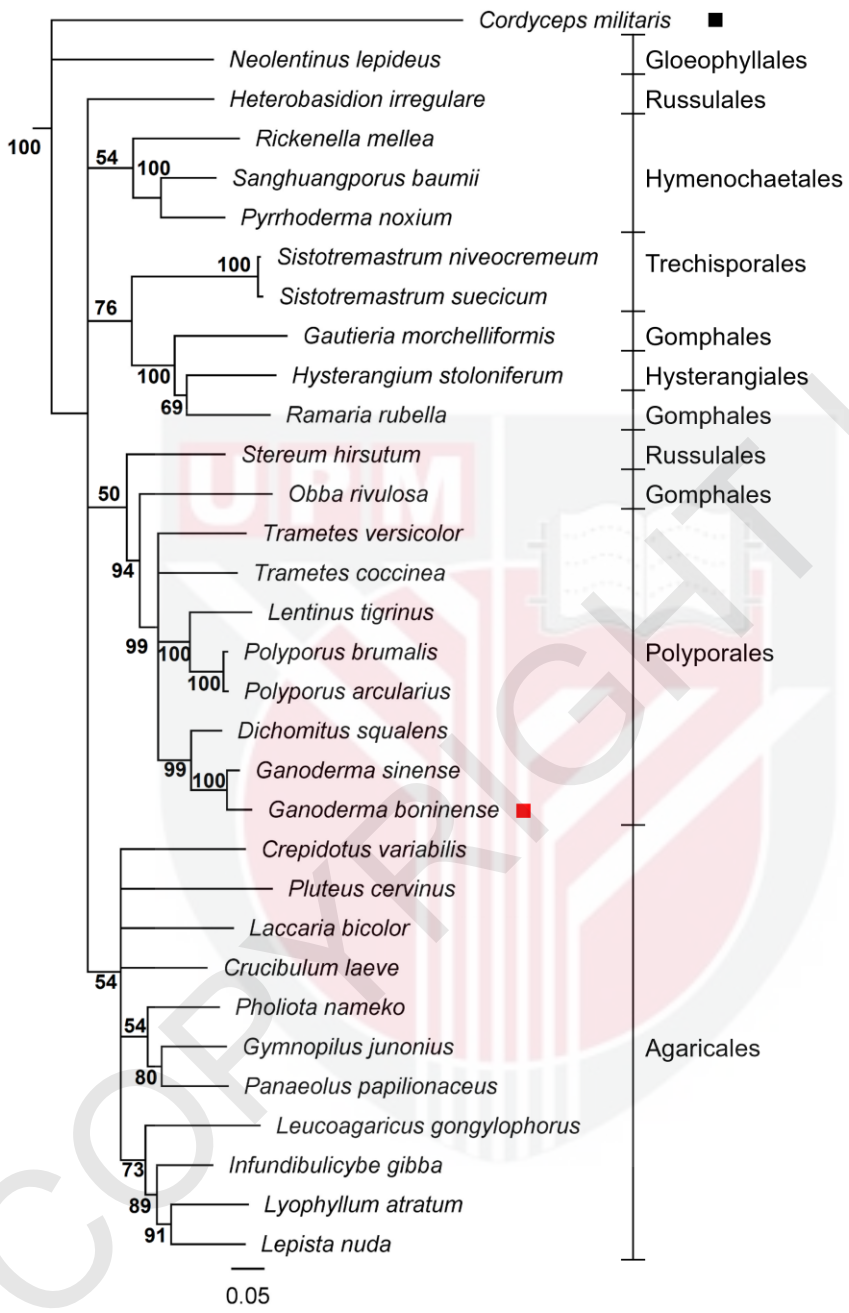


Figure 4.14 : Phylogenetic tree inferred from *U42611* amino acid sequence using the neighbour-joining method. The bootstrap support % based on 1000 replicates is indicated next to the node and the scale bar corresponds to 0.05 substitutions per site. The outgroup from Ascomycota division is labelled with '■'. The sequence obtained from this study is labelled with '■', otherwise were retrieved from GenBank. Details of sequences obtained from GenBank are included in Appendix A

U42611 is mapped to two homologs in the PHI-base which were experimentally validated (Table 4.6). The pathogenicity of these gene mutants was not affected. Alternative or multiple peptidase action was suggested for host-pathogen interaction where aspartic protease was not the only component for host epidermal penetration (Plummer *et al.*, 2004; Ten Have *et al.*, 2010). Therefore, the role of *U42611* in *G. boninense* remains to be functionally characterized.

Table 4.6 : Sequence homologs of *U42611* involved in pathogenesis

PHI No.	Gene	Pathogen/ Host	Gene Mutant
PHI:697	*ugt51E1*	*Leptosphaeria maculans/ Brassica juncea*	unaffected pathogenicity
PHI:697	*ugt51E1*	*Leptosphaeria maculans/ Brassica napus*	unaffected pathogenicity

Briefly, *U42611* encodes for a putative secreted aspartic protease in the pepsin A family which clustered with shelf fungi often associated with wood rot. Owing to great interest in the aspartic protease enzymatic roles in microbial gene regulation and industrial application, cloning of the gene has been carried out extensively since 1986 (Ammerer *et al.*, 1986; Gray *et al.*, 1986; Rothman *et al.*, 1986; Tonouchi *et al.*, 1986; Woolford *et al.*, 1986). This study presents the first cloning of aspartic protease in *G. boninense* as part of the efforts to understand its role in fungal virulence. The transcript sequence of *U42611* was deposited in GenBank with the accession number ON922905.

4.3.3 Putative lipase *(Unigene 56931)*

Candidate transcript *U56931* encoding for lipase was first amplified by gradient PCR to identify the optimum T_a. The expected size of PCR product was ~1,000 bp. When a range of T_a from 58.8–63.0°C were used for gradient PCR, a single, specific band was observed (Figure 4.15). The brightest band was found at T_a 58.8 °C. Large-scale amplification of *U56931* amplicon was then conducted at 58.8 °C and purified (Figure 4.16).

The purified PCR product was ligated into pTA plasmid (pTA/*U56931*), transformed to *E. coli*, and confirmed by colony PCR (Figure 4.17). Five out of six of the selected bacterial colonies contained *U56931* insert of the expected size. The correct gene insertion into the multiple cloning sites region of the plasmid was confirmed by restriction enzyme digestion analysis. The insert was released from the cloning vector upon digestion by *Hind*III flanking the cloning site (Figure 4.18). The pTA/*U56931* plasmid was confirmed by sequencing.

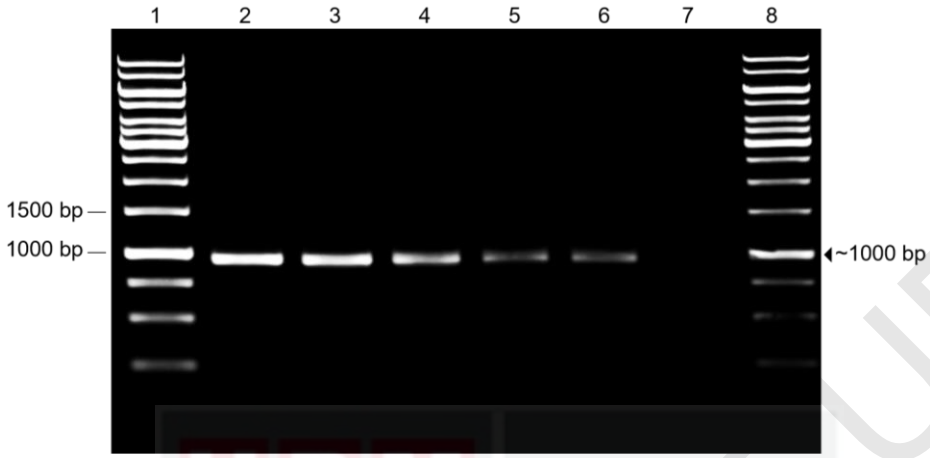


Figure 4.15 : Gel image of PCR products encoding *U56931* amplified from *G. boninense* BLSM5B at different annealing temperatures. Lanes 1 and 8: 1 kb DNA Ladder, Lanes 2–6: PCR products amplified at T_a of 58.8 °C, 60.4 °C, 61.7 °C, 62.5 °C, and 63.0 °C respectively, and Lane 7: negative control

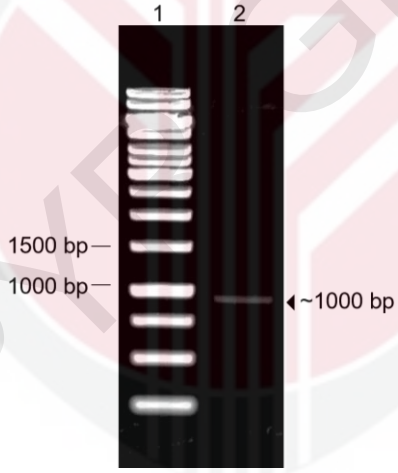


Figure 4.16 : Gel image of *U56931* purified PCR products. Lane 1: 1 kb DNA Ladder, Lane 2: purified PCR product

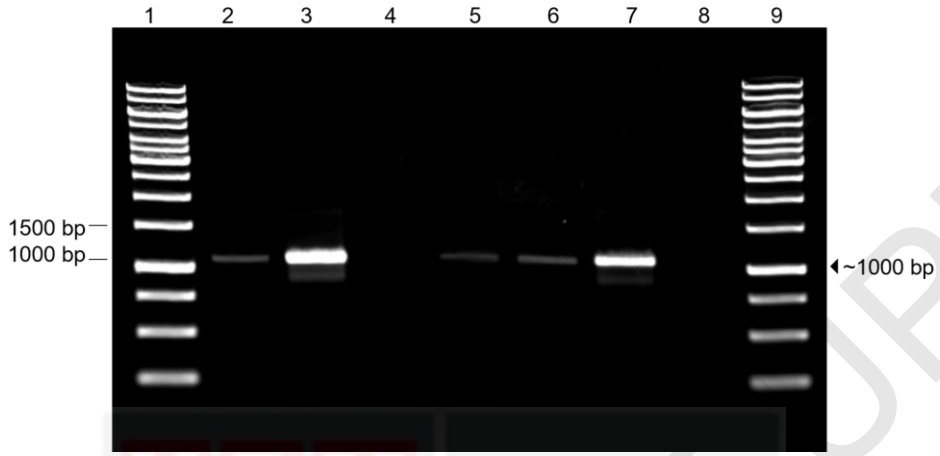


Figure 4.17 : Gel image of *U56931* colony PCR products. Lanes 1 and 9: 1 kb DNA Ladder, Lanes 2–7: colonies 1–6, and Lane 8: negative control

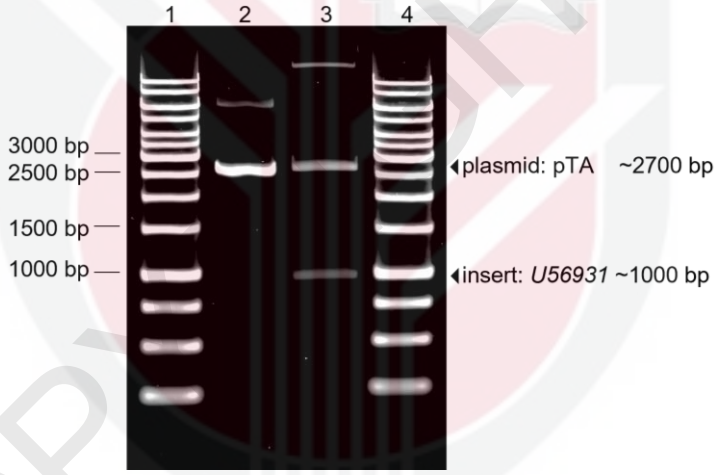


Figure 4.18 : Gel image of *U56931* purified plasmid DNA and products of *Hind*III restriction enzyme analysis. Lanes 1 and 4: 1 kb DNA Ladder; Lane 2: Undigested pTA/*U56931* plasmid DNA; and Lane 3: *Hind*III-treated plasmid DNA

From the sequencing result, the full-length CDS was found to be 939 bp encoding 312 amino acids. BLASTx analysis showed that the cloned *U56931* shares 86% sequence similarities to a sequence homolog from *G. sinense*, and up to 74–65% to other white-rot fungi such as *D. squalens*, *P. brumalis*, and *L. tigrinus* (Table 4.7).

Table 4.7 : BLASTx results for *U56931* encoding lipase isolated from *G. boninense* BLSM5B

Description	E-value	Identity	Accession
Ganoderma sinense ZZ0214-1	1e-141	86%	PIL23397.1
Dichomitus squalens LYAD-421 SS1	2e-127	74%	XP_007367670.1
Polyporus brumalis	1e-102	65%	RDX40351.1
Lentinus tigrinus ALCF2SS1-7	1e-102	65%	RPD75617.1
Colletotrichum gloeosporioides Cg-14	8e-80	54%	EQB47005.1

The translated amino acid sequence of *U56931* was predicted to contain a signal peptide cleavage site at position 26–27, with no transmembrane domains. It was predicted to be localised extracellularly (Figure 4.19). Four conserved domains found are COG3240: phospholipase/lecithinase/hemolysin (amino acids: 33–311), cd01846: fatty acyltransferase-like subfamily of the SGNH hydrolases (amino acids: 41–308), pfam00657: GDSL-like lipase/acylhydrolase (amino acids: 41–306) which contains motif GDSX (amino acids: 45–48), and PRK15381: type III secretion system effector (amino acids: 30–154) (Lu *et al.*, 2019).

COG3240 was predicted to be involved in lipid transport and metabolism (Lu *et al.*, 2019), whereas cd01846, pfam00657, and PRK15381 are members of cl01053: SGNH hydrolase superfamily (also known as GDSL hydrolase) which consists primarily of carbohydrate-modifying enzymes that play diverse biological roles including virulence, signalling, and biomass conversion (Anderson *et al.*, 2022). The signature Ser-Gly-Asn-His (SGNH) conserved residues were also found in *U56931* isolated from *G. boninense* BLSM5B (Figure 4.19). GDSL (previously known as GDSLS) lipase was first found in bacteria, a new subfamily different from the true lipase, where GDSL lipase has a Gly-Asp-Ser-(Leu or Ile) motif near the N-terminus, which confers flexibility at the active site and allows for broad substrate specificity and regiospecificity (Akoh *et al.*, 2004; Bender & Flieger, 2010; Upton & Buckley, 1995).

On the other hand, motif for type III secretion system (T3SS) effector was related to bacterial virulence (Greene *et al.*, 2021; Hardy *et al.*, 2021; Lian *et al.*, 2021; Sheppe *et al.*, 2021; Soules *et al.*, 2020; Wagner *et al.*, 2018). T3SS was known to translocate bacterial effectors into the host, which is essential for the replication and survival of the bacteria in the host (Galán, 2001; Hueck, 1998). Additionally, T3SS secreted proteins were shown to be functionally redundant

and capable of suppressing plant innate immune responses, contributing to successful infection (Jha *et al.*, 2007; Sinha *et al.*, 2013).

Functionally redundant T3SS proteins are able to complement single gene knockout. This is advantageous to the pathogen as the loss of single gene or protein often does not attenuate the virulent properties of the pathogen (Hotinger *et al*., 2021). Despite T3SS proteins were secreted into the plant cell, their actual targets and mechanisms were diverse and largely unknown. In the *Xanthomonas oryzae* pv. *oryzae*-rice pathosystem, suppression of quadruple T3SS proteins (XopN, XopX, XopZ, and XopQ) was required for complete suppression of host defence (Sinha *et al*., 2013). In addition, Long *et al*. (2018) reported additive PTI suppressing ability of XopZ, XopN, and XopV, in preventing the activation of peptidoglycan-triggered mitogen-activated protein kinase (MAPK). On the other hand, XopU, XopV, XopP, XopG, and AvrBs2 were secreted by the pathogen to suppress host defence response which was triggered by the interaction of XopQ and XopX (Deb *et al*., 2022). T3SS effector motif of this protein could suppress plant basal defence and is different from lipase of type II secretion system (T2SS) which often induces host innate immunity upon damaging the host cell wall (Jha *et al.*, 2007; Nascimento *et al.*, 2016; Rajeshwari *et al.*, 2005; Sinha *et al.*, 2013). However, further investigations are necessary to validate if the lipase encoded by *U56931* plays a role in oil palm immune response suppression.

Phylogeny analysis revealed that *U56931* from *G. boninense* BLSM5B clustered with the fungal lipase amino acid sequences from the order Polyporales of the division Basidiomycota (Figure 4.20). Of the eight sequence homologs from the Polyporales order, the most similar sequence to *U56931* is from *G. sinense* with 86% sequence similarity.

To sum up, *U56931* encodes for a putative secreted lipase under the GDSL-like subfamily. It clustered with shelf fungi often associated with wood rot. This is the first isolation of GDSL-like lipase from *G. boninense*. Despite the lack of information from this lipase subfamily, the presence of a T3SS effector motif in this sequence suggests its plant immune response suppressive ability, making *U56931* an interesting candidate to be investigated further. The transcript sequence of *U56931* was deposited in GenBank with the accession number ON922906.

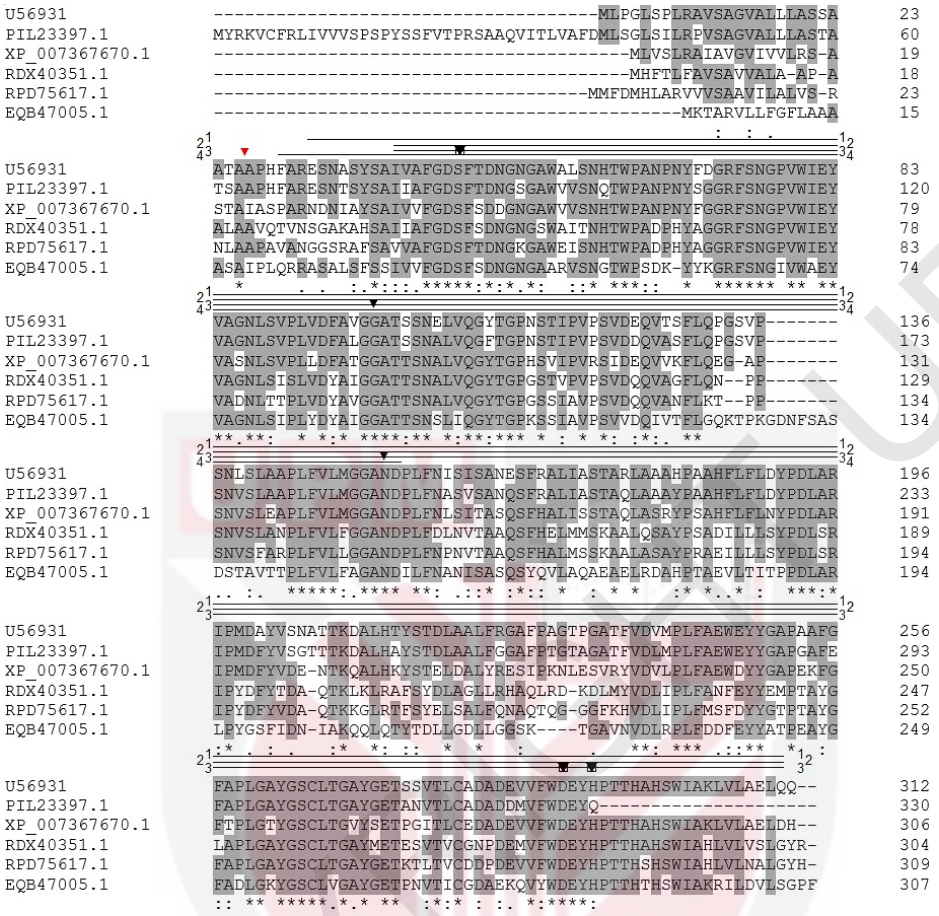


Figure 4.19 : Multiple sequence alignment of the *U56931* lipase based on BLASTX result. Fully conserved amino acid residues are labelled with '*', strongly similar properties (Gonnet PAM 250 matrix > 0.5) are labelled with ':', weakly similar properties (Gonnet PAM 250 matrix < 0.5 and > 0) are labelled with '.', residues conservation >50% are shaded in grey. The active site is indicated by '▼', catalytic triad by '□', and signal peptide cleavage site is indicated by '▼'. 1: phospholipase/lecithinase/hemolysin, 2: fatty acyltransferase-like subfamily of the SGNH hydrolases, 3: GDSL-like lipase/acylhydrolase, and motif 4: type III secretion system effector

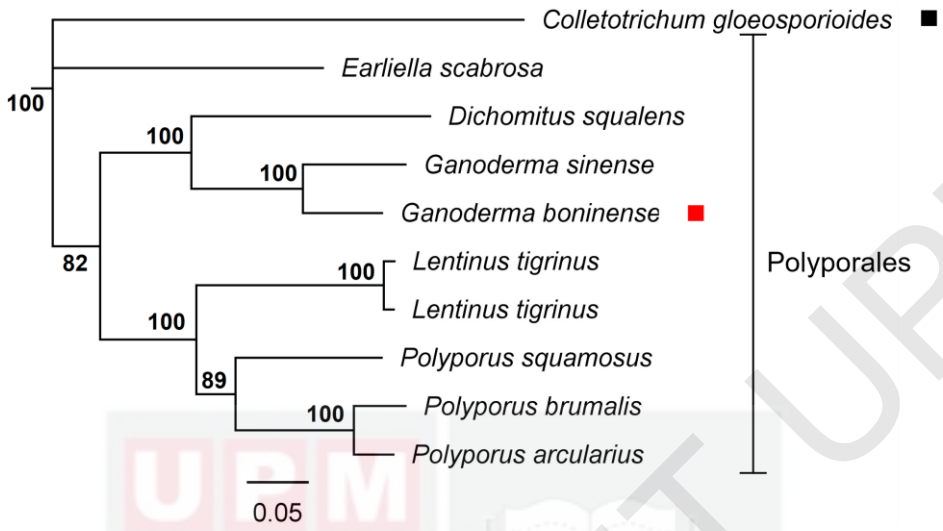


Figure 4.20 : Phylogenetic tree inferred from *U56931* amino acid sequence using the neighbour-joining method. The bootstrap support % based on 1000 replicates is indicated next to the node and the scale bar corresponds to 0.05 substitutions per site. The outgroup from Ascomycota division is labelled with '■'. The sequence obtained from this study is labelled with '■', otherwise were retrieved from GenBank. Details of sequences obtained from GenBank are included in Appendix A

4.3.4 Putative polysaccharide deacetylase (*Unigene 128397*)

Candidate transcript *U128397* encoding for polysaccharide deacetylase was first amplified by gradient PCR to identify the optimum T_a. The expected size of PCR product was ~1,400 bp. When a T_a within the range of 62–66.4°C was used for gradient PCR, a single, specific band was observed (Figure 4.21). The brightest band was found at T_a 62.7 °C indicating the optimum T_a. Large-scale amplification of *U128397* amplicon was then conducted at 62.7 °C and purified (Figure 4.22). The purified PCR product was ligated to pTA plasmid (pTA/*U128397*), transformed into *E. coli*, and confirmed by colony PCR (Figure 4.23). All six of the selected bacterial colonies contained the *U128397* insert of interest at ~1,400 bp. The gene insertion into the multiple cloning sites region of the plasmid was confirmed by restriction enzyme digestion analysis. The insert of ~1,400 bp was released from the cloning vector upon digestion by Hind*III* flanking the cloning site (Figure 4.24). The pTA/*U128397* plasmid was confirmed by sequencing.

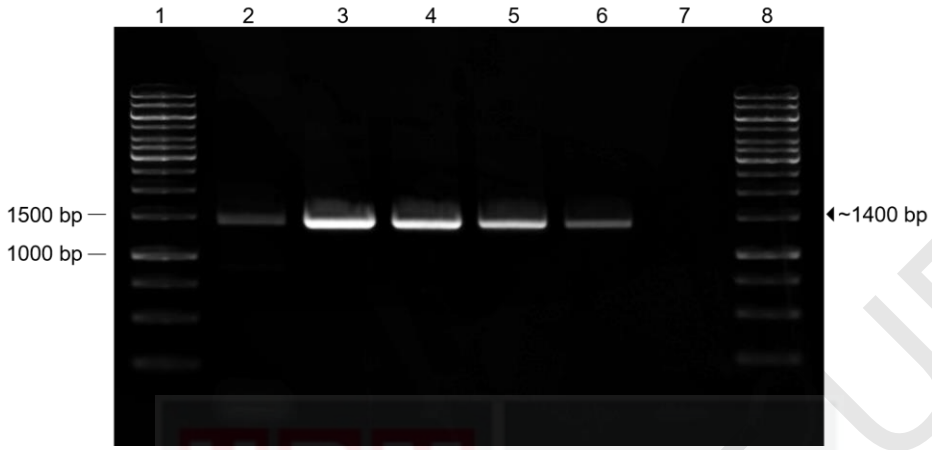


Figure 4.21 : Gel image of PCR products encoding *U128397* amplified from *G. boninense* BLSM5B at different annealing temperatures. Lanes 1 and 8: 1 kb DNA Ladder, Lanes 2–6: PCR products amplified at T_a of 62.0 °C, 62.7 °C, 63.7 °C, 64.8 °C, and 66.4 °C respectively, and Lane 7: negative control

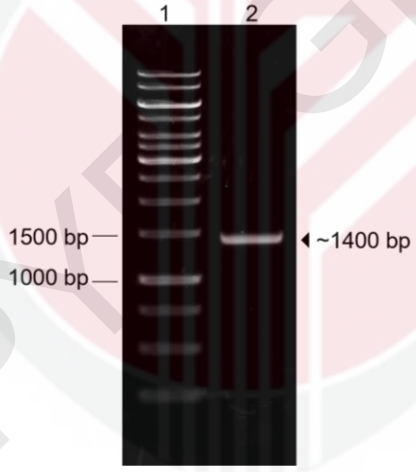


Figure 4.22 : Gel image of *U128397* purified PCR products. Lane 1: 1 kb DNA Ladder, Lane 2: purified PCR product

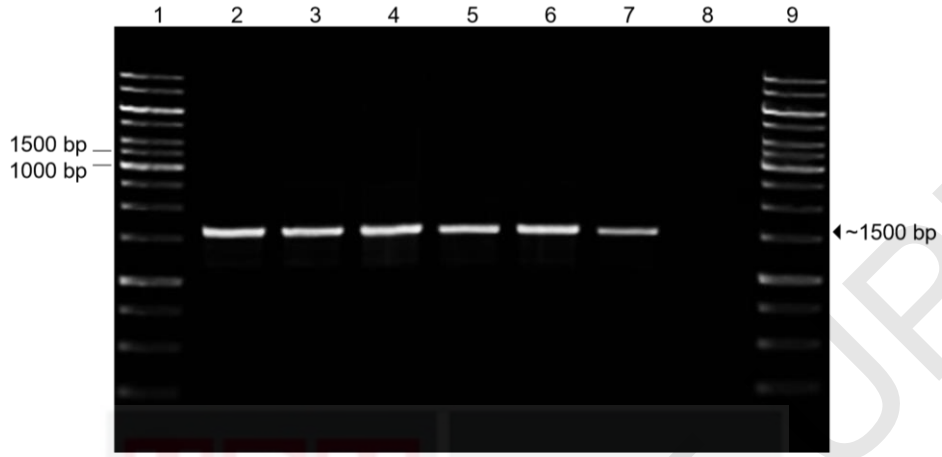


Figure 4.23 : Gel image of *U128397* colony PCR products. Lanes 1 and 9: 1 kb DNA Ladder, Lanes 2–7: colonies 1–6, and Lane 8: negative control

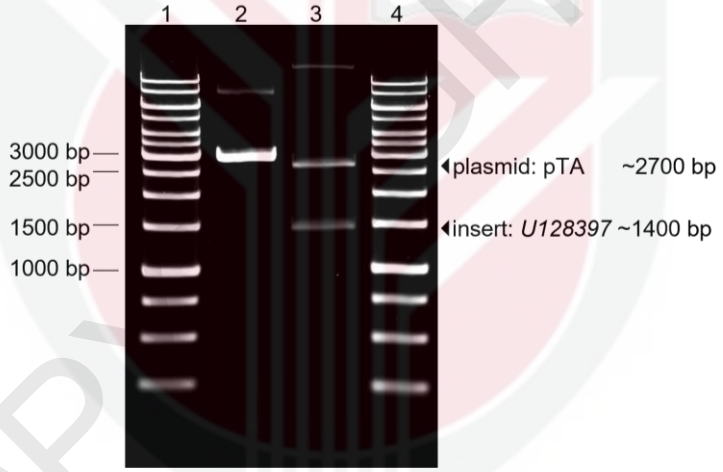


Figure 4.24 : Gel image of *U128397* purified plasmid DNA and products of *Hind*III restriction enzyme analysis. Lanes 1 and 4: 1 kb DNA Ladder; Lane 2: Undigested pTA/*U128397* plasmid DNA; and Lane 3: *Hind*III-treated plasmid DNA

From the sequencing result, the full-length CDS was found to be 1,413 bp encoding for 470 amino acids. BLASTx analysis showed that the cloned *U128397* shares 94% sequence similarities to a sequence homolog from *G. sinense*, and up to 81–73% to sequences from other white-rot fungi such as *D. squalens* and *T. coccinea* (Table 4.8).

Table 4.8 : BLASTx results for *U128397* encoding polysaccharide deacetylase isolated from *G. boninense* BLSM5B

Description	E-value	Identity	Accession
Ganoderma sinense ZZ0214-1	0.0	94%	PIL29657.1
Dichomitus squalens	0.0	81%	TBU47379.1
Dichomitus squalens LYAD-421 SS1	0.0	81%	XP_007360000.1
Dichomitus squalens	0.0	81%	TBU64757.1
Trametes coccinea BRFM310	0.0	73%	OSD05919.1

The translated amino acid sequence of *U128397* was predicted to contain a signal peptide cleavage site at position 17–18, with no transmembrane domains. It was predicted to be localised extracellularly (Figure 4.25). Three conserved domains found in this sequence are pfam01522: polysaccharide deacetylase (amino acids: 191–310), COG0726: peptidoglycan/xylan/chitin deacetylase, PgdA/CDA1 family (amino acids: 192–389), and cd10952: catalytic NodB homology domain (amino acids: 196–373).

COG0726 is a chitin deacetylase domain which is involved in the transport and metabolism of carbohydrates, as well as the biogenesis of cell wall, membrane, or envelope (Choi *et al.*, 2013; Lu *et al.*, 2019). On the other hand, pfam01522 and cd10952 are members of cl15692 carbohydrate esterase 4 superfamily. The carbohydrate esterase 4 superfamily encompasses chitin deacetylases, bacterial peptidoglycan N-acetylglucosamine deacetylases, and acetylxylan esterases having the signature NodB domain and a conserved zinc binding triad of His-His-Asp which are also observed in *U128397* (amino acid residues 203, 252, and 256, Figure 4.25) (Blair *et al.*, 2005; Lu *et al.*, 2019). Additionally, the active sites of chitin deacetylase were also conserved in this sequence, i.e., Tf/yDD (amino acids: 200–203) and Ra/pPY (amino acids: 290–293) (Kuroki *et al.*, 2017; Liu *et al.*, 2017). Thus, it is suggestive that *U128397* is a chitin deacetylase of the polysaccharide deacetylase carbohydrate esterase 4 superfamily.

Phylogeny analysis revealed that *U128397* from *G. boninense* BLSM5B clustered together with fungal chitin deacetylase amino acid sequences from other Polyporales of the division Basidiomycota (Figure 4.26), whereby the most similar sequence is from *G. sinense*, sharing a 94% sequence similarity.

Figure 4.25 : Multiple sequence alignment of the *U128397* polysaccharide deacetylase based on BLASTX result. Fully conserved amino acid residues are labelled with '*', strongly similar properties (Gonnet PAM 250 matrix > 0.5) are labelled with ':', weakly similar properties (Gonnet PAM 250 matrix < 0.5 and > 0) are labelled with '.', residues conservation >50% are shaded in grey. The active sites are indicated by '▼', catalytic triad by '□', and signal peptide cleavage site by '▼'. 1: peptidoglycan/ xylan/chitin deacetylase, 2: catalytic NodB homology domain, and 3: polysaccharide deacetylase

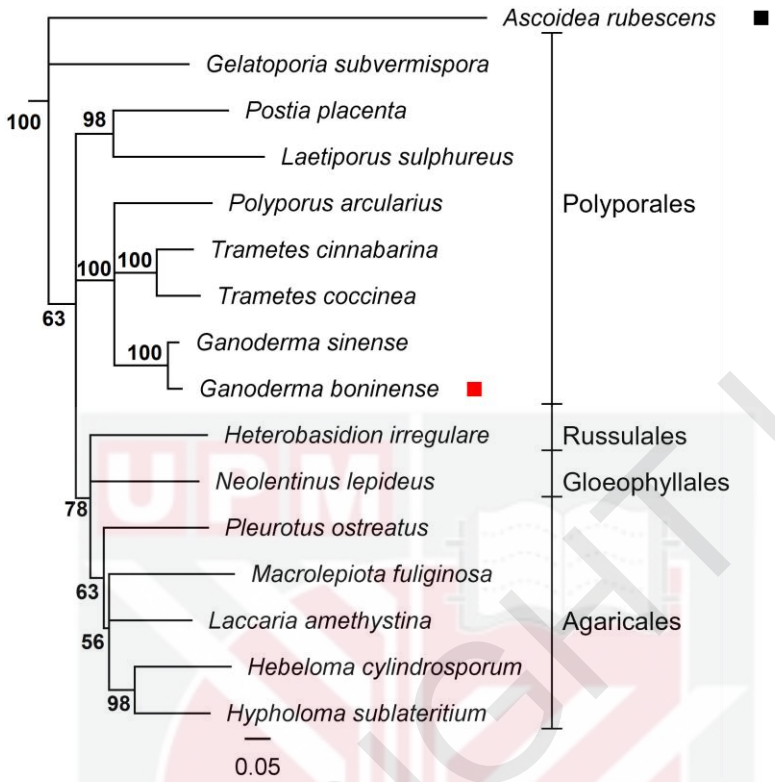


Figure 4.26 : Phylogenetic tree inferred from *U128397* amino acid sequence using the neighbour-joining method. The bootstrap support % based on 1000 replicates is indicated next to the node and the scale bar corresponds to 0.05 substitutions per site. The outgroup from Ascomycota division is labelled with '■'. The sequence obtained from this study is labelled with '■', otherwise were retrieved from GenBank. Details of sequences obtained from GenBank are included in Appendix A

Apart from that, *U128397* was mapped to experimentally characterised homologs, where the gene mutants had variable effects on fungal virulence upon gene disruption (unaffected pathogenicity, reduced or increased virulence, loss of pathogenicity) (Table 4.9). This indicated that the gene may have different importance in pathogenicity in each pathosystem. The members of a chitin deacetylase gene family in an organism may be functionally redundant, some may have exceptional specificity, not limited to virulence but also for vitality (Rizzi *et al.*, 2021). The role of *U128397* as a putative virulence factor in *E. guineensis*-*G. boninense* interaction has to be elucidated to better understand the mechanism of interaction during the initial stage of plant infection.

Table 4.9 : Sequence homologs of *U128397* involved in pathogenesis

PHI No.	Gene	Pathogen/ Host	Gene Mutant
PHI:11211	*cda1*	*Ustilago maydis/ Zea mays*	unaffected pathogenicity
PHI:11211	*cda1*	*Ustilago maydis/ Zea mays*	reduced virulence
PHI:11216	*cda6*	*Ustilago maydis/ Zea mays*	unaffected pathogenicity
PHI:11216	*cda6*	*Ustilago maydis/ Zea mays*	reduced virulence
PHI:11212	*cda2*	*Ustilago maydis/ Zea mays*	unaffected pathogenicity
PHI:11212	*cda2*	*Ustilago maydis/ Zea mays*	reduced virulence
PHI:11213	*cda3*	*Ustilago maydis/ Zea mays*	unaffected pathogenicity
PHI:11213	*cda3*	*Ustilago maydis/ Zea mays*	reduced virulence
PHI:11214	*cda4*	*Ustilago maydis/ Zea mays*	unaffected pathogenicity
PHI:11214	*cda4*	*Ustilago maydis/ Zea mays*	reduced virulence
PHI:6391	*CDA2*	*Magnaporthe oryzae/ Oryza sativa*	unaffected pathogenicity
PHI:4639	*Cbp1*	*Magnaporthe oryzae/ Oryza sativa*	loss of pathogenicity
PHI:4639	*Cbp1*	*Magnaporthe oryzae/ Hordeum vulgare*	loss of pathogenicity
PHI:2166	*CBP1*	*Magnaporthe oryzae/ Oryza sativa*	unaffected pathogenicity
PHI:2166	*CBP1*	*Magnaporthe oryzae/ Hordeum vulgare*	unaffected pathogenicity
PHI:6390	*CBP1*	*Magnaporthe oryzae/ Oryza sativa*	unaffected pathogenicity
PHI:3972	*CDA*	*Colletotrichum gloeosporioides/ Persea americana*	unaffected pathogenicity
PHI:11215	*cda5*	*Ustilago maydis/ Zea mays*	unaffected pathogenicity
PHI:11528	*Pst_13661*	*Puccinia striiformis/ Triticum aestivum*	reduced virulence
PHI:6392	*CDA3*	*Magnaporthe oryzae/ Oryza sativa*	unaffected pathogenicity

In summary, *U128397* encodes for a putative secreted polysaccharide deacetylase, possibly a chitin deacetylase. Previous studies have shown the role of chitin deacetylase in the formation of fungal fruiting body, ascospore wall, and appressorium, as well as in fungal invasion (Christodoulidou *et al.*, 1996; Kuroki *et al.*, 2017; Yamada *et al.*, 2008; Yang *et al.*, 2022). Therefore, the cloning of chitin deacetylase from *G. boninense* could facilitate the understanding on plant-pathogen interaction in the future. Transcript sequence of *U128397* was deposited in GenBank with the accession number ON922907.

4.4 Gene expression analysis of selected putative virulence factors during fungal growth and plant-pathogen interactions

Gene specific primers for gene expression analysis were designed based on transcript sequence retrieved from the sequencing result, targeting for an amplicon which is 50–150 bp in length (Table 3.1). Standard curve analysis showed that all the primers have an amplification efficiency of 90–110%. The slope of the curve was approximately -3.3 with a correlation coefficient, R^2>0.99 (Appendix B). The melt curve analysis generated a single peak indicating the specificity of the amplification. The primers were found suitable for the gene expression analysis (Thermo Fisher Scientific Inc, 2023b).

4.4.1 Gene expression in fungal samples harvested at different time-points

The transcript abundance was measured on Day-14, Day-21, and Day-28 in relative to that of the actively growing *G. boninense* BLSM5B fungal mycelia of Day-7, to analyze the regulation of the selected transcripts during fungal vegetative growth. *U56931* was found to be significantly up-regulated at Day-21 (12.1-fold) and Day-28 (48.4-fold). The gene expression of *U42611* was down-regulated at Day-28 (0.36-fold) in comparison with that of the *G. boninense* harvested at Day-7 (Figure 4.27, Appendix C).

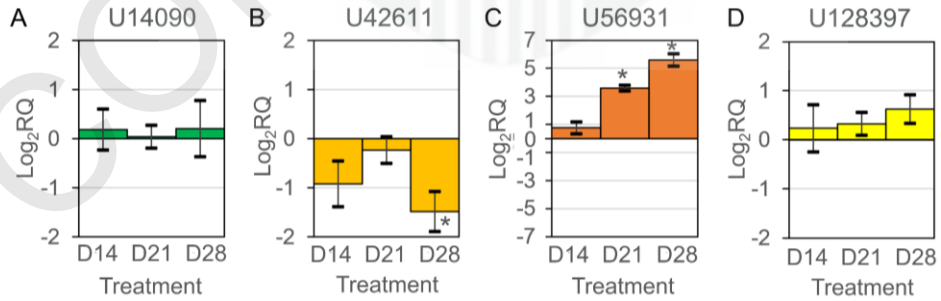


Figure 4.27 : The normalised transcripts expression for fungal samples harvested at different time-points. Asterisk (*) represents significant expression (|log_2RQ≥1|, t-test with p<0.05). Error bar was derived from standard deviation of three technical replicates

Nawawi and Ho (1990) reported that the *G. boninense* in axenic culture, had an initial lag phase for the first 3 days, followed by an exponential growth on Day 6–18, which declined and autolysed after Day-21. They found this growth pattern to be linked to the pH fluctuation, whereby the acidification of culture media (pH 3.8) was recorded at the maximal mycelial growth. The pH of the media became neutral during prolonged incubation (pH 7.3 at Day-27) as the mycelial growth decreased. *G. boninense* exhibited ideal mycelial growth at a slightly acidic media (pH 3.7–5) (Nawawi & Ho, 1990; Peng *et al.*, 2019). Since the culture media used in this experiment was at pH 5, it may promote optimal growth of *G. boninense*. Significant transcript regulation (*U56931* and *U42611*) was only observed at prolonged incubation at Day-21 and Day-28. At this stage, the fungal growth could have declined in the culture media as described by Nawawi and Ho (1990). *U56931* and *U42611* may play a role in fungal survival under an undesirable environment when the pH became neutral during prolonged incubation. Aspartic protease accumulation happened at acidic culture condition (Li *et al.*, 2012), this may account for the down-regulation of *U42611* at Day-28 since it encodes for a putative aspartic protease.

Additionally, nutrient depletion could happen at Day-21 and Day-28 during prolonged incubation. The significant up-regulation of *U56931* during the prolonged incubation may reflect the potential role of the putative lipase in the vegetative growth of *G. boninense* through nutrient assimilation under nutrient depletion conditions. In *F. graminearum*, lipase was responsible for fungal growth and aerial hyphae production. Other than virulence, it could cause the degradation of storage lipids during starvation (Nguyen *et al.*, 2011a; Nguyen *et al.*, 2011b). Similarly, bacterial lipase was depicted as a nutritional virulence determinant that helps to scavenge food from the host for the survival of the pathogen and virulence (Kaur & Kaur, 2019; Kuhn *et al.*, 2021).

The transcript abundance of *U128397* and *U14090* did not change significantly during the fungal growth. The constitutive expression of these genes could be vital to provide a higher fitness to *G. boninense* in response to environmental stress (Geisel, 2011; Masnoddin *et al.*, 2022). It will be interesting to examine the roles of these transcripts in *G. boninense*.

4.4.2 Gene expression *in-planta*

For this experiment, the five-month-old oil palm seedlings were in direct contact with *G. boninense* BLSM5B in a flask using the dip inoculation method. The transcript abundance of fungal samples in contact with oil palm was compared to that of fungal samples without oil palm and maintained in the same condition, to analyse the plant-pathogen interaction at 12-, 24-, and 48-hour post inoculation (hpi). The experiment was repeated twice (designated as *in-planta* 1 and *in-planta* 2, respectively). Each of these experiments consisted of three biological replicates for each time-point. Only consistent and significant gene expression in all biological replicates is discussed here.

There were no detectable changes in both the host and the pathogen at each time-point of sample collection, possibly due to the short duration of incubation. The infection process could be inferred from previous studies, it was reported in Nusaibah *et al*. (2016) on cell wall degradation by *G. boninense* at 24-hpi. Also, Abdul Wahab (2016) observed the adherence of basidiospores on cut oil palm roots at 24- and 48-hpi, followed by basidiospore germination at 48-hpi and >72-hpi on white roots and brown roots, respectively. Later, germ tube extension happened by 96-dpi in both white and brown roots. Subsequently, penetration and root epidermis degradation were observed at 72–96 hpi, despite the absence of infection structure. The molecular interaction between oil palm-*G. boninense* during early invasion stages described by Nusaibah *et al*. (2016) and Abdul Wahab (2016) remains unknown. On the other hand, the invasion of hemibiotrophic pathogen *Phytophthora infestans* was recognised by the tomato host at 24-hpi (Rezzonico *et al*., 2017). In addition, rice blast fungus *Magnaporthe oryzae* was found to invade the host tissue at 24–38-hpi (Eseola *et al*., 2021).

qPCR analysis having the advantage of being a fast and highly sensitive analytical approach, was able to capture minor changes in expression, to understand the underlying interactions between oil palm-treated *G. boninense* in relative to the untreated *G. boninense*. *U14090* was consistently up-regulated in all three biological replicates of oil palm-treated *G. boninense* in relative to the untreated *G. boninense* at 24-hpi and 48-hpi for both experiments (*in-planta* 1: 2.23–3.26-fold and 5.32–24.7-fold, at 24-hpi and 48-hpi, respectively; *in-planta* 2: 2.14–8.21-fold and 3.71–5.65-fold, at 24-hpi and 48-hpi, respectively) (Figure 4.28, Appendices D-F).

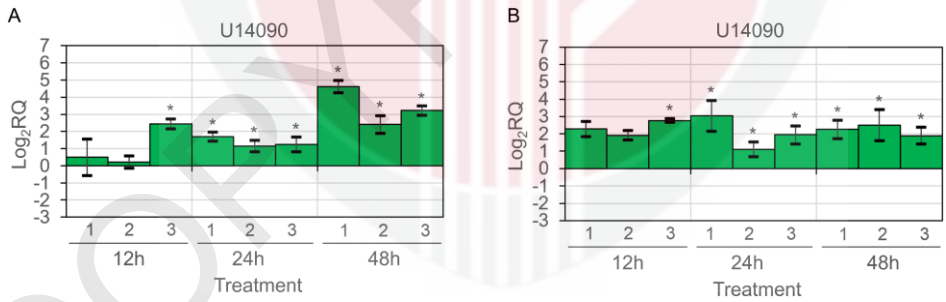


Figure 4.28 : The normalised transcripts expression for *U14090* in oil palm *in-planta* experiments. Asterisk (*) represents significant expression ($|\log_2 RQ \geq 1|$, t-test with $p<0.05$), whereas error bar represents standard deviation of the three technical replicates from each biological replicate (1, 2, and 3, respectively). A. *in-planta* 1 and B. *in-planta* 2

Early interaction between *G. boninense* and *E. guineensis* was observed by Nusaibah *et al.* (2016) at 24-hpi, where *G. boninense* degraded host cell wall before hyphal colonization happened. The expression of *U14090* metalloproteinase could be essential for host colonisation and disease

progression during the biotrophic phase (Sanz-Martín *et al.*, 2016). The up-regulation of *U14090* which is putatively a hydrolytic metalloproteinase could be a strategy to degrade the host cell wall for colonisation and nutrient uptake (Ramzi *et al.*, 2019). Similarly, early induction (18-hpi) of fungal metalloproteinase was reported in the wheat-*Rhizoctonia cerealis* pathosystem (Pan *et al.*, 2020b). Hydrolytic enzymes or their products may diffuse through the plant cell wall to the plasma membrane before the establishment of direct contact (Vorwerk *et al.*, 2004). It is possible that *G. boninense* secreted an arsenal of hydrolytic enzymes including *U14090* to degrade host cell wall before actual mycelial colonisation.

Additionally, M36 metalloproteinase may breach plant defence through host chitinase degradation, hence protects the fungal cell wall from chitinase hydrolysis, and prevents the release of chitin oligomers (elicitors) from the fungal cell wall or chitin-triggered immunity in the host (Jashni *et al.*, 2015; Naumann *et al.*, 2011; Ökmen *et al.*, 2018; Volk *et al.*, 2019). The expression of *U14090* during the early interaction stage may help *G. boninense* to avoid host recognition and contribute to virulence. Several studies have confirmed the presence of chitinase and its encoding genes in the oil palm host during *G. boninense* infection (Aditama *et al.*, 2019; Naher *et al.*, 2012; Yeoh *et al.*, 2012). However, the presence of chitinase degrading activity in *U14090* may need further investigation.

For *U56931*, the transcript abundance increased 9.74–29.49-fold at 48-hpi in oil palm-treated *G. boninense* in relative to the untreated *G. boninense* (control) in experiment *in-planta* 1 (Figure 4.29, Appendices D-F). However, the up-regulation of *U56931* was detected at much earlier time-points in the treated *G. boninense* in relative to control in *in-planta* 2 (30.02–41.52-fold and 10.62–18.39-fold at 12-hpi and 24-hpi, respectively). The increase of *U56931* transcript abundance during early host exposure could be explained by its role as a lipase in plant cell wall adhesion and penetration, which is induced in the presence of its host (Comménil *et al.*, 1999; Comménil *et al.*, 1998; Feng *et al.*, 2011; Feng *et al.*, 2009). The importance of GDSL-lipase in the utilisation and modification of host innate immunity-related fatty acid has been reported (Chimalapati *et al.*, 2020). The loss of lipase activity often results in reduced virulence (Bravo-Ruiz *et al.*, 2013; Nascimento *et al.*, 2016; Rosenberg *et al.*, 2022).

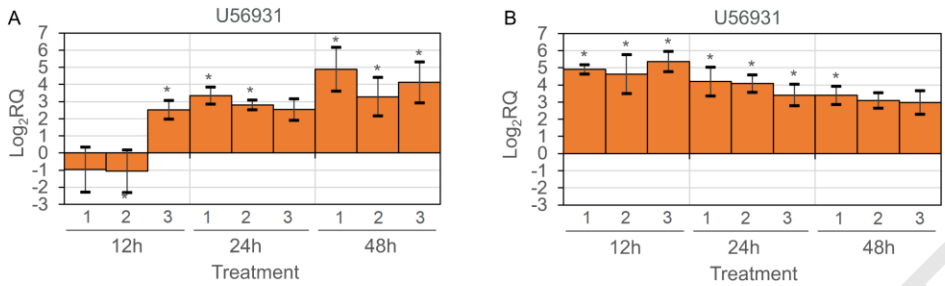


Figure 4.29 : The normalised transcripts expression for *U56931* in oil palm *in-planta* experiments. Asterisk (*) represents significant expression ($|\log_2 RQ \geq 1|$, t-test with $p<0.05$), whereas error bar represents standard deviation of the three technical replicates from each biological replicate (1, 2, and 3, respectively). A. *in-planta* 1, and B. *in-planta* 2

The transcript abundance of *U128397* increased >2-fold only at 48-hpi in *in-planta* 1, but it increased >2-fold at 24-hpi in *in-planta* 2 (Figure 4.30, Appendices D-F). The increase of *U128397* transcript abundance in *G. boninense* in contact with oil palm could due to its chitin deacetylase activity in suppressing the elicitor-induced plant defence (Tsigos & Bouriotis, 1995). Chitin deacetylase causes the hydrolysis and conversion of chitin oligomers (elicitors) into chitosan, which may not be recognised by the pattern recognition receptors (PRRs), hence prevents the reactive oxygen species generation and chitin-triggered immunity by the host plant (Cord-Landwehr *et al.*, 2016; Gao *et al.*, 2019; Xu *et al.*, 2020). In addition, chitin deacetylase is preferentially involved in the formation of fungal fruiting body, ascospore wall, and appressorium, as well as in fungal invasion (Christodoulidou *et al.*, 1996; Kuroki *et al.*, 2017; Yamada *et al.*, 2008; Yang *et al.*, 2022). Thus, this may explain for increase in the transcript abundance of *U128397* upon host contact.

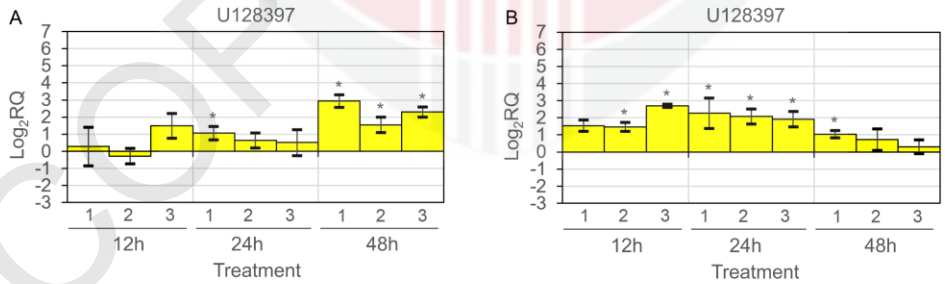


Figure 4.30 : The normalised transcripts expression for *U128397* in oil palm *in-planta* experiments. Asterisk (*) represents significant expression ($|\log_2 RQ \geq 1|$, t-test with $p<0.05$), whereas error bar represents standard deviation of the three technical replicates from each biological replicate (1, 2, and 3, respectively). A. *in-planta* 1, and B. *in-planta* 2

The gene expression of *U42611* was down-regulated 0.08–0.14-fold only at 48-hpi in *in-planta* 2 (Figure 4.31, Appendices D-F). Aspartic protease was found to degrade grape chitinase in a few wine making study (Theron *et al.*, 2017, 2018; Van Sluyter *et al.*, 2013). *U42611* could be a pathogenic determinant in *G. boninense* which is capable of breaking down chitinase, a host pathogenesis-related protein during host invasion while avoiding the host defence elicitation. *G. boninense* may down-regulate its pathogenic determinant to escape from the host defence during *in-planta* interaction (Khairi *et al.*, 2022).

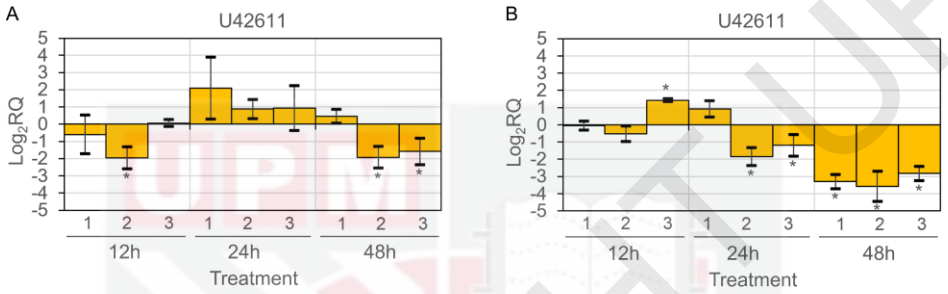


Figure 4.31 : The normalised transcripts expression for *U42611* in oil palm *in-planta* experiments. Asterisk (*) represents significant expression ($|\log_2 RQ \geq 1|$, t-test with $p<0.05$), whereas error bar represents standard deviation of the three technical replicates from each biological replicate (1, 2, and 3, respectively). A. *in-planta* 1, and B. *in-planta* 2

The gene expression during *in-planta* experiments in this study is different from the previous study (Ho *et al*., 2016) which showed that the selected candidates were overexpressed in *G. boninense*-infected oil palm roots. This could be due to the differences in the experimental parameters as tabulated below.

Table 4.10 : Differences in experimental parameters

Parameters	Previous study (Ho *et al*., 2016)	Current study
1. Planting material	Sime Darby GH 500 Series (*Dura* x *Pisifera*)	FGV Yangambi ML161 (*Deli* x *Yangambi ML161*)
2. Fungal inoculum	*G. boninense* PER71 colonised-rubber wood block	*G. boninense* BLSM5B liquid culture
3. Incubation period	3-, 6-, and 12-week	12-, 24-, and 48-hour
4. Incubation condition	Green house	Indoor laboratory
5. Gene expression analysis	RNA-sequencing on infected oil palm roots	qPCR analysis on fungal samples
6. Transcript regulation	Overexpressed	Upregulation of *U14090*, *U56931*, and *U128397*; Downregulation of *U42611*

There are a few limitations in this experiment. Firstly, the transcript abundance of *U56931*, *U128397*, and *U42611* showed a delayed transcript regulation in *in-planta* 1 as compared to that in *in-planta* 2. This could be due to lack of environmental control and monitoring of room temperature and air humidity where the samples were incubated. The experiment could be carried out in a controlled growth chamber with all the variables well-monitored to ensure the reproducibility of the results.

Secondly, the transcript abundance of *U56931*, *U42611*, and *U128397* was inconsistent among *G. boninense* samples in contact with oil palm in relative to the *G. boninense* samples not in contact with oil palm. The experiment could be limited by the unknown genetic heterogeneity of the oil palm seedlings. The phenotypic variations of oil palm seedlings were minimised by selecting plant materials of the same breed with similar physical characteristics and maintained at the same condition. However, each oil palm seedling is an individual with unique genotype, having different degree of resistance or susceptibility towards pathogenic attack thus may cause different interactions with the pathogen. In order to reduce this, oil palm clones which possess identical genetic composition could be used.

Thirdly, this experiment only provides a limited overview of the transcriptional regulation in the four candidate genes during early interaction with the oil palm host. The understanding on the molecular interaction during early host-pathogen recognition and signalling could be largely improved by carrying out dual RNA-sequencing on both the plant and pathogen.

The interpretation of the gene expression results remains the biggest challenge of this study. Given that *G. boninense* is a hemibiotroph, reprogramming of regulatory network happens as it switches from biotrophy into necrotrophy lifestyle, the putative virulence factors could take part at different stages of infection.

4.4.3 Gene expression upon phytohormone and elicitor treatments

SA, JA, and H_2O_2 are important components in the plant immune signalling. They were hypothesised to mediate oil palm defence at different stages of *G. boninense* infection (Ho & Tan, 2015; Ho *et al.*, 2016). Under phytohormone/elicitor treatments, only *U56931* which encodes a putative lipase was significantly down-regulated by SA at 0.34-fold, as compared to the untreated control. No significant expression was observed in other transcripts under the SA, JA, and H_2O_2 treatment (Figure 4.32, Appendix G). The role of SA in mediating early defence response in asymptomatic oil palm infected by *G. boninense* was reported (Mohan *et al.*, 2022). The down-regulation of *U56931* under SA treatment may mimic the oil palm host defence action against the *G. boninense*. Lipase activity is crucial for fungal virulence (Bravo-Ruiz *et al.*, 2013; Nascimento *et al.*, 2016; Rosenberg *et al.*, 2022). The SA inhibitory effect against pancreatic lipase was non-competitive, lowering the maximum reaction rate independent of the substrate concentration (Hamdan *et al.*, 2019; Karamać & Amarowicz, 1996; Liu *et al.*, 2020a). Reduced lipase activity resulted in decreased fungal growth (Nguyen *et al.*, 2011b). Likewise, SA of equivalent concentration has been shown to be suppressive towards *G. boninense* mycelial growth, in an isolate-dependent manner (Ong *et al.*, 2021; Ong *et al.*, 2018; Rahamah Bivi *et al.*, 2012; Saputra *et al.*, 2022; Surendran *et al.*, 2017). However, the mycelial growth promoting or inhibiting activity of JA (Ong *et al.*, 2018) did not affect the transcript abundance of *U56931* in this study.

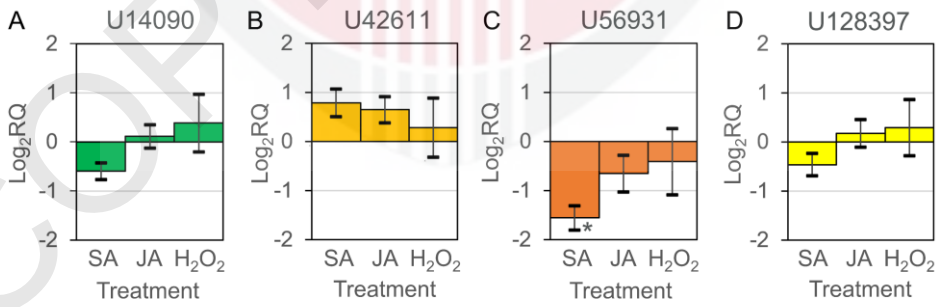


Figure 4.32 : The normalised transcripts expression under phytohormone and elicitor treatments. Asterisk (*) represents significant expression ($|\log_2 RQ \geq 1|$, t-test with $p<0.05$). Error bar was derived from standard deviation of three technical replicates

4.5 TFBS analysis of putative virulence factors in selected *Ganoderma* species

Based on the JASPAR 2022 fungi database (https://jaspar.genereg.net/), the dominant classes of TF predicted to bind to TFBS in the promoter sequence of the selected putative virulence factors are from the C2H2 zinc finger factors and C6 zinc cluster factors which make up 20–36% of the overall predicted TFBS (Figure 4.33, Appendix I). For *U14090*, 69 TFBSs were found in all three selected *Ganoderma* species, whereas only 7, 13, and 8 unique TFBSs were found in *G. boninense*, *G. sinense*, and *G. lucidum*, respectively. The unique TFBSs in *G. boninense U14090* promoter sequence were predicted to bind to ECM23, GAT3, GAT4, IME1, INO4, RDR1, and SWI5. For *U42611*, 73 TFBSs were found in all three selected *Ganoderma* species, where only 6, 5, and 7 unique TFBSs were found in *G. boninense*, *G. sinense*, and *G. lucidum*, respectively. The unique TFBSs in *G. boninense U42611* promoter sequence were predicted to bind to CEP3, HAC1, MET4, RSC30, SUT2, and nuc-1.

For *U56931*, 67 TFBSs were found in all three selected *Ganoderma* species, where only 11, 6, and 11 unique TFBSs were found in *G. boninense*, *G. sinense*, and *G. lucidum*, respectively. The unique TFBSs in *G. boninense U56931* promoter sequence were predicted to bind to ARG81, AZF1, CEP3, HSF1, INO4, LEU3, PDR8, RPH1, SPT15, YNR063W, and YRR1. For *U128397*, 74 TFBSs were found in all three selected *Ganoderma* species, where only 11, 4, and 10 unique TFBSs were found in *G. boninense*, *G. sinense*, and *G. lucidum*, respectively. The unique TFBSs in *G. boninense U128397* promoter sequence were predicted to bind to DAL80, ECM23, GAT1, GZF3, SRD1, STP2, SWI5, NCU02182, nuc-1, wc-1, and NRG1 (Figure 4.34).

Of these unique TFBSs in *G. boninense*, ECM23 and SWI5 binding sites were found in both *U14090* and *U128397*, INO4 binding site was found in both *U14090* and *U56931*, CEP3 binding site was found in both *U42611* and *U56931*, while nuc-1 binding site was found in both *U42611* and *U128397*. Despite GAT1, HSF1, and NRG1 were virulence factors in human fungal pathogen (Childers & Kadosh, 2015; Nicholls *et al.*, 2011), there is no direct evidence between these unique TFBSs to the virulence of plant pathogen.

Recent publications suggested that *G. boninense*, *G. zonatum* and *G. miniacinctum* as the fungal pathogens causing BSR (Rakib *et al.*, 2017; Wong *et al.*, 2012). *G. lucidum* and *G. sinense* are both edible mushrooms cultivated for their medicinal values (Zhang *et al.*, 2019), which were not reported as pathogen which causes BSR. The main reason for selecting *G. lucidum* and *G. sinense* in addition to *G. boninense* for TFBS analysis is due to the availability of their complete promoter sequence in GenBank database as of May 2021. This analysis provided an overview on the frequency of TFBS in the promoter sequence of putative virulence factors in three *Ganoderma* species. In addition, the unique TFBSs can provide information on the different binding proteins which possibly lead to distinct regulation of the candidates in *G. boninense* as

compared to *G. lucidum* and *G. sinense*. Since there is no disease infection study done using *G. lucidum* and *G. sinense* on oil palm, it is necessary to test whether the unique TFBSs are involved in the virulence determination.

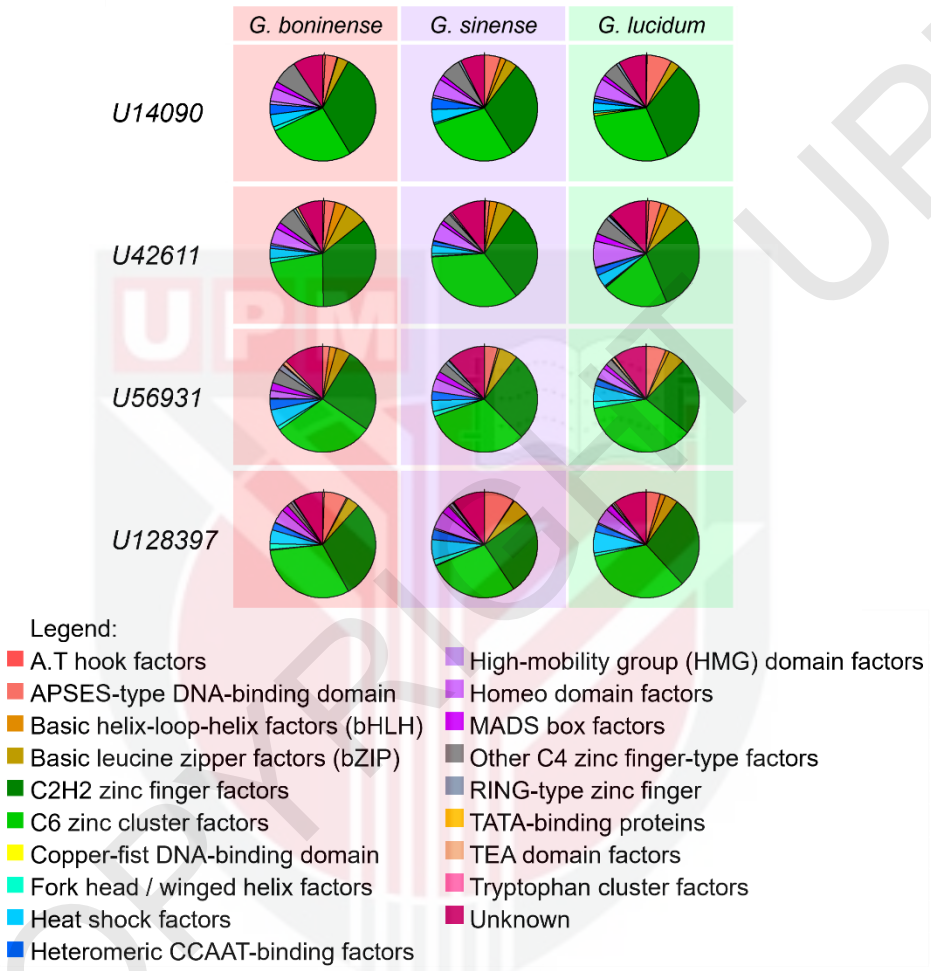


Figure 4.33 : Abundance of TFBS predicted in promoter sequence of respective candidate genes

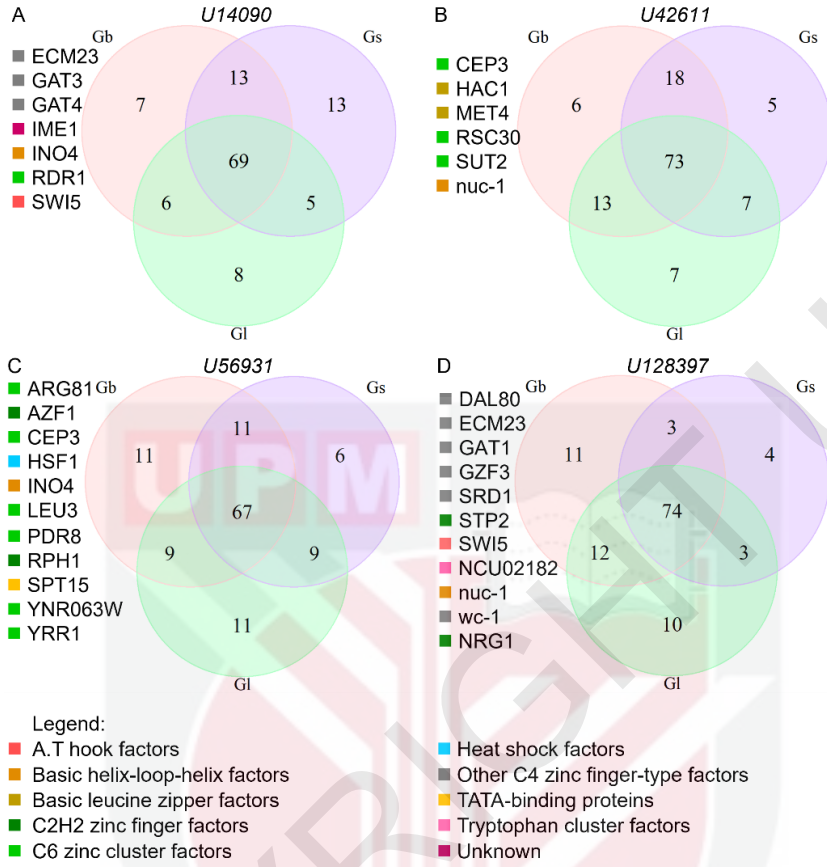


Figure 4.34 : Venn diagram of TFBS detected in promoter region of candidate transcripts in selected *Ganoderma* species. A. *U14090*, B. *U42611*, C. *U56931*, and D. *U128397*. Gb, *G. boninense*; Gs, *G. sinense*; Gl, *G. lucidum*. ARG81, Arginine metabolism regulation protein II; AZF1, Asparagine-rich zinc finger protein AZF1; CEP3, Centromere DNA-binding protein complex CBF3 subunit B; DAL80, Nitrogen regulatory protein DAL80; ECM23, Protein ECM23; GAT1, Transcriptional regulatory protein GAT1; GAT3, Protein GAT3; GAT4, Protein GAT4; GZF3, Protein GZF3; HAC1, Transcriptional activator HAC1; HSF1, Heat shock transcription factor; IME1, Meiosis-inducing protein 1; INO4, Protein INO4; LEU3, Regulatory protein LEU3; MET4, Transcriptional activator of sulfur metabolism MET4; NCU02182, MYB DNA-binding domain-containing protein; NRG1, Transcriptional regulator NRG1; nuc-1, Phosphorus acquisition-controlling protein; PDR8, Transcription factor PDR8; RDR1, Protein RDR1; RPH1, DNA damage-responsive transcriptional repressor RPH1; RSC30, Chromatin structure-remodeling complex protein RSC30; SRD1, Pre-rRNA-processing protein SRD1; SUT2, Sterol uptake protein 2; SPT15, TATA-box-binding protein; STP2, Transcription factor STP2; SWI5, Transcriptional factor SWI5; wc-1, White collar 1 protein; YNR063W, Uncharacterised transcriptional regulatory protein YNR063W; YRR1, Zinc finger transcription factor YRR1

CHAPTER 5

SUMMARY, CONCLUSION, AND RECOMMENDATIONS FOR FUTURE RESEARCH

In this study, the full-length CDS of four putative virulence factors (*U14090*, metalloproteinase; *U42611*, aspartic protease; *U56931*, lipase; *U128397*, polysaccharide deacetylase) were cloned and verified by sequencing. The deduced amino acid sequences of *U14090*, *U42611*, *U56931*, and *U128397* have the highest sequence similarities to their homologs in *G. sinense* in the same genus. In addition, they contain a signal peptide, without transmembrane domains, and are predicted to be localised extracellularly. Based on the conservation of sequence and signature domain of the candidates, they may have the same functions as their homologs.

The gene expression of these candidates in *G. boninense* at vegetative growth stages, in *in-planta* host interaction, and in phytohormone/elicitor treatment was analysed to understand their transcriptional regulation during fungal growth and plant-pathogen interactions. The transcript abundance of *U56931* was increased at Day-21 and Day-28 in relative to that in Day-7, whereas the transcript abundance of *U42611* was decreased at Day-28 compared to that in Day-7. This may reflect the involvement of these transcripts in nutrient acquisition and response towards environmental stress (e.g., nutrient depletion). *U14090* was consistently induced in *G. boninense* in contact with oil palm seedlings at 24-hpi and 48-hpi as compared to untreated *G. boninense*. In relative to the untreated *G. boninense*, *U56931* and *U128397* were also up-regulated during 12–48-hpi, while *U42611* was down-regulated at 48-hpi in *G. boninense* in contact with the host. The gene expression may reflect an early fungal response in host recognition, and it may assist the fungus to breach the host defence. On the other hand, the suppression of *U56931* by SA may suggest fungal response to phytohormone in the host.

Based on the TFBS analysis, the C2H2 zinc finger factors and C6 zinc cluster factors are the main TF classes predicted to bind to TFBS spanning the promoter sequence of the selected virulence factors and their homologs in *G. boninense*, *G. sinense*, and *G. lucidum*. Unique TFBSs to each candidate in *G. boninense* were identified. In conclusion, the identification of fungal virulence factors and their key regulators will help to develop a precise crop improvement programme in selecting oil palm traits capable of protecting the plant against pathogenic attack, leading to a longer productive lifespan while facilitating the design of chemical fungicides with enhanced efficacy.

The study on the molecular interaction between oil palm and *G. boninense* can be improved by carrying out the experiment using clonal palms in a controlled growth chamber. For future research, the function of those putative virulence factors in *G. boninense* should be validated by either gene silencing or gene overexpression, to better understand the interplay during the disease infection

and to ease the development of a precise disease control strategy. In addition, highly specific fungicides can be tailored to inhibit the putative virulence factors once their roles in virulence are confirmed. Together, these will contribute to a novel *Ganoderma* disease control strategy using genetic information from the pathogen counterpart which is currently lacking.

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APPENDICES

Appendix A

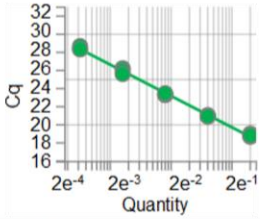
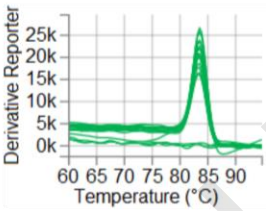
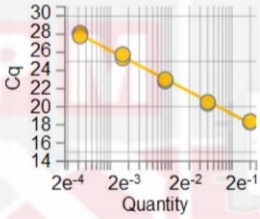
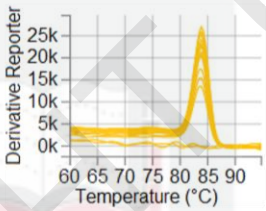
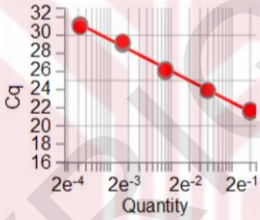
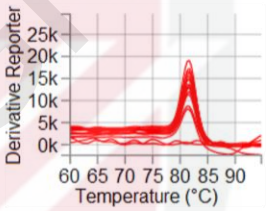
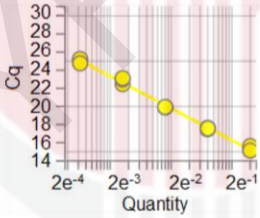
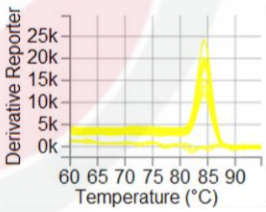
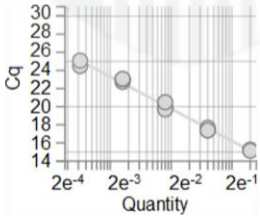
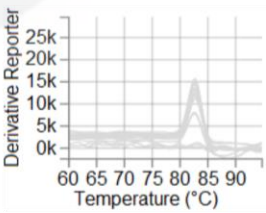
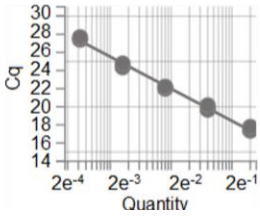
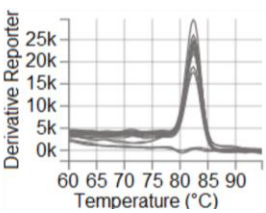
Accession number of amino acid sequences used for phylogenies construction

GenBank Accession Number	Organism
U14090	
KAF2679942.1	*Lentithecium fluviatile* CBS 122367
PAV14924	*Pyrrhoderma noxium*
KAF9492360	*Pleurotus eryngii*
RDB21981	*Hypsizygus marmoreus*
KNZ78975	*Termitomyces* sp. J132
KAF8063400	*Lyophyllum atratum*
KAF8804118	*Cortinarius glaucopus*
KAF8151674	*Crassisporium funariophilum*
KAF6747561	*Coprinellus angulatus*
XP_001838249	*Coprinopsis cinerea* okayama7#130
TFK22535	*Coprinopsis marcescibilis*
KAF9267163	*Marasmius fiardii* PR-910
ESK95467	*Moniliophthora roreri* MCA 2997
KAF7361365	*Mycena sanguinolenta*
KAF7328920	*Mycena venus*
KAF7293235	*Mycena chlorophos*
XP_037213939	*Mycena indigotica*
KAF7329057	*Mycena kentingensis*
KAF8476407	*Russula ochroleuca*
XP_007305972	*Stereum hirsutum* FP-91666 SS1
KAA1470881	*Dentipellis* sp. KUC8613
XP_027616528	*Sparassis crispa*
OBZ72764	*Grifola frondosa*
OJT06757	*Trametes pubescens*
U14090 (ON922904)	*Ganoderma boninense* BLSM5B
PIL32564	*Ganoderma sinense* ZZ0214-1
U42611	
ATY65989.1	*Cordyceps militaris*
KZT18689	*Neolentinus lepideus* HHB14362 ss-1
XP_007299149	*Stereum hirsutum* FP-91666 SS1
XP_009541068	*Heterobasidion irregulare* TC 32-1
TDL28098	*Rickenella mellea*
PAV22065	*Pyrrhoderma noxium*
OCB85625	*Sanghuangporus baumii*
KZT43813	*Sistotremastrum suecicum* HHB10207 ss-3
KZS93385	*Sistotremastrum niveocremeum* HHB9708
KAF8497321	*Gautieria morchelliformis*
KAF8588745	*Ramaria rubella*

GenBank Accession Number	Organism
OCH89539	*Obba rivulosa*
RPD58932	*Lentinus tigrinus* ALCF2SS1-6
TFK81321	*Polyporus arcularius* HHB13444
RDX41559	*Polyporus brumalis*
XP_008043424	*Trametes versicolor* FP-101664 SS1
OSD00664	*Trametes coccinea* BRFM310
XP_007367947	*Dichomitus squalens* LYAD-421 SS1
U42611 (ON922905)	*Ganoderma boninense* BLSM5B
PIL25289	*Ganoderma sinense* ZZ0214-1
TFK77430	*Pluteus cervinus*
TFK41838	*Crucibulum laeve*
XP_001881739	*Laccaria bicolor* S238N-H82
KAF9535626	*Crepidotus variabilis*
BAL02925	*Pholiota nameko*
KAF9055852	*Panaeolus papilionaceus*
KAF8910133	*Gymnopilus junonius*
AHA86292	*Leucoagaricus gongylophorus*
KAF8899074	*Infundibulicybe gibba*
KAF9469814	*Lepista nuda*
U56931	
EQB47005	*Colletotrichum gloeosporioides* Cg-14
KAI0716638	*Earliella scabrosa*
XP_007367670	*Dichomitus squalens* LYAD-421 SS1
U56931 (ON922906)	*Ganoderma boninense* BLSM5B
PIL23397	*Ganoderma sinense* ZZ0214-1
RPD75617	*Lentinus tigrinus* ALCF2SS1-7
RPD61344	*Lentinus tigrinus* ALCF2SS1-6
KAI0703659	*Polyporus squamosus*
TFK80986	*Polyporus arcularius* HHB13444
RDX40351	*Polyporus brumalis*
U128397	
XP_020044804.1	*Ascoidea rubescens* DSM 1968
EMD41637	*Gelatoporia subvermispora* B
XP_024344625	*Postia placenta* MAD-698-R-SB12
KZT06317	*Laetiporus sulphureus* 93-53
U128397 (ON922907)	*Ganoderma boninense* BLSM5B
PIL29657	*Ganoderma sinense* ZZ0214-1
TFK93558	*Polyporus arcularius* HHB13444
OSD05919	*Trametes coccinea* BRFM310
CDO70236	*Trametes cinnabarina*
XP_009540296	*Heterobasidion irregulare* TC 32-1
KDQ30470	*Pleurotus ostreatus* PC15
KAF9452563	*Macrolepiota fuliginosa* MF-IS2
KIK05550	*Laccaria amethystina* LaAM-08-1
KJA26117	*Hypholoma sublateritium* FD-334 SS-4

Appendix B

Standard curve plot and melt curve plot for qPCR primers

		Standard Curve Plot	Melt Curve Plot
Target	***U14090***		
Slope	-3.47		
R^2	0.997		
Efficiency (%)	94.37		
Error	0.056		
Target	***U42611***		
Slope	-3.51		
R^2	0.997		
Efficiency (%)	92.71		
Error	0.054		
Target	***U56931***		
Slope	-3.45		
R^2	0.996		
Efficiency (%)	94.96		
Error	0.064		
Target	***U128397***		
Slope	-3.54		
R^2	0.995		
Efficiency (%)	91.54		
Error	0.070		
Target	**eef2**		
Slope	-3.55		
R^2	0.992		
Efficiency (%)	91.22		
Error	0.091		
Target	**α-tubulin**		
Slope	-3.55		
R^2	0.995		
Efficiency (%)	91.45		
Error	0.073		

Appendix C

Expression data for fungal samples harvested at different time-point treatment

Cq_Control 1	2	3	Mean	SD	Cq 1	2	3	Mean	SD	p-val	RQ	log_2 RQ
D7					**D14**							
U14090												
23.81	23.80	23.68	23.76	0.07	23.23	23.15	23.14	23.17	0.05	**0.00**	1.14	**0.19**
U42611												
23.43	23.42	23.29	23.38	0.08	23.98	23.94	23.79	23.90	0.10	**0.00**	0.53	**-0.92**
U56931												
27.18	26.78	26.99	26.98	0.20	25.88	25.82	25.79	25.83	0.05	**0.01**	1.68	**0.75**
U128397												
20.85	20.89	20.92	20.89	0.03	20.23	20.37	20.14	20.25	0.11	**0.01**	1.18	**0.24**
D7					**D21**							
U14090												
23.81	23.80	23.68	23.76	0.07	25.61	25.67	25.70	25.66	0.04	**0.00**	1.03	**0.04**
U42611												
23.43	23.42	23.29	23.38	0.08	25.63	25.52	25.51	25.55	0.07	**0.00**	0.85	**-0.23**
U56931												
27.18	26.78	26.99	26.98	0.20	25.31	25.32	25.33	25.32	0.01	**0.00**	12.1	**3.60**
U128397												
20.85	20.89	20.92	20.89	0.03	22.49	22.54	22.46	22.50	0.04	**0.00**	1.25	**0.33**
D7					**D28**							
U14090												
23.81	23.80	23.68	23.76	0.07	26.00	26.09	26.42	26.17	0.22	**0.00**	1.16	**0.21**
U42611												
23.43	23.42	23.29	23.38	0.08	27.56	27.32	27.56	27.48	0.14	**0.00**	0.36	**-1.48**
U56931												
27.18	26.78	26.99	26.98	0.20	24.01	23.83	24.16	24.00	0.16	**0.00**	48.4	**5.60**
U128397												
20.85	20.89	20.92	20.89	0.03	22.80	22.92	22.91	22.88	0.07	**0.00**	1.54	**0.63**

Appendix D

Expression data under oil palm *in-planta* experiment (12-hpi)

Cq_Control 1	2	3	Mean Cq	SD	Cq 1	2	3	Mean Cq	SD	p-val	RQ	log_2 RQ
12h_*in-planta* 1												
U14090												
23.53	23.55	23.57	23.55	0.02	23.18	23.24	23.45	23.29	0.15	**0.07**	1.42	**0.51**
23.53	23.55	23.57	23.55	0.02	23.87	23.90	23.96	23.91	0.05	**0.00**	1.16	**0.21**
23.53	23.55	23.57	23.55	0.02	22.95	22.99	23.12	23.02	0.09	**0.01**	5.43	**2.44**
U42611												
22.09	22.19	22.26	22.18	0.09	22.85	22.97	23.26	23.03	0.21	**0.01**	0.66	**-0.60**
22.09	22.19	22.26	22.18	0.09	24.54	24.62	24.95	24.71	0.22	**0.00**	0.26	**-1.95**
22.09	22.19	22.26	22.18	0.09	24.00	24.02	24.06	24.02	0.03	**0.00**	1.05	**0.06**
U56931												
25.17	25.23	25.25	25.22	0.04	26.14	26.33	26.81	26.43	0.34	**0.02**	0.51	**-0.97**
25.17	25.23	25.25	25.22	0.04	26.32	26.97	27.25	26.85	0.48	**0.02**	0.48	**-1.06**
25.17	25.23	25.25	25.22	0.04	24.34	24.71	24.75	24.60	0.22	**0.03**	5.75	**2.52**
U128397												
20.98	21.21	21.33	21.17	0.18	21.02	21.03	21.39	21.15	0.21	**0.77**	1.21	**0.27**
20.98	21.21	21.33	21.17	0.18	21.94	21.98	22.16	22.03	0.12	**0.00**	0.82	**-0.28**
20.98	21.21	21.33	21.17	0.18	21.42	21.44	21.95	21.60	0.30	**0.06**	2.79	**1.48**
12h_*in-planta* 2												
U14090												
24.35	24.37	25.02	24.58	0.38	24.21	23.98	23.90	24.03	0.16	**0.20**	4.84	**2.28**
24.35	24.37	25.02	24.58	0.38	23.91	23.83	23.87	23.87	0.04	**0.08**	3.78	**1.92**
24.35	24.37	25.02	24.58	0.38	22.44	22.51	22.49	22.48	0.04	**0.01**	6.79	**2.76**
U42611												
26.43	26.48	26.52	26.48	0.05	28.27	28.28	28.17	28.24	0.06	**0.00**	0.97	**-0.04**
26.43	26.48	26.52	26.48	0.05	28.15	28.07	28.37	28.20	0.15	**0.00**	0.70	**-0.51**
26.43	26.48	26.52	26.48	0.05	25.70	25.71	25.74	25.72	0.02	**0.00**	2.69	**1.43**
U56931												
28.24	28.27	28.44	28.32	0.11	25.21	25.07	25.13	25.14	0.07	**0.00**	30.02	**4.91**
28.24	28.27	28.44	28.32	0.11	25.19	25.10	24.38	24.89	0.45	**0.01**	24.94	**4.64**
28.24	28.27	28.44	28.32	0.11	23.38	23.57	23.88	23.61	0.25	**0.00**	41.52	**5.38**
U128397												
22.26	22.32	22.38	22.32	0.06	22.52	22.39	22.61	22.51	0.11	**0.09**	2.90	**1.54**
22.26	22.32	22.38	22.32	0.06	22.07	22.02	22.09	22.06	0.04	**0.02**	2.76	**1.47**
22.26	22.32	22.38	22.32	0.06	20.31	20.31	20.25	20.29	0.03	**0.00**	6.49	**2.70**

Appendix E

Expression data under oil palm *in-planta* experiment (24-hpi)

Cq_Control 1	2	3	Mean	SD	Cq 1	2	3	Mean	SD	p-val	RQ	log_2 RQ
24h_*in-planta* 1												
U14090												
23.76	23.95	24.00	23.91	0.12	24.19	24.33	24.34	24.29	0.09	**0.00**	3.26	**1.70**
23.76	23.95	24.00	23.91	0.12	24.67	24.81	24.86	24.78	0.10	**0.00**	2.23	**1.16**
23.76	23.95	24.00	23.91	0.12	24.78	24.81	24.88	24.83	0.05	**0.00**	2.38	**1.25**
U42611												
23.74	23.80	24.02	23.86	0.15	23.42	23.44	24.67	23.84	0.72	**0.97**	4.27	**2.10**
23.74	23.80	24.02	23.86	0.15	24.82	24.96	25.25	25.01	0.22	**0.00**	1.84	**0.88**
23.74	23.80	24.02	23.86	0.15	24.80	24.83	25.67	25.10	0.49	**0.03**	1.90	**0.92**
U56931												
26.60	26.65	27.02	26.75	0.23	25.27	25.59	25.61	25.49	0.19	**0.01**	10.19	**3.35**
26.60	26.65	27.02	26.75	0.23	25.93	25.98	26.04	25.98	0.05	**0.02**	6.98	**2.80**
26.60	26.65	27.02	26.75	0.23	26.18	26.46	26.54	26.40	0.19	**0.05**	5.77	**2.53**
U128397												
21.59	21.80	21.96	21.79	0.18	22.69	22.76	22.97	22.81	0.15	**0.00**	2.08	**1.06**
21.59	21.80	21.96	21.79	0.18	23.06	23.13	23.36	23.18	0.16	**0.00**	1.55	**0.63**
21.59	21.80	21.96	21.79	0.18	23.28	23.32	23.74	23.45	0.25	**0.00**	1.42	**0.51**
24h_*in-planta* 2												
U14090												
24.14	24.21	24.23	24.19	0.04	22.67	22.88	22.89	22.81	0.12	**0.00**	8.21	**3.04**
24.14	24.21	24.23	24.19	0.04	24.56	24.66	24.72	24.65	0.08	**0.00**	2.14	**1.10**
24.14	24.21	24.23	24.19	0.04	23.70	23.81	23.98	23.83	0.14	**0.03**	3.84	**1.94**
U42611												
25.29	25.38	25.81	25.49	0.27	26.22	26.22	26.33	26.26	0.06	**0.03**	1.91	**0.93**
25.29	25.38	25.81	25.49	0.27	28.59	28.59	28.74	28.64	0.09	**0.00**	0.28	**-1.85**
25.29	25.38	25.81	25.49	0.27	28.16	28.16	28.41	28.25	0.14	**0.00**	0.44	**-1.19**
U56931												
26.74	27.06	27.19	27.00	0.23	24.39	24.44	24.52	24.45	0.07	**0.00**	18.39	**4.20**
26.74	27.06	27.19	27.00	0.23	24.31	24.48	24.60	24.47	0.14	**0.00**	16.92	**4.08**
26.74	27.06	27.19	27.00	0.23	24.97	25.17	25.37	25.17	0.20	**0.00**	10.62	**3.41**
U128397												
21.96	21.97	22.09	22.01	0.07	21.26	21.39	21.53	21.39	0.13	**0.00**	4.81	**2.27**
21.96	21.97	22.09	22.01	0.07	21.39	21.48	21.57	21.48	0.09	**0.00**	4.22	**2.08**
21.96	21.97	22.09	22.01	0.07	21.61	21.62	21.78	21.67	0.09	**0.00**	3.78	**1.92**

Appendix F

Expression data under oil palm *in-planta* experiment (48-hpi)

Cq_Control 1	2	3	Mean	SD	Cq 1	2	3	Mean	SD	p-val	RQ	log_2 RQ
48h_*in-planta* 1												
U14090												
24.41	24.63	24.83	24.62	0.21	22.18	22.29	22.35	22.28	0.09	**0.00**	24.70	**4.63**
24.41	24.63	24.83	24.62	0.21	23.09	23.22	23.27	23.19	0.10	**0.00**	5.32	**2.41**
24.41	24.63	24.83	24.62	0.21	22.82	22.90	22.93	22.88	0.06	**0.00**	9.36	**3.23**
U42611												
25.25	25.51	25.61	25.46	0.18	27.16	27.26	27.40	27.28	0.12	**0.00**	1.37	**0.46**
25.25	25.51	25.61	25.46	0.18	28.18	28.35	28.55	28.36	0.19	**0.00**	0.26	**-1.92**
25.25	25.51	25.61	25.46	0.18	28.17	28.67	28.76	28.53	0.32	**0.00**	0.33	**-1.59**
U56931												
28.67	29.02	29.37	29.02	0.35	25.81	26.67	26.79	26.42	0.53	**0.00**	29.47	**4.88**
28.67	29.02	29.37	29.02	0.35	26.21	26.96	26.99	26.72	0.44	**0.00**	9.74	**3.28**
28.67	29.02	29.37	29.02	0.35	25.87	26.42	26.88	26.39	0.50	**0.00**	17.40	**4.12**
U128397												
22.44	22.46	22.83	22.58	0.22	21.85	21.88	22.01	21.91	0.09	**0.01**	7.68	**2.94**
22.44	22.46	22.83	22.58	0.22	22.00	22.01	22.03	22.01	0.02	**0.04**	2.92	**1.55**
22.44	22.46	22.83	22.58	0.22	21.68	21.78	21.83	21.77	0.08	**0.01**	4.92	**2.30**
48h_*in-planta* 2												
U14090												
22.20	22.38	22.54	22.37	0.17	22.91	23.01	23.16	23.03	0.13	**0.00**	4.79	**2.26**
22.20	22.38	22.54	22.37	0.17	22.58	23.09	23.28	22.98	0.36	**0.03**	5.65	**2.50**
22.20	22.38	22.54	22.37	0.17	23.21	23.48	23.57	23.42	0.19	**0.00**	3.71	**1.89**
U42611												
22.43	22.43	22.65	22.50	0.13	28.73	28.95	29.00	28.89	0.15	**0.00**	0.10	**-3.29**
22.43	22.43	22.65	22.50	0.13	29.04	29.13	29.47	29.21	0.23	**0.00**	0.08	**-3.57**
22.43	22.43	22.65	22.50	0.13	28.33	28.36	28.42	28.37	0.05	**0.00**	0.14	**-2.82**
U56931												
25.52	25.62	25.87	25.67	0.18	25.04	25.23	25.29	25.18	0.13	**0.01**	10.56	**3.40**
25.52	25.62	25.87	25.67	0.18	25.60	25.61	25.82	25.68	0.12	**0.88**	8.58	**3.10**
25.52	25.62	25.87	25.67	0.18	25.45	25.48	25.95	25.63	0.28	**0.60**	7.88	**2.98**
U128397												
19.11	19.26	19.45	19.27	0.17	21.30	21.32	21.39	21.34	0.05	**0.00**	2.05	**1.04**
19.11	19.26	19.45	19.27	0.17	21.64	21.70	21.74	21.69	0.05	**0.00**	1.64	**0.72**
19.11	19.26	19.45	19.27	0.17	22.00	22.00	22.06	22.02	0.04	**0.00**	1.23	**0.30**

Appendix G

Expression data under phytohormones and elicitor treatment

Cq_Control 1	2	3	Mean	SD	Cq 1	2	3	Mean	SD	p-val	RQ	log_2 RQ
SA												
U14090												
24.51	24.51	24.42	24.48	0.05	25.57	25.45	25.50	25.51	0.06	**0.00**	0.66	**-0.60**
U42611												
24.76	25.02	24.99	24.92	0.14	24.44	24.66	24.60	24.57	0.11	**0.00**	1.72	**0.78**
U56931												
26.32	26.08	25.97	26.12	0.18	28.15	28.00	28.18	28.11	0.10	**0.00**	0.34	**-1.56**
U128397												
20.92	21.06	20.99	20.99	0.07	21.83	21.83	21.99	21.88	0.09	**0.01**	0.73	**-0.46**
JA												
U14090												
24.70	24.54	24.53	24.59	0.09	24.96	24.98	25.09	25.01	0.07	**0.04**	1.08	**0.11**
U42611												
24.95	25.03	24.96	24.98	0.04	24.79	24.85	24.96	24.87	0.09	**0.18**	1.56	**0.64**
U56931												
26.23	26.16	26.12	26.17	0.05	27.29	27.52	27.26	27.35	0.14	**0.01**	0.63	**-0.66**
U128397												
20.87	20.93	21.00	20.93	0.07	21.17	21.35	21.33	21.29	0.10	**0.01**	1.13	**0.17**
H_2O_2												
U14090												
24.13	24.23	24.29	24.22	0.08	23.43	23.33	23.45	23.40	0.06	**0.01**	1.00	**0.00**
U42611												
25.35	25.39	25.00	25.25	0.22	24.50	24.63	24.46	24.53	0.09	**0.02**	1.22	**0.28**
U56931												
26.10	26.18	26.15	26.14	0.04	26.02	26.03	26.30	26.12	0.16	**0.80**	0.75	**-0.41**
U128397												
20.92	21.16	21.04	21.04	0.12	20.33	20.34	20.27	20.31	0.04	**0.01**	1.22	**0.29**

Appendix H

R Scripts

R scripts for TFBS analysis

```
library(JASPAR2020)
library(TFBSTools)
library(Biostrings)
library(ggplot2)
library(GenomicRanges)


#Retrieve from JASPAR2020_Database

optsF<-list()
optsF[["tax_group"]]<-"fungi"
PFMatrixListF<-getMatrixSet(JASPAR2022,optsF)
pwmlist_sc<-c(PFMatrixListF)
pwmlist_sc<-toPWM(c(PFMatrixListF))


#Sequence input and analysis

seq14090.Gb.1000.90<-
DNAString("ACCGGATTGTCCAGCCAATCTTCAAGTGAAATTGGTATGTGG
ACGGGGGGCTGGGACTCGGACATGGCAAGCAGTGCGTTGAGACTCGAG
AGCGACAAGGTTCAGCGGGAAGGGCTTCAAAATGATAGTTCCAAGAAATG
TTCGTTCCCGACCAGAATCGAACACACGGCCTGACGCAGAGCCCAACAG
AAGCCCTGTGCACCAACCAACTGAGCCATCCGCCCTGCATTTTGGGCGC
GTAAAATAAGCCCTATGTTGCTGTGTGCTTAAACCTGACGACGATCGGAA
ACAGCCCCAGCTTGTCGGACGATCGTGCAGTGTCAGTCTGACAGTCAGT
GGTCCTCCAGTACGCTTGCAACCATCCTTACGCTGAACAATTTTTTTCGAT
TGGGAGCCTCGGTCAGGAATCGGTTTCACCCACCAGTGCACCAATGTCC
ATGCTGGTCCATGACTCACCTAAATATCTATGGCGATTTTCACTTGCGAGA
TAAGAGACCTTCCGCAAAGAGCGACAGTAAGGCCGCCGGCATCGTCTCC
AAAACAGCGCAAACCAGGCGACTGGGCATATGTGGGGTGGGTGGAAGG
GACCCCGGCCCTAATACACAGCTTCGGACTACAGTCGCGGGCGTCGGCG
TAAAGACTATCGCACGGCCAGCTCACCTTGTCGGAGTATCATTGGAGGCG
TGCACCGCCAGATCCGGGTTGCTCTTCGAGATAAGAGCAGGCTGTGACG
CAGCAAGTTGCCCGATGTGGCTCGAAAGGAAGTGGACGTGCGCAGAAGC
AGCGTGAAGGAAGTGTGCGGGGCGTGTGACAGAAGCCGGCTCGGCGCT
CGGGGTGCCCAATATGGGGTGCCACCGGATGACGGGGACGGTCGCGTG
CAGGATAAGAAGGGACTGTCCCGCAGCCGCCTCTTTCCTCCCCACCATC
ACCTCCCCCTCTTCATCGTCGGGGAGCGTCTTCTGCACCCTCTCTCAACC
CGCTATCCTCTCTCCTGCCTAGT")
```

```
class(seq14090.Gb.1000.90)
as.character(seq14090.Gb.1000.90)
siteset_sc.14090.Gb.1000.90 <- searchSeq(pwmlist_sc, seq14090.Gb.1000.90,
    seqname="seq14090.Gb", strand="*", min.score="90%")
sitesetList_sc.14090.Gb.1000.90 <- searchSeq(pwmlist_sc,
    seq14090.Gb.1000.90, seqname="seq14090.Gb",
    strand="*",min.score="90%", mc.cores=1L)
as(siteset_sc.14090.Gb.1000.90, "data.frame")
as(siteset_sc.14090.Gb.1000.90, "DataFrame")
as(siteset_sc.14090.Gb.1000.90, "GRanges")
write.csv(siteset_sc.14090.Gb.1000.90,"2022.2.scores14090.Gb.1000.90.csv")
```

#Repeat for the following by replacing the DNA sequence and sequence identifier for the following sequences

>seq14090.Gb.1000.90
ACCGGATTGTCCAGCCAATCTTCAAGTGAAATTGGTATGTGGACGGGGG
GCTGGGACTCGGACATGGCAAGCAGTGCGTTGAGACTCGAGAGCGACAA
GGTTCAGCGGGAAGGGCTTCAAAATGATAGTTCCAAGAAATGTTCGTTCC
CGACCAGAATCGAACACACGGCCTGACGCAGAGCCCAACAGAAGCCCTG
TGCACCAACCAACTGAGCCATCCGCCCTGCATTTTGGGCGCGTAAAATAA
GCCCTATGTTGCTGTGTGCTTAAACCTGACGACGATCGGAAACAGCCCCA
GCTTGTCGGACGATCGTGCAGTGTCAGTCTGACAGTCAGTGGTCCTCCA
GTACGCTTGCAACCATCCTTACGCTGAACAATTTTTTTCGATTGGGAGCCT
CGGTCAGGAATCGGTTTCACCCACCAGTGCACCAATGTCCATGCTGGTCC
ATGACTCACCTAAATATCTATGGCGATTTTCACTTGCGAGATAAGAGACCT
TCCGCAAAGAGCGACAGTAAGGCCGCCGGCATCGTCTCCAAAACAGCGC
AAACCAGGCGACTGGGCATATGTGGGGTGGGTGGAAGGGACCCCGGCC
CTAATACACAGCTTCGGACTACAGTCGCGGGCGTCGGCGTAAAGACTATC
GCACGGCCAGCTCACCTTGTCGGAGTATCATTGGAGGCGTGCACCGCCA
GATCCGGGTTGCTCTTCGAGATAAGAGCAGGCTGTGACGCAGCAAGTTG
CCCGATGTGGCTCGAAAGGAAGTGGACGTGCGCAGAAGCAGCGTGAAG
GAAGTGTGCGGGGCGTGTGACAGAAGCCGGCTCGGCGCTCGGGGTGCC
CAATATGGGGTGCCACCGGATGACGGGGACGGTCGCGTGCAGGATAAG
AAGGGACTGTCCCGCAGCCGCCTCTTTCCTCCCCACCATCACCTCCCCCT
CTTCATCGTCGGGGAGCGTCTTCTGCACCCTCTCTCAACCCGCTATCCTC
TCTCCTGCCTAGT

>seq14090.Gs.1000.90
AGGGGCACTGATTCTTGGATGTAGTGGAACATTGACAAGCAGTGAGTTGA
CGGAGGTTGACGGCAGGTTGAGCTGCAGAGGGAGGGACACCCGGTCGC
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TGCACCAACCAACTGAGCCATCCAAGCTGTAATTGGGCGCTCGAAATCAA
CCAATTTATTCTTGCAGTGGGCCCTGAGTGGACACAACCAGGCGACGATC
GGAAACAGCCTCAGGTTGACAACTCGGATCGTACGCTCCAATATACAACA
AACCTTACTTTACACTGAACGGATTTTGCCTACCACAGCTGATATCGATAG
GAGACCTCGGTCAGGAATCGGTTCCACCCAGGTTTCACCTGTCAATGCTA
GCTGCAGCAAAATGTCCGTGACCATGTTCATGTGCAATATAAGAGGACTT

CTGCCAAGCGCGGTCGACAGTAAGACCGCCGGCATCGTCCCCCAATGGC
AAGAGCGCAAACCGGGCAACTGGGGATATGTGGGGTTGGTGGAAAGAAC
CCCGGCTCGTGTGATGCACGCAAGACATGGCGGAGATGACACAACTTAC
CCTACGGGACCAACCACATACACTCGCCGGCGGCGACGAAAACGTTATC
GCCCGGCCAGTGCTTGCTTTGTCGGAGTGTCATTGGATGCGTGCACTGC
CAGATTAGGGTGAGTCTTTGATAAGGGCAGACTGCAACGCGGCAACTCG
TCCGATGTGGCTCGAAAGGCAATCGACGTGCGCAGAGGCAGCGTGAAGG
AAGTGTGCGGGGCGTGTGAAAGAAGCCGGCTCGGCGCTCGGGGTGCCA
AATATGGGGTGCCACCGGATGACGGGGACTGTCGTGTGCAGGATAAGAA
GGGGACAGTCCCGCAGCCGTCCCGTCTCTCCCCACCATCACCTCCCCCT
CTTCATCGTCGGGGAGCATCTTCTACACCCTCTCTCAACCCGCTGTCCTC
TCGTTTACCCAGT

>seq14090.Gl.1000.90
ATATAAATCCTAGAAAGTCGTAGCAGTCGAGGTTTGTTATGTTGTGATAGA
GAGGAAACAAAATCAGTCATGGCGTTGAGCAGACACTGGGCGCTAGAGC
CTTCTCGCGGTCGCGGCACCCTCATGCCCTCGCGCACTGTGCGCGCGCC
ATCGAAATCATTTCAGTCGTGTATAGTAAGCCATAATGAATATATTATGTAC
TCGCGAAGTCGTATAGGTCCATAGTCGAAGAACAGGAGGAGGGGGAAAG
GCGCCGGCGCGGGTGTCCCATTATTTTTGGCGGCAACTGTACATAGAGC
AAAAGAGAGATACTGTACTCATGTAAGTACTGTATCGTAAGTAAGGATGGA
AGGATTTGCGAATACATTGTGAATCTGTTCCTATTACAGTCGATAACGGCA
ATCAGGCATCGGTTTCACATTACCAGACTCCAGTCAAGGACGACCGCCG
GTATCATCCCCCAAACGGCGCAAAGCGGTTGACTGGGGATATGTGGGGC
TGGCTGCGGCTGGCTGGATTCGAAGGTACCCCGGCTCGTGTGATCCACG
CAAGACATGGTGGAAATGGCAACAACTTGCCCAACGATGCACAGCTTGCC
ACATACACTCGCGGGCGTCGGCGTAAACGTTACCGCCCGCGCAAATACA
ATTTTGATCACCCGGAGTATCGTTGGCCGTGGGCTCTGCCGGATAGGGG
TCCCAGTTCTTCGATAAGAGAGCAGAGCCACTTTGTGCGATGAGACTAAA
AAGGGAGTCGAGGCGTGAAGTCCTGAGAGATTGAGTCCGAGTCAGCGCG
TAGCAGGGGCGTGTGAAAGAAGCCGGCTCGGCGCTCGGGGTGCCAAAT
ATGGGGTGCCACCGGATGACGGAGCTGTCGCGTGCAGGATAAGAAGGG
CTCCGTCCCGCAGCCGCCCCTTCTCCCCCAACCATCACCTCCCCCCCTT
CATCGTCGGGGAGCATCTTCTACTCCTTCCCTCTACCCGCTAGCCTCTCT
TTTACGCAAC

>seq42611.Gb.1000.90
TGCCCCACCGGAGCACATGAACTCCACACCCGTGCGGTCAGGACCTGTC
CTTCAACTCACTCTTGTCAGAAGTCCATTTGAAGATCCTTTTAGTCTCTCTA
TTCTGGGCTGCTGCGCAATCCTCTTTATGTTCACCGGGCTCCCTGACATC
TTGCGTCCGGTGGAATTATTATGTCGTACTCGATTCTCACAGCCAGGTGG
TACACATGGCATCGCATCCCATAACGTGAGGCTCATCGATCGTCAGCGGC
ACCTTTCGCTCTGCACGCCACGACGTTGAGCTTCCGTCCGCACAAATGG
GCGAGGATCTCCTTCTATCTGTATGTCAGGTATAGTGATGTTCTTGTATCC
CTATAACAACAGTCGCTCATGTCTTGAGCTGTCTCTGACTCTCTGAAGTCT
CGCAGATTCCCCGAAGAAGCTCACAGCGCTAGTCAGTAGAGTTTTCTCAG
TTACGTGAGTCGGCTTTCCTCCCACAGGTGTTGAGTTAAAATTACACGTG
GTTCCCTTTACAAGGTATGGCCATATGCTGTTACTGCTTCTATGAAAGGCT

TACTCCGGGACCCCCGACTCCCTAGTAGCTTCTACAGACACGGCCACTGT
ACCTGCCCGTTGATGCTCAGAAGCTGTACAAGGGGATCAACGAGATATGT
ACCCAGGATGCACCATTGAAGATGGAACACAGGGGGTTGTCCTTGCCTAA
CACGACTTTTCTGTATAATCACTTGCCCACCCCCCGCCAAAACTCCGTATA
CCTCATGCCAAGTCCAGCAACCCAAGTTCGGGGTCCCACGATGCTTCTCC
GTCGCGGCCATGACCTTTCGGAACTTCGTTCTGGGTGCCTGGTGGCATG
ATCTGGGGAATGGATCCGACGGACTCCCAGAGGATCCTTCGCTATAAAAA
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R scripts to identify unique TFBS

```
library(VennDiagram)
library(ggbio)
library(ggplot2)

#Read file
scores14090.Gb.1000.90 <- read_csv("2022.2.scores14090.Gb.1000.90.csv")
scores14090.Gs.1000.90 <- read_csv("2022.2.scores14090.Gs.1000.90.csv")
scores14090.Gl.1000.90 <- read_csv("2022.2.scores14090.Gl.1000.90.csv")

#Select data for TF
TF14090.Gb.1000.90<-scores14090.Gb.1000.90$TF
TF14090.Gs.1000.90<-scores14090.Gs.1000.90$TF
TF14090.Gl.1000.90<-scores14090.Gl.1000.90$TF

#Draw Venn diagram
set1<-c(TF14090.Gb.1000.90)
set2 <-c(TF14090.Gs.1000.90)
set3 <- c(TF14090.Gl.1000.90)

#List out elements in Venn Diagram
overlap <- calculate.overlap(x = list(set1, set2, set3))
sink("overlapTF.1000.14090.90.GbGsGl.csv")
print(overlap)
sink()

#Repeat for the other candidates by replacing the sequence/file identifier
#Elements in a1 are unique for G. boninense
```

Appendix I

Abundance of TF classes predicted in promoter sequence

No	Class	*U14090*			*U42611*			*U56931*			*U128397*		
		Gb	Gs	Gl	Gb	Gs	Gl	Gb	Gs	Gl	Gb	Gs	Gl
1	A.T hook factors	0.8	0.0	0.2	0.5	0.3	0.9	0.0	0.0	0.0	0.4	0.0	0.0
2	APSES-type DNA-binding domain	3.6	4.9	7.7	3.3	1.3	3.8	2.1	4.0	6.1	7.0	9.2	4.2
3	Basic helix-loop-helix factors (bHLH)	0.3	1.8	0.0	3.6	2.3	2.3	2.1	0.6	1.2	0.4	0.3	1.6
4	Basic leucine zipper factors (bZIP)	3.3	3.6	2.8	6.9	5.4	6.9	4.5	5.8	4.9	3.8	5.6	4.0
5	C2H2 zinc finger factors	33.4	30.8	32.7	35.6	30.4	29.8	25.9	27.2	23.7	30.3	25.7	28.2
6	C6 zinc cluster factors	26.5	28.8	29.2	22.4	34.3	20.2	30.7	32.1	36.4	31.4	27.7	33.4
7	Copper-fist DNA-binding domain	0.0	0.0	0.7	0.0	0.3	0.3	0.0	0.0	0.0	0.2	0.3	0.0
8	Fork head / winged helix factors	1.3	0.5	0.7	1.3	0.8	0.3	1.2	1.5	2.0	1.8	1.8	0.9
9	Heat shock factors	3.9	4.1	2.6	3.1	2.6	3.8	5.4	3.4	4.9	4.3	6.1	6.6
10	Heteromeric CCAAT-binding factors	3.1	3.6	1.4	1.0	1.3	2.3	3.3	2.8	2.0	2.2	3.1	2.4
11	High-mobility group (HMG) domain factors	1.0	0.8	0.7	0.5	0.3	0.3	0.3	0.0	0.3	0.2	0.5	0.5
12	Homeo domain factors	4.1	5.1	5.6	4.8	5.4	8.1	2.4	4.0	3.5	4.3	5.3	4.7
13	MADS box factors	2.1	1.5	1.4	2.0	1.3	2.3	2.4	2.4	1.4	2.0	2.5	1.9
14	Other C4 zinc finger-type factors	7.5	6.2	4.9	5.6	2.6	5.2	4.5	3.1	2.0	1.3	0.5	0.2
15	RING-type zinc finger	0.0	0.8	0.9	0.8	0.5	1.2	1.8	1.2	0.3	0.9	1.0	0.7
16	TATA-binding proteins	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0
17	TEA domain factors	0.0	0.0	0.0	0.8	0.0	0.3	1.2	0.3	0.9	0.2	0.3	0.5
18	Tryptophan cluster factors	0.0	0.0	0.0	0.0	0.5	0.3	0.0	0.0	0.3	0.0	0.3	0.0
19	Unknown	9.3	7.5	8.6	7.9	10.6	11.8	12.2	11.6	10.1	9.2	9.9	10.1
	Total Percentage (%)	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

BIODATA OF STUDENT

Jee Mui Sie was born on 25 November 1993 in Kuching, Sarawak. She received her primary education from Sekolah Jenis Kebangsaan (Cina) Sungai Apong (2000-2005) and continued her secondary education and pre-university education in Sekolah Menengah Kebangsaan Pending (2006-2010; 2011-2012). She pursued her tertiary education at Universiti Malaysia Sarawak (UNIMAS) in 2013 and graduated with a Bachelor of Science with Honours (Resource Biotechnology) in November 2016. In the same year, she joined Sarawak Tropical Peat Research Institute (TROPI) as an Assistant Research Officer. Then, she was given an opportunity to further post-graduate study on *Ganoderma* research. In September 2019, she enrolled in a Master of Science degree programme at the Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, under the supervision of Prof. Dr. Ho Chai Ling.

LIST OF PUBLICATIONS

Journal article accepted for publication

Jee, M. S., Ho, C.-L., Yusof, M. T., Lau, S. Y. L, Midot, F., Lo, M. L., Chin, M.-Y., Melling, L. (2025). Gene expression of transcripts encoding putative secreted proteins from an oil palm fungal pathogen *Ganoderma boninense*. *Physiological and Molecular Plant Pathology*, 102715. https://doi.org/10.1016/j.pmpp.2025.102715.

Poster presented

Jee, M. S., Ho, C.-L., Yusof, M. T., Lau, S. Y. L., Midot, F., Lo, M. L., Chin, M.-Y., Melling, L. (2023). *Molecular cloning and characterization of putative virulence factors of Ganoderma boninense* [Poster]. PIPOC 2023 International Palm Oil Congress and Exhibition: Navigating Uncertainties Building Resilience, November 7-9, 2023; Kuala Lumpur, Malaysia.

Jee, M. S., Ho, C.-L., Yusof, M. T., Lau, S. Y. L., Midot, F., Lo, M. L., Chin, M.-Y., Melling, L. (2023). *Molecular cloning and characterization of putative virulence factors of Ganoderma boninense* [Poster]. New Phytologists Next Generation Scientists, July 2-5, 2023; National University Singapore, Singapore.