GENOME-WIDE IDENTIFICATION AND FUNCTIONAL CHARACTERISATION OF β-1,3-GLUCANASE GENES DURING BIOTIC AND ABIOTIC STRESSES IN *Hevea brasiliensis*

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by

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LIST OF SYMBOLS AND ABBREVIATIONS

3’	3-prime
5’	5-prime
ABA	Abscisic acid
A_{260}	Absorbance at 260 nm
A_{280}	Absorbance at 280 nm
α	Alpha
ANOVA	Analysis of variance
&	And
et al.	And others
bp	base pair
BLAST	Basic Local Alignment Search Tool
β	Beta
GLU	Beta-1,3-Glucanase
β-ME	Beta-mercaptoethanol
CBM	Carbohydrate Binding Module
cm	Centimetre
cDNA	Complementary DNA
CDD	Conserved Domain Database
CLF	Corynespora Leaf Fall
CTS	C-terminal sequence
℃	Degree Celsius
dNTP	Deoxynucleotide triphosphate
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
DEPC	Diethyl pyrocarbonate

dPCR	Digital polymerase chain reaction
DTT	Dithiothreitol
ddH_2O	Double-distilled water
U/μL	Enzyme unit per microliter
GH-17	Glycoside hydrolase family 17
g	Gram
GC	Guanine-cytosine
g/t/t	Gutta ("drop")
HSP	Heat shock proteins
HSD	Honestly Significant Difference
HR	Hypersensitive responses
ID	Identical
IgE	Immunoglobulin E
IAA	Indole-3-acetic acid
pI	Isoelectric point
IPP	Isopentenyl pyrophosphate
JA	Jasmonic acid
kDa	Kilodalton
Tm	Melting temperature
Tm	Melting temperature
mRNA	Messenger RNA
m	Metre
μL	Microliter
μM	Micromolar
mg	Milligram
mg/dm^3	Milligram per cubic decimetre
mL	Millilitre

mM	Millimolar
−	Minus
NAC tf	NAM, ATAF1/2, and CUC2 transcription factor
ng/μL	Nanogram per microliter
nM	Nanomolar
NCBI	National Center for Biotechnology Information
NR	Natural rubber
NTS	N-terminal signal peptide
NBS	nucleotide-binding site
OD_{260}	Optical density measured at 260 nm
PR	Pathogenesis-related
PME	Pectin methylesterase
%	Percentage
±	Plus-minus
PCR	Polymerase chain reaction
pH	Power of hydrogen
ROS	Reactive oxygen species
RT-PCR	Reverse-transcriptase polymerase chain reaction
rpm	Revolution per minute
RNase	Ribonuclease
RNA	Ribonucleic acid
REF	Rubber elongation factor
RPA	Rubber particle aggregation
SA	Salicylic acid
spp.	Several species
SMART	Simple Modular Architecture Research Tool
SRPP	Small rubber particle protein

SALB	South American leaf blight
sp.	Species
SE	Standard error
SPSS	Statistical Package for the Social Sciences
SAR	Systemic acquired resistance
×	Times
× g	Times gravity
TAE	Tris Acetate-EDTA
UV	Ultraviolet
V	Volt
w/v	Weight per volume
XTH	Xyloglucan endotransglucosylase

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PENGENALPASTIAN SELURUH GENOM DAN PENCIRIAN KEFUNGSIAN GEN B-1,3-GLUCANASE SEMASA TEKANAN BIOTIK DAN ABIOTIK DALAM *Hevea brasiliensis*

ABSTRAK

Getah asli (NR) merupakan salah satu bahan mentah industri yang paling banyak digunakan, manakala *Hevea brasiliensis* merupakan spesies yang paling eksklusif untuk menghasilkan NR berkualiti tinggi secara komersial. Walau bagaimanapun, penanaman *H. brasiliensis* menghadapi cabaran yang akan menurunkan pengeluaran getah asli, seperi keperluan persekitaran pertumbuhannya yang khusus dan mudah terdedah kepada jangkitan kulat. Oleh itu, kajian mengenai gen berkaitan ketahanan terhadap penyakit dan pembiakan varieti yang dapat menahan penyakit baru adalah penting untuk mengekalkan peranan *H. brasiliensis* sebagai sumber getah asli yang menjanjikan. β-1,3-glucanase (GLU) merupakan salah satu protein berkaitan patogenesis yang termasuk dalam keluarga protein PR-2 dan boleh ditemui dengan banyaknya dalam *H. brasiliensis*. Selain signifikannya dalam proses fisiologi dan perkembangan, protein ini adalah penting dalam pertahanan terhadap serangan mikrob patogen dengan kesan fungisidnya secara langsung. Beberapa jujukan nukleotida yang mengandungi GLU telah dikenal pasti dalam *H. brasiliensis* dengan peningkatan pengekspresan mereka sebagai gerak balas terhadap beberapa patogen. Walau bagaimanapun, cabaran utama untuk peningkatan kualiti *H. brasiliensis* melalui GLUs adalah kekurangan kurasi gen GLU yang dikumpulkan dan dicirikan serta pemahaman mengenai ekspresi gen masing-masing dalam keadaan fisiologi yang berbeza. Dalam kajian ini, gen GLU telah dikumpulkan daripada beberapa pangkalan data dan dikurasikan. Pencirian gen dan analisis pengekspresan

gen telah dijalankan dalam pelbagai keadaan: peringkat daun yang berbeza, tekanan abiotik dan biotik. Empat belas jujukan penuh telah dipilih dan dikategorikan kepada empat kelas (I–IV), diikuti dengan pengelompokan ke dalam enam klad utama (I–VI) dalam analisis filogenetik. Didapati bahawa GLU yang diekspresikan dalam daun mencapai tahap ekspresi tertinggi dalam peringkat III. Dalam keadaan kekeringan dan banjir, peningkatan GLU 1 dan GLU 5 yang signifikan di dalam daun menunjukkan peranan penting GLU dalam mekanisme tindak balas terhadap tekanan abiotik tersebut. Apabila dijangkiti oleh strain *C. cassiicola* CLN 16, ekspresi GLU 1 telah menunjukkan peningkatan dalam kedua-dua klon yang toleran dan terdedah. Peningkatan GLU 5 dalam klon yang toleran dan penurunannya dalam klon yang terdedah telah mencadangkan bahawa GLU 5 sebagai gen calon yang sesuai untuk membangunkan klon baru dengan ketahanan yang lebih tinggi terhadap *C. cassiicola* CLN 16.

GENOME-WIDE IDENTIFICATION AND FUNCTIONAL CHARACTERISATION OF B-1,3-GLUCANASE GENES DURING BIOTIC AND ABIOTIC STRESSES IN *Hevea brasiliensis*

ABSTRACT

Natural rubber (NR) is one of the most widely used industrial raw materials while *Hevea brasiliensis* is the most exclusive species to produce high-quality NR commercially. However, the cultivation of *H. brasiliensis* faces numerous challenges which can negatively impact the production of natural rubber, such as the specific growth environment requirement of *H. brasiliensis* and its high susceptibility to fungal infections. Hence, the studies on disease-resistance related genes as well as breeding new disease-resistant varieties are crucial to maintain the role of *H. brasiliensis* as the promising source of natural rubber. β-1,3-glucanases (GLUs) are pathogenesis-related proteins that belong to the family of PR-2 proteins and can be abundantly found in *H. brasiliensis*. Besides their significance in physiological and developmental processes, they are vital in defending against microbial pathogen invasion with direct fungicidal effects. A few nucleotide sequences encoding GLUs have been identified from *H. brasiliensis* with their overexpression in response to a few fungal pathogens. However, the main challenges for utilising GLUs in the crop improvement include the lack of curated and characterised GLU gene sequences and understanding regarding the respective gene expression under different physiological conditions. In the current study, GLU sequences deposited in various databases were obtained, manually curated, analysed the gene features and the gene expression analysis under various conditions: different stages of leaves, abiotic and biotic stress. Fourteen full-length sequences were obtained and categorised into four distinct classes (I–IV), which

formed six major clades (I–VI) in phylogenetic analysis. It was observed that GLUs expressed in leaves achieved the highest expression level in stage III leaves with apple green colour. Under both drought and flooding conditions, significant upregulations of GLU 1 and GLU 5 were observed in leaves, indicating the significant role of both GLUs in the plants' response mechanism towards respective abiotic stress. Upon infection by *C. cassiicola* strain CLN 16, GLU 1 showed upregulation in both tolerant and susceptible clone whereas GLU 5 exhibited upregulation in tolerant clone but downregulation in susceptible clone. The results suggest that GLU 5 might be the potential candidate gene to develop novel clone with higher resistance to the fungus *C. cassiicola* CLN 16.

CHAPTER 1

INTRODUCTION

1.1 Introduction

Natural rubber (NR) is a remarkable material with a complex molecular structure, primarily composed of long polymer chains of isoprene units (Archer *et al*., 1963; Lau *et al*., 2016; Yeang *et al*., 2002). NR can be derived commercially from the latex of the *Hevea brasiliensis* (Nair, 2021). NR has been a valuable material for humanity for centuries as its unique molecular structure has contributed to its impressive elasticity and resilience properties. NR becomes even more durable during vulcanisation, retaining its elasticity over a wide temperature range (Cataldo, 2000; Cornish, 2001). Its unique properties and versatile applications have played a pivotal role in shaping various manufacturing industries and improving our daily lives (Nair, 2021).

H. brasiliensis (Para rubber tree) is the most exclusive species for commercial production of NR (Men *et al*., 2019). Originating from Brazil, the cultivation of *H. brasiliensis* is currently concentrated in tropical regions, particularly Southeast Asia, due to the ideal climate for growth. Countries such as Thailand, Indonesia, Malaysia, and Vietnam are some of the largest NR producers (Archer & Audley, 1987; Lau *et al*., 2016; Priyadarshan, 2017). Despite being the most promising source of NR, *H. brasiliensis* faces numerous challenges that can highly impact the NR yield and latex prices (Radhakrishnan *et al*., 2021; Yeang *et al*., 2002). These challenges include monoculture, climate change, labour intensity, market price volatility and, most importantly, the high susceptibility of *H. brasiliensis* to pests and diseases (Mooibroek & Cornish, 2000).

Corynespora Leaf Fall (CLF), caused by the fungal pathogen *Corynespora cassiicola,* is one of the major fungal diseases of *H. brasiliensis* in Asian and African countries (Déon *et al*., 2013). CLF was first identified in India in 1958 (Ramakirishnan & Pillai, 1961) and then in Malaysia in 1960 (Newsam, 1960; Pu et al., 2007). *C. cassiicola* can affect both young and old leaves, leading to extensive defoliation, delayed maturation of rubber trees and even death (Pu *et al*., 2007). Despite measurements such as quarantine measures, hygiene practice and fungicides, the most effective strategy for managing fungal disease is developing novel clones with enhanced pathogen or disease resistance.

Abiotic stress refers to adverse environmental conditions that can negatively affect the growth and productivity of plants. Common types of abiotic stress include drought, salinity, temperature, flooding, and pollution. Since *H. brasiliensis* is a tropical plant which is adapted to a humid and consistently moist environment, the exposure of *H. brasiliensis* to severe drought conditions will result in adverse effects such as stunted growth and reduced latex production (Anjum *et al*., 2017; Farooq *et al*., 2009). On the other hand, as *H. brasiliensis* is adapted to well-drained soils, flooding can result in severe damage to the plants, including oxygen deprivation, hormonal imbalance and increased vulnerability to diseases (Rouhier & Jacquot, 2008; Sasidharan *et al*., 2011; Voesenek *et al*., 2006).

β-1,3-glucanases (GLUs) are pathogenesis-related (PR) proteins which will be induced upon pathogenic infection (Radhakrishnan *et al*., 2021). It can be commonly found in the cell walls of bacteria, fungi, viruses, and various plant species (Leubner-Metzger & Meins, 1999; Xu *et al*., 2016). GLUs are well-known for their antifungal activity and significant role in pathogen defence mechanisms (Doxey *et al*., 2007). Degrading β-1,3-glucans in fungal cell walls can eventually suppress the growth of

invading fungi in plants (Doxey *et al*., 2007; Mølhøj *et al*., 2002). Due to its potent antifungal properties, GLUs have gained significant attention in biotechnological applications as a potential tool in managing fungal diseases. Some researchers have investigated the use of genetically modified plants that express higher levels of GLUs to enhance their resistance to fungal infections (Tsabary *et al*., 2003). Besides, with its significant role as cell wall-modifying proteins, GLUs will also be involved in plant's cell wall modification upon abiotic stress such as drought and flooding (Sasidharan *et al*., 2011; Spollen *et al*., 2008). Under drought condition, GLUs can alter the extensibility of the cell wall and promote root growth, assisting the plant adaptation to water deficit (Sasidharan *et al*., 2011; Spollen *et al*., 2008). Besides, upregulation of cell wall-modifying proteins has also been found in flooding tolerant plant as they can aid in cellular expansion and accelerate the shoot growth to emerge from water (Voesenek *et al*., 2006).

1.2 Problem Statement

Since GLUs play a significant role in defending against fungal disease and responding to abiotic stress, it is significant for identifying and characterising different GLU genes in *H. brasiliensis* to develop novel clones with enhanced disease resistance. Despite several studies on the characterisation of GLU genes in various bacteria and plants such as rice, pea and cotton, the characterisation of GLU genes in *H. brasiliensis* is least documented, and most of the GLU sequences still need to be characterised. This might be due to the research focus in *Hevea brasiliensis* has traditionally been on genes directly related to natural rubber biosynthesis. Therefore, the current work aims to identify and characterise GLU genes in *H. brasiliensis* while comparing the expression of GLU genes under various biotic and abiotic conditions.

1.3 Rationale of study

In the present study, a complete set of curated GLU gene sequences in the whole genome of *H. brasiliensis* was identified and characterised using various bioinformatics tools. Expression analysis of GLU genes in different stages *of H. brasiliensis* leaves was conducted to determine the significance of GLUs in the regulation of leaf development and to identify the critical leaf developmental stage likely to be more susceptible or resistant to fungal infections. Besides, expression of GLU genes under abiotic and biotic stresses were identified to determine the significance of GLUs expression in plant's defense mechanisms against respective biotic and abiotic stresses. Biotic stress being discussed is fungal infection with fungus *Cornyspora cassiicola* while abiotic stresses being discussed are drought and flooding. In this study, differential expression analysis of GLUs was conducted by identifying the absolute expression GLUs using digital PCR. Absolute expression analysis can quantify the actual number of transcripts present in the respective sample and provide a highly accurate and quantifiable understanding of gene activity without relying on a standard curve or reference gene.

1.4 Objectives

a) To identify the complete set of curated GLU gene sequences in the whole genome of *H. brasiliensis*.

b) To characterise GLU gene sequences in *H. brasiliensis* using various bioinformatics tools.

c) To determine the absolute expression of GLU genes in the different stages of *H. brasiliensis* leaves.

d) To determine the absolute expression of GLU genes in *H. brasiliensis* under abiotic stress (drought and flooding) and biotic stress (fungal infection).

CHAPTER 2

LITERATURE REVIEW

2.1 Natural rubber

Based on the source, rubber can be divided into two categories: synthetic and natural rubber (Men *et al*., 2019). Synthetic rubber is polymers made of alkenes or dienes generated from the petrochemical industry. In contrast, natural rubber (NR) comprises biopolymers derived from rubber-producing plants, primarily *H. brasiliensis* (Men *et al*., 2019). NR is a complex molecular structure primarily composed of long hydrocarbon polymer chains of isoprene units (Archer *et al*., 1963; Lau *et al*., 2016; Yeang *et al*., 2002). It is a secondary metabolite (cis 1, 4-polyisoprene) that originates in the laticifer cells, which are the latex vessels in rubber-producing plants interconnected with tangential rings (Nair, 2021; Yeang *et al*., 2002), present in the secondary phloem (Venkatachalam *et al*., 2013). NR can be derived commercially from latex, the living cytoplasm of laticifer cells.

Even with the advent of synthetic rubber, NR is irreplaceable in manufacturing industries due to its specific molecular structure and exceptional properties: high resilience, elasticity, resistance to abrasion and impact, efficient heat dissipation, water resistance and malleability at low temperatures (Cataldo, 2000; Cornish, 2001). NR becomes more durable during vulcanisation, retaining its elasticity over a wide temperature range (Cataldo, 2000; Cornish, 2001). As one of the most versatile biopolymers, NR has played a pivotal role and is extensively utilised in the automotive, electrical, and medical industries (Nair, 2021). It has been heavily employed to produce heavy-duty tyres as it provides better grip, reduced wear, and improved overall performance. NR is also used to manufacture surgical gloves due to its biocompatibility, flexibility, and hypoallergenic properties (Lau *et al*., 2016; Men *et al*., 2019).

Attributable to the Industrial Revolution and the development of the rubber manufacturing process, commercial trade and commercial cultivation of rubber plants were stimulated in the nineteenth-century (Nair, 2021). Southeast Asian countries are the leading producers of NR due to their monsoon climate, which is perfect for the vegetative growth of rubber plants (Nair, 2021). Thailand was the biggest producer and exporter of NR in 2022, followed by Indonesia, Côte d'Ivoire, Vietnam and Malaysia (Statista Research Department, 2023).

In Malaysia, even though NR production had declined by 41.03% from 639.8 thousand tonnes (2019) to 377.3 thousand tonnes (2022), the consumption of NR still showed an decrement of 15.36% from 501.3 thousand tonnes (2019) to 424.3 thousand tonnes (2022) (Malaysian Rubber Board, 2022). The decline in NR production could be attributed to several factors, including land shortage and the nation's economic development, leading to labour scarcity (Ali *et al*., 2021). Due to its unstable supply, the rubber market has experienced significant fluctuations over time (Nair, 2021).

2.2 *Hevea brasiliensis*

2.2.1 Origin and botany of *H. brasiliensis*

Even though NR can be found in the latex of around 2, 500 plant species, most of them are unfavourable for the commercial production of NR due to their limited productivity (Van Beilen & Poirier, 2007). To overcome problems such as latex allergies and increasing demands for NR, alternative NR sources such as *Parthenium argentatum* Gray, *Euonymus alata, Taraxacum koksaghyz, Ficus carica* and fungi *Lactarius chrysorrheus* have been extensively studied. However, the commercial harvest of NR from alternative sources has faced limitations such as slow growth, low yield and low abundance of rubber particles (Venkatachalam *et al*., 2013).

H. brasiliensis (Para rubber tree), which belongs to *Euphorbiaceae* family is the most exclusive species among all eleven species in the family for NR production (Clément-Demange *et al*., 2007). The eleven species of the genus *Hevea* are *H. brasiliensis, H. bethamiana, H. camporum, H. camargoana, H. guianensis, H. microphylla, H. nitida, H. pauciflora, H. paludosa, H. rigidifolia* and *H. spruceana* (Lau *et al*., 2016). Originally from Brazil, *H. brasiliensis* is a woody, monoecious, cross-pollinated tropical tree which can grow up to 40 m in height. *H. brasiliensis* is mainly grown in tropical Southeast Asia countries, such as Malaysia, Indonesia, India, Sri Lanka and Thailand (Archer & Audley, 1987; Lau *et al*., 2016; Priyadarshan, 2017).

H. brasiliensis consists of three main parts: crown, stem and root. The crown contains leaves attached to the branch through petioles which will defoliate annually from January to March (Haji Ahmad, 2009). As flowering plants, *H. brasiliensis* produces inflorescence and unisexual flowers (male and female) with insects as its main pollination agents. The stem of *H. brasiliensis* consists of three main layers: soft outermost bast with latex-bearing vessels, cambium, and medulla rays. *H. brasiliensis* has three distinct parts of root systems: tap roots, feeder roots and root hairs. Tap root penetrates deep into the soil and keeps the plant upright. Feeder roots are significant for water absorption, while root hairs have their role in nutrient uptake (Haji Ahmad, 2009).

H. brasiliensis leaves are oval shaped with pointed tips and rounded bases, which can vary in size but are generally large (several inches long individually). As compound leaves, they are around 30 to 60 cm long with three outward-pointing boat-shaped leaflets joined to a long stalk. The leaves have prominent veins with a pinnate pattern and are generally smooth with a glossy upper surface which can help in shedding water and prevent excessive transpiration (Bernadete *et al*., 2003).

H. brasiliensis is accounted for producing most of the NR worldwide as it can commercially produce viable amounts of high-quality NR (Men *et al*., 2019; Venkatachalam *et al*., 2013). Besides NR, *H. brasiliensis* has been exploited commercially for ancillary products such as rubberwood to further maximize rubber returns (Nair, 2021; Venkatachalam *et al*., 2013). Moreover, *H. brasiliensis* also provides environmental benefits as a renewable resource. NR production requires less oil than synthetic rubber, while rubber plantations serve as efficient carbon sinks (Haji Ahmad, 2009).

To ensure promising yield, *H. brasiliensis* needs to have the following characteristics: high seed vigour, good girth increase, broad leaves with bright colour, well-extended main branches, balanced side branches, thick virgin bark for the ease of tapping, fast-recovered bark for retaping, high yielding, latex production with the stable flow and high dry rubber content and high resistance to stresses (biotic and abiotic) (Haji Ahmad, 2009).

2.2.2 Tapping of *H. brasiliensis* for latex

Latex vessels of *H. brasiliensis* run spirally and latex can be harvested by tapping (Haji Ahmad, 2009). Rubber tree-tapping for latex can only be performed after they reach maturity at around seven years old and minimum girth size (50 cm). The timing of tapping is based on factors such as the tree's growth rate, climate, and soil condition, as the timing of first tapping determines its profitability (Venkatachalam *et al*., 2013). Tapping must be done carefully to ensure the tree's health and maximize latex yield. Tapping is carried out at dawn because the transpiration rate is low and cell turgidity is high with high efficiency of latex flow.

Vessels are cut from high left to low right, which is the opposite direction of latex flow. The trunk bark is periodically incised or tapped to generate latex flow (Nair,

2021). The tapping angle is crucial as it can affect the length and bark consumption, eventually influencing the product yield (Haji Ahmad, 2009). Rubber trees are tapped regularly (every 2 to 3 days), and the tapping intensity is commonly stimulated by the application of ethephon, an ethylene-generating compound (Abraham, 1968). Ethylene is a crucial plant growth factor which is synthesized in response to wounding (Haji Ahmad, 2009).

2.2.3 Latex

Latex is a milky white fluid that flows through latex vessels in the bark of *H. brasiliensis*. Latex is the by-product of *H. brasiliensis* which contains a large amount of water. Dry rubber content is the rubber particle content found in latex, which can be processed to produce NR and is greatly influenced by season, climates, clone types, ages, soil conditions and tapping systems (Haji Ahmad, 2009).

Latex comprises three main fractions that can be separated through ultracentrifugation: top fraction (rubber phrase), C-serum, and bottom fraction. Top fraction is the site of rubber biosynthesis which comprises mainly rubber particles, which is the site of rubber biosynthesis while C-serum is an aqueous medium with suspended latex organelles (Chye & Cheung, 1995). Latex C-serum is the laticifer cytoplasm with various cellular metabolism-associated proteins and glycolytic enzymes. On the other hand, bottom fraction is known as the lutoid-body fraction of latex with lutoids as its main constituent (Sunderasan *et al*., 1995; X. Wang *et al*., 2013; Yeang *et al*., 2002).

Rubber particles, also known as polymeric hydrocarbon cis-polyisoprene are the main constituent (20 to 45%) in latex and NR is synthesized on the surface of rubber particles. Rubber particles are typically spherical and coated with adsorbed protein and phospholipids for protection (Archer *et al*., 1963; Dickenson, 1965). Other than rubber particles, latex contains large amounts of water and non-rubber particles like carbohydrates, proteins, lipids, inorganic materials and other minor components (Archer *et al*., 1963; Lau *et al*., 2016; Yeang *et al*., 2002). Examples of proteins present in rubber particles are rubber elongation factor (REF) and small rubber particle protein (SRPP) while C-serum contains proteins such as profilin and enolase (Yeang *et al*., 2002).

Bottom fraction contains B-serum which has a higher amount of protein than C-serum. The proteins found in rubber particles and C-serum are mostly acidic (Linthorst *et al*., 1990). In comparison, lutoidic proteins from B-serum can be both acidic and basic, with the examples such as GLU and hevein (Yeang *et al*., 2002). There are three main biological functions of lutoidic proteins: pH maintenance and ion homeostasis; defence response against microbial pathogen attacks; facilitating rubber particle aggregation (RPA) and latex coagulation. Notable, there are approximately 1 to 2% of proteins in latex with a small portion of protein indicated as a factor of latex allergy (Yeang *et al*., 2002). Due to the heterogeneous nature of NR latex, proteins are not distributed evenly (Yeang *et al*., 2002).

In general, rubber biosynthesis starts with the active transport of sucrose from photosynthesis into laticiferous cells where it is hydrolysed into glucose and fructose. Glycolysis converts these sugars into acetyl-CoA, which is then condensed into mevalonic acid and converted to isopentenyl pyrophosphate (IPP). Polymerization of IPP molecules via the association of enzyme rubber transferase and REF will result in

high molecular weight rubber particles. Genes expressed in the latex can be categorised into different groups based on the encoding proteins: rubber biosynthesis-related proteins, defence/stress-related proteins, and latex allergen proteins (Venkatachalam *et al*., 2013).

2.2.4 Breeding and conservation of *H. brasiliensis*

To obtain maximum variability, germplasm conservation is carried out via both *in-situ* and *ex-situ* conservation, excluding cryopreservation and *in vitro* conservation. At present, propagation of *H. brasiliensis* has been performed via generative and vegetative methods through seeds and axillary bud-grafting. Seeds are utilised mainly to produce rootstocks which can be grown directly in the plantation field (Venkatachalam *et al*., 2013). Nonetheless, propagation of rubber plants can also be carried out via tissue culture techniques such as somatic embryogenesis using anther and leaf explants. Due to apomixis and lack of control over sexual reproduction, preserving the genetic stocks of chosen clones through tissue culture is crucial (Venkatachalam *et al*., 2013).

The primary goal of *H. brasiliensis* breeding is to develop superior clones with faster tree growth, high rubber yield, capable of withstanding high tapping intensities and higher resistance to environmental stresses (Annamma *et al*., 1990; Varghese *et al*., 1992; Venkatachalam *et al*., 2013). Previously, *H. brasiliensis* breeding has been carried out via selective hybridisation, mutation breeding and polyploidization. In the present day, biotechnologies combined with conventional propagation have been a promising tool for producing desired clones efficiently (Wang *et al*., 2023). Studying the regulation and expression of the genes involved in NR biosynthesis is essential to meet the increasing demand for rubber. Identifying novel clones with higher

profitability is essential to secure a consistent supply of NR and reduce our reliance on petroleum-based synthetic rubber (Priya *et al*., 2006; Venkatachalam *et al*., 2009).

2.2.5 Current challenges of natural rubber industry

Currently, the NR industry of *H. brasiliensis* faces numerous challenges due to its specific growth environments, susceptibility to various fungal infections, tedious harvesting and allergic response to the proteins in latex (Mooibroek & Cornish, 2000). In the recent research by Malaysian Rubber Board (Rasyidah *et al*., 2023), the selection of latex clone and latex timber clone was carried out based on their stability of latex production and trunk growth. Eight clones showed potential for selection of Latex Timber Clones with their high girth measurement (more than 60 cm) at tenth year of planting and high latex yield (more than 50 g/t/t): RRIM 3001, RRIM 2024, RRIM 2002, RRIM 2001, PB 374, PB 371, RRIM 2007, and PB 260. Meanwhile, clone RRIM 2004 have the potential to be recommended as Latex Clone due to its high latex yield and stable performance, however with a low trunk girth.

Due to the inefficiency of new rubber-producing plants, omics analysis and agrobacterium-mediated genetic transformation have been carried out to increase rubber yield (Blanc *et al*., 2006; Iaffaldano *et al*., 2016; Stolze *et al*., 2017; Tang *et al*., 2013).

2.3 Abiotic stress

Abiotic stress refers to the adverse environmental conditions that can negatively affect growth, yield and performance (Huang & Pan, 1992; Sethuraj *et al*., 1984; Sreelatha *et al*., 2011). Abiotic stress can be natural or human-induced, and plants are continually subjected to various abiotic stresses, such as drought, flooding, light insufficiency, and low temperature (Mohamed Sathik *et al*., 2018). Differential gene

expression studies by Luke *et al*., (2015, 2017) and Mohamed Sathik *et al*., (2012) reported that different *H. brasiliensis* genotypes have variable transcript expression levels and various degrees of abiotic stress tolerance. Identifying genotypes with a higher degree of tolerance to withstand unfavourable environments is required to achieve maximum growth and productivity (Silva *et al*., 2014).

The studies reported by Luke *et al*., (2017) and Mohamed Sathik *et al*., (2018) have revealed several transcripts strongly associated with drought and cold stress tolerance in *H. brasiliensis*. Drought-responsive transcripts include ferritin, DNA-binding protein, NAC transcription factor (NAC tf) and aquaporin, while cold-responsive transcripts include ethylene-responsive transcription factor. In the cultivation of *H. brasiliensis*, cold stress (between 0 to 10 °C) can result in growth reduction and reduced latex production (Jacob *et al*., 1999; Priyadarshan *et al*., 2005). Adverse condition of low temperature with high light intensity has led to the inhibition of photosynthesis and chlorophyll bleaching due to the damage of Photosystem II and photosynthetic apparatus (Annamalainathan *et al*., 2010). Besides, it was found that young *H. brasiliensis* plants are more susceptible to abiotic stress (Jacob *et al*., 1999).

2.3.1 Drought

Drought stress is a critical environmental factor that significantly impacts plant growth and development (Farooq *et al*., 2009). As climate change continues to escalate, the frequency and intensity of drought events have increased, making it crucial to comprehend the plant's mechanisms to respond to drought stress (Anjum *et al*., 2017). Drought stress, also known as prolonged water deficiency, can lead to severe water stress in plants, resulting in wilting, reduced growth, and yield loss (Farooq *et al*., 2009; Harris *et al*., 2002; Kaya *et al*., 2006).

Mechanisms adopted by plants to cope with drought stress can be categorised into morphological adaptations, physiological responses, molecular signalling, and gene regulation. Plants exhibit a range of morphological adaptations such as root structural modification and leaf appearances, to optimise water use and conservation (Chakraborty *et al*., 2022). Deeper root systems might be developed to access water deeper in the soil for higher water absorption efficiency (Comas *et al*., 2013; Passot *et al*., 2016). Meanwhile, other species may shed leaves to minimise transpiration and water loss (Anjum *et al*., 2017). By staying green, crops can extend the photosynthesis period by prolonging the senescence of their leaves (Vadez *et al*., 2013). Leaf-rolling and wilting assist the plants in water conservation by reducing leaf surface area and slowing down the transpiration rate (Cal *et al*., 2019).

At the physiological level, drought-stressed plants activate different protective mechanisms involving stomata closure to reduce water loss through transpiration (Anjum *et al*., 2017; Yoo *et al*., 2010). Molecular signalling pathways have orchestrated the plant's response to drought stress with plant hormones such as abscisic acid (ABA) (Akiyama & Pillai, 2001). ABA plays a central role in signalling stress, initiating various adaptive processes of protective proteins, antioxidants, and osmoprotectants and regulating the expression of stress-associated genes (Farooq *et al*., 2009; Nakashima *et al*., 2009). Under drought stress, glutathione reductase activity in the roots and leaves can be enhanced to facilitate the defence against the accumulation of reactive oxygen species (ROS) (Gallé *et al*., 2013; Mittler, 2002). Nonetheless, heat shock proteins (HSP) have also enhanced high-temperature tolerance by serving as molecular chaperones in heat-responsive pathways that effectively regulate protein unfolding and denaturation (Chaturvedi *et al*., 2015; López-Hernández & Cortés, 2019).

Under drought conditions, cell wall modifying proteins alter the extensibility of the cell wall to maintain root growth, assisting the plant adaptation to water deficit. Plants adapt inhibition of shoot growth to enhance root growth and reach water-rich zones (Sasidharan *et al*., 2011). Proteomic analysis by Zhu *et al*., (2007) on water-stressed maize roots proposed several drought-induced cell wall-modifying proteins. While experiencing water deficiency, upregulation of cell wall-modifying proteins, including expansin and GLU, were determined in the roots' actively growing apical tip region (Spollen *et al*., 2008).

2.3.2 Flooding

Flooding is a severe environmental stress as the less efficient gas diffusion environment underwater can severely restrict plant photosynthesis and aerobic respiration. Excessive water due to flooding can lead to oxygen deprivation in roots, reducing plant vigour, retarding growth, and lack of ability to absorb nutrients and water, which can lead to disease outbreaks. Under flooding stress, leaves turn yellow, and lenticels become swollen. Smaller plants are sometimes killed under prolonged flooding while cracking bark and oozing latex are present on larger plants. Meanwhile, roots can be infected by stinking root rot (Haji Ahmad, 2009).

Cellular expansion and the acceleration of shoot growth are attributable to cell wall modification controlled by cell wall-modifying proteins to aid plants in surviving flooding. Some flood-tolerant plants adopt rapid shoot elongation regulated by expansins to emerge from water (Voesenek *et al*., 2006). The adaptation is supported by the finding of expansin upregulation, along with petiole elongation in flooding-tolerant species *Rumex palustris* (Vriezen *et al*., 2000). The high amount of ethylene was determined during flooding, indicating its implications with elongation response (Jackson, 2008).

2.4 Biotic stress

Biotic stress is the damage caused by the interaction between plants and living organisms. Examples of biotic stress are pathogens, pests, herbivores, and parasitic plants (Mazlan *et al*., 2019). *H. brasiliensis* is comparably less affected by pests compared to pathogens such as bacteria, fungi and viruses, which can cause diseases through infection and result in plant disease, which will then reduce plant growth and crop yield (Clément-Demange *et al*., 2007; Mazlan *et al*., 2019). Meanwhile, pests are animals that feed on plants, which can cause damage and transmission of diseases among plants (Mazlan *et al*., 2019). Pests that attack *H. brasiliensis* include leaf-eating beetles, mites, termites, scale insects, mealy bugs, thrips caterpillars and weevils (Haji Ahmad, 2009).

2.4.1 Susceptibility of *H. brasiliensis* to fungal disease

The susceptibility of *H. brasiliensis* to fungal diseases is determined by the climatic conditions and cultural techniques practiced (Nair, 2021). Examples of common fungal diseases which are infectious to *H. brasiliensis* are abnormal leaf fall (ALF), South American leaf blight (SALB), powdery mildew, CLF, leaf blight, pink disease, white root disease, brown rot disease (Clément-Demange *et al*., 2007). Most fungal diseases have specific target parts of the plant. For instance, white root disease and brown rot disease infect root systems while pink disease infects the lower part of *H. brasiliensis*. On the other hand, SALB, CLF and powdery mildew affect the leaves.

ALF disease caused by *Phytophthora* spp. was the primary leaf fall disease experienced by India's rubber plantation, and it resulted in a significant yield loss (38–56%) when fungicides were not applied throughout the disease season (Radhakrishnan *et al*., 2021). It has a wide range of hosts and typically causes green pod rot, shoot dieback, abnormal leaf fall, and black stripe diseases in rubber trees. Starting with

immature green pods and small leaf lesions with black globules of latex, the stem will then be infected, and leaves will turn reddish-brown and fall from the petioles. Meanwhile, shoot infection is commonly leading to shoot dieback (Erwin & Ribeiro, 1996; Mazlan *et al*., 2019).

Even though SALB caused by *Microcyclus ulei* is exclusively found in the tropical Americas, Asian countries must also prioritize the development and selection of SALB-resistant clones because of its ideal climatic environment for the fungus growth (Nair, 2021). The attack of *M. ulei* is aggressive and often targets the young leaves exclusively. *M. ulei* will attach their spores to young leaves and stick to the cuticle of the leaves after germination (Mazlan *et al*., 2019). Penetration of hyphae will later result in the formation of appressoria and small lesions with ring-like structures (Mazlan *et al*., 2019).

Pink disease, caused by the fungus *Corticium salmonicolor*, is another significant disease in rubber that causes the drying of immature plants' main stems and branches (Nair, 2021). Powdery mildew is a fungal infection caused by *Oidium heveae* and is commonly observed in cold environments. It often results in discoloration, defoliation of young shoots, and curling of older leaves. Plant susceptibility to the disease is significantly impacted by leave stages, with the immature leaves the most susceptible stage. Flowers are more susceptible than rubber leaves (Mazlan *et al*., 2019). Biotic stresses are commonly managed via measures such as using fungicides or pesticides, crop rotation, planting disease-resistant crop varieties, and sanitation practices. However, leaf diseases are mostly untreatable by fungicides (Clément-Demange *et al*., 2007). The list of common fungal diseases which are infectious to *H. brasiliensis* with their name of the pathogen and parts of the plant affected was tabulated in Table 2.1.

Table 2.1 List of common fungal diseases which are infectious to *H. brasiliensis* with their name of the pathogen and parts of the plant affected.

Name of the disease	Name of the pathogen	Parts of the plant affected
Abnormal leaf fall	*Phytophthora* spp.	Leaves, stem
South American leaf blight	*Microcyclus ulei*	Leaves
Powdery mildew	*Oidium heveae*	Leaves, young shoots
Cornyspora Leaf Fall	*Cornyspora cassiicola*	Leaves
Leaf blight	*Fusicoccum* spp.	Leaf
Pink disease	*Corticium salmonicolor*	Stem, branches
White root disease	*Rigidosporus microsporus*	Root
Brown rot disease	*Phellinus noxius*	Root

2.4.2 Disease-related genes and pathways in *H. brasiliensis*

Disease resistance genes (R genes), making up 0.57% of all *H. brasiliensis* genes, are the members of the nucleotide-binding site (NBS) resistance gene family and are responsible for disease resistance mechanisms (Lau *et al*., 2016). The finding of 483 disease resistance-related genes in *H. brasiliensis* is notable since the expansion of R genes indicates greater resistance to fungal infections (Lau *et al*., 2016). Fang *et al*., (2016) reported the distinct defence mechanisms existing in young and mature leaves of *H. brasiliensis* by *de novo* transcriptome analysis.

Pathogens have utilised cell wall modification mechanisms to facilitate their penetration by altering cell wall structure (Sasidharan *et al*., 2011). During cell expansion, cell wall modifications are mediated by GLU by breaking down β-1,4 glucosidic linkages of the cell wall's primary structure (del Campillo, 1999; Rose & Bennett, 1999; Tsabary *et al*., 2003). Other than GLU, cell wall loosening also involves various enzymes, including xyloglucan endotransglucosylases (XTH), pectin methylesterase (PME) and expansin, jasmonic acid (JA), ethylene, and salicylic acid (SA) (Anderson *et al*., 2004; Sasidharan *et al*., 2011; Tsabary *et al*., 2003). Expansin and XTH are found abundantly in growing regions of a plant to regulate cell elongation (Cho & Kende, 1997b, 1997a; Shin *et al*., 2006; Vissenberg *et al*., 2000).

Pathogen infections are associated with the upregulation of expansin expression and mediated by enhanced auxin levels upon infection (Ding *et al*., 2008; Domingo *et al*., 2009). Despite being well-known for inducing shoot growth and cell elongation, auxin is also a pathogen virulence factor that plays a significant role in pathogenesis and disease resistance (Ding *et al*., 2008; Domingo *et al*., 2009; Kazan & Manners, 2009; Navarro *et al*., 2006). Upon bacterial infection, a high level of auxin can induce the accumulation of indole-3-acetic acid (IAA), results in the upregulation of expansin

associated with cell wall loosening (Romero *et al*., 1998). Subsequently, the loosened cell wall will increase the plant susceptibility to pathogen infection (Ding *et al*., 2008; Woodward & Bartel, 2005). In contrast, inhibition of auxin signalling and repression of auxin biosynthesis has significantly enhances its resistance to fungal pathogen (Chen *et al*., 2007; Romero *et al*., 1998).

Additionally, nutrient leakage due to loosened cell walls might provide a favourable growing environment for pathogens. In contrast, transgenic plants with low auxin levels showed suppressed expansin expression and increased pathogenic disease resistance (Ding *et al*., 2008). The reduced expression level of expansin will result in a more rigid cell wall structure, which will act as a physical barrier against biotic agents, thereby enhancing plant disease resistance (Domingo *et al*., 2009; Sasidharan *et al*., 2011).

Pectins are complex carbohydrates in cell walls, while PME is a pectin-modifying enzyme via pectin methylesterification (Sasidharan *et al*., 2011). In *Arabidopsis*, overexpression of PME inhibitor proteins reduced pectin methylesterification, subsequently enhancing its resistance to *Botrytis cinerea*, a fungus with efficient pectinolytic machinery (Lionetti et al., 2007; Van Kan, 2006). Fruit ripening has also been found to significantly affect plants susceptibility to pathogen infection as ripening fruits will undergo irreversible cell wall disassembly, leaving the plant more vulnerable to pathogen invasions (Rose & Bennett, 1999; Sasidharan *et al*., 2011). Pathogen infection is beneficial during the fruit ripening as it can speed up cell wall disassembly and dispersal of mature seeds (Sasidharan *et al*., 2011). The study by Cantu *et al*., (2008) revealed that suppressing polygalacturonase and expansin can delay fruit softening and decrease ripening-associated susceptibility to *B. cinerea*.

Cyanogenesis of *H. brasiliensis* is significant for its resistance to fungal infection as its mechanically wounded leaves will release free cyanide. A high amount of free hydrogen cyanide (HCN) was detected in infected leaves of susceptible clones, while very low level for resistant clones. It indicates that cyanogenesis is associated with impairing plant resistance to fungal infection (Clément-Demange *et al*., 2007).

2.4.3 *Cornyspora cassiicola*

Cornyspora Leaf Fall (CFL) caused by the fungus *C. cassiicola* is one of the primary diseases responsible for a substantial production loss in rubber plants in Southeast Asian countries, especially regions with frequent rainy seasons such as Malaysia and Indonesia (Clément-Demange *et al*., 2007; Mazlan *et al*., 2019). This disease was first detected in India in 1958 (Ramakirishnan & Pillai, 1961) and then in Malaysia (Newsam, 1960).

C. cassiicola infects young and old leaves along the veins, causing defoliation and delaying rubber tree maturation (Roy *et al*., 2019). The initial symptoms of the disease are dark spots on the leaf lamina, which later enlarge into circular lesions encircled by a yellow halo. Discoloured vein shows fish bone or railway track symptoms. As the leaves turn yellow, defoliation occurs (Haji Ahmad, 2009; Priyadarshan, 2017; Pu *et al*., 2007). Dieback on the shoot tips can also result from severe infection (Mazlan *et al*., 2019). RRIC 103, one of the most popular high-yielders, has to be removed from the planting recommendation due to the high prevalence of Corynespora leaf spot disease in Sri Lanka, forcing a large-scale replantation (Liyanage *et al*., 1991).

2.5 β-1,3-glucanases

PR proteins are a collection of proteins that can be induced by plants upon pathogenic infection and the release of elicitors and hormonal changes (Lee *et al*., 2011; Leubner-Metzger & Meins, 1999; Radhakrishnan *et al*., 2021). GLUs (E.C. 3.2.1.39) are PR proteins belonging to the PR-2 family, commonly found in bacteria, fungi, viruses and various plant species, including *Arabidopsis*, rice, tobacco, soybean and *H. brasiliensis* (Leubner-Metzger & Meins, 1999; X. Xu *et al*., 2016). GLU was first discovered, characterised and cloned in the rice by Romero *et al*., (1998).

GLU is recognised as a lutoid protein because it can be found abundantly in bottom fractions of latex of *H. brasiliensis* that comprise mainly lutoids (Chye & Cheung, 1995). In *H. brasiliensis*, a higher expression of GLU was discovered in the stem than in the leaf due to the presence of higher amount of laticifer in stem (Chye & Cheung, 1995). Based on the previous study by Thanseem *et al*. (2005), GLUs were found to play a major role in combating the abnormal leaf fall disease (ALF) caused by the oomycete *Phytophthora* spp. in *H. brasiliensis* as there was differential gene expression in tolerant and susceptible clones of *H. brasiliensis*. Even though GLUs are present in both tolerant and susceptible clones, only tolerant clones showed prolonged gene expression (Radhakrishnan *et al*., 2021).

2.5.1 Different roles of GLU in plant

GLUs are known for their antifungal activity and significant role in plant defence mechanisms against invasion of microbial pathogens (Doxey *et al*., 2007; Mølhøj *et al*., 2002; Romero *et al*., 1998). They function as a catalyst in the hydrolysis of 1,3-β-D-glucosidic linkages in β-1,3-glucans, a critical structural element of fungal cell walls (Legentil *et al*., 2015; X. Xu *et al*., 2016). Tsabary *et al*., (2003) discovered that GLUs have involved in hormonal mechanisms in which their gene expression can

regulate the cell wall growth and architecture. Gene expression of GLUs can be regulated by environmental stress and specific plant hormones such as ABA, IAA and JA (Domingo *et al*., 2009). It was found that GLUs can work together with chitinase, another member of PR proteins for more effective degradation of fungal cell walls and inhibit its growth (Broekaert *et al*., 2000; Mauch *et al*., 1988).

Despite their implications in pathogen defence mechanisms, GLUs are also implicated in a broad range of physiological and developmental processes that require cell wall modification, such as microsporogenesis, flowering, seed germination, wound callose removal, and fruit ripening (Goellner *et al*., 2001). GLUs break down the callose-rich cell wall during microsporogenesis for pollen maturation (McCormick, 1993; Romero *et al*., 1998). Meanwhile, GLUs can degrade the cell walls during seed germination and enable other hydrolases to enter and mobilize stored reserve materials (Romero *et al*., 1998). Due to their diverse physiological roles, GLUs present in a variety of structural isoforms and are categorised into different classes according to their messenger RNA (mRNA) expression profile and various properties, including size, isoelectric point, primary structure, cellular localisation, and regulatory patterns (Radhakrishnan *et al*., 2021; Yeang *et al*., 1995). Different isoforms of GLUs are characterised with various molecular mass ranging from 34-38 kDa (Yeang *et al*., 2002).

Studies by Alenius *et al*., (1996) and Sunderasan *et al*., (1995) had reported GLUs as allergenic proteins which respond to Immunoglobulin E (IgE) binding and skin reactivity. Unlike other common allergenic proteins which are acidic, GLUs are basic proteins with pI ranging from 9.5 to 9.8 (Sunderasan *et al*., 1995; Yeang *et al*., 2002). Based on the study by Yeang *et al*., (2002), with the enzymatic capability of recombinant protein retained, the binding of IgE antibodies from sera of latex-allergic