Malaysian Applied Biology (2022) 51(5): 271-280
https://doi.org/10.55230/mabjournal.v51i5.2343

Research Article

In silico Genome-Wide Computational Profiling of Non-Coding RNA in Oil Palm *Elaeis guineensis* and its Pathogen *Ganoderma boninense*

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ABSTRACT

Oil palm plantation was first established in Malaysia in 1917. Since then, the oil palm industry in Malaysia flourished especially following the shifting of Deli Dura palm to Tenera palm in the 1960s, which contributed to a 30% increase in yield. However, the outbreak of basal stem rot disease caused by *Ganoderma boninense* has caused substantial yield losses. With no known cure to date, extensive molecular studies were conducted to better understand the underlying mechanism of *G. boninense* infection and the role of protein-coding genes as regulators in oil palms against *G. boninense*. The studies have demonstrated the importance of non-coding RNAs (ncRNAs) in the interaction between oil palm and *G. boninense*. However, there is still limited genome-scale identification for ncRNAs in oil palm (*Elaeis guineensis* jacq.) and its pathogen, *G. boninense*. In this study, we focused on the identification of small and medium-sized non-coding RNA using a computational approach and managed to predict 2,233 ncRNAs and 369 ncRNAs in the *E. guineensis* and *G. boninense* genomes, respectively. The identified ncRNAs include transfer RNA (tRNA), ribosomal RNA (rRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), and microRNA (miRNA). Although the number may be far fewer than the real number, the predicted ncRNAs here represent an almost complete dataset of small and medium-sized ncRNA in both the *E. guineensis* and *G. boninense* genomes. The information obtained may be useful to tackle the issue of *G. boninense* attack on oil palm plantations.

Key words: *Elaeis guineensis* Jacq., *Ganoderma boninense*, Non-coding RNA

Article History
Accepted: 6 December 2022
First version online: 25 December 2022

Cite This Article:
Othman, F.N., Azizi, N., Mohd-Zim, N.A., Ping, L.Y. & Sulaiman, S. 2022. *In silico* genome-wide computational profiling of non-coding RNA in oil palm *Elaeis guineensis* and its pathogen *Ganoderma boninense*. Malaysian Applied Biology, 51(5): 271-280. https://doi.org/10.55230/mabjournal.v51i5.2343

INTRODUCTION

The history of oil palm in Malaysia can be traced back to the introduction of oil palm by the British as an ornamental plant in the early 1870s (Malaysian Palm Oil Council, n.d.). Later, in 1917, oil palm began to be planted for commercial gain which took place at Tennamaran Estate (Malaysian Palm Oil Council, n.d.). Presently, the majority of the oil palm planted in Malaysia is *Elaeis guineensis*. *Elaeis guineensis* (African oil palm) is known for its capability to yield more palm oil compared to *Elaeis oleifera* (American oil palm) but is also known to be more susceptible to infection (Cochard *et al*., 2005; Astorkia *et al*., 2020).

The majority of the deadly infection that affects perennial crops is linked to wood rot disease (Roozbeh *et al*., 2013). The causal agent for this disease is often associated with pathogenic fungi by lethal wood rotting. In oil palm particularly, this disease is highly associated with *Ganoderma boninense* infection which causes basal stem rot (BSR) and upper stem rot (USR) that consequently results in progressive loss of root system and reduction in oil yield. The yield losses caused by these fungi are estimated to be around 50–80% (Corley & Tinker, 2015).

Currently, it is nearly impossible to manage a pathogen-free field in an oil palm plantation (Siddiqui *et al*., 2021), but a significant reduction in disease spread could be accomplished

by reducing tree wounds, removing old trees before they reach their maximum age susceptibility and improving treatment and harvesting operations (Flood *et al*., 2000). Essentially, an in-depth understanding of the underlying mechanism of the host-pathogen interaction is also being investigated concurrently through molecular studies. Molecular studies such as transcriptional profiling of *G. boninense* (Dhillon *et al*., 2021) and pan-genome study of *Ganoderma* sp. (Sulaiman *et al*., 2018) sheds light on understanding the possible evasion mechanism of host immune response.

While 90% of the eukaryotic genome is transcribed, only around 2% of the genome codes for proteins, implying that the genomic output is predominantly non-coding RNA (ncRNA) (Pauli *et al*., 2011). The declining proportion of protein-encoded genes is said to correspond to the increasing organism complexity (Amaral & Mattick, 2008). ncRNA can be defined as ribonucleic acid (RNA) transcribed from deoxyribonucleic acid (DNA) that does not get translated into proteins. They were previously referred to as "junk RNA" or "dark matter" since they were thought to be non-functional (Brosius, 2005). However, it was later discovered that the ncRNA possesses functions in different levels of gene regulation, including epigenetic, transcriptional, and post-transcriptional (Mattick & Amaral, 2022). ncRNA can be categorized into two main categories; structural RNAs and regulatory RNAs. The RNAs that belong to structural RNAs are transfer RNA (tRNA), ribosomal RNA (rRNA), small nucleolar RNA (snoRNA), and small nuclear RNA (snRNA). Regulatory RNAs are RNAs such as micro RNA (miRNA) (Bhogireddy *et al*., 2021). Currently, knowledge of ncRNA is still limited. Even so, the critical functions that these molecules play in almost all biological processes have boosted interest in ncRNA research. For instance, a study of miRNAs in the plant pathogenic fungus *Fusarium oxysporum* f. sp. niveum (Jiang *et al*., 2017) provides insight into how pathogenic fungi miRNAs participate in toxin biosynthesis, and a study of long ncRNA (lncRNA) in tomato by Jiang *et al*.(2019) shed insight into how lncRNA regulates tomato resistance to *Phytophthora infestans*.

With recent advances in transcriptomic studies and RNA sequencing technologies, elucidation of non-coding RNA structures and functions has been realized (Xin *et al*., 2011). This will help to improve our understanding of the regulatory roles of ncRNA in such interactions. However, there are no such reports on oil palm and its infamous pathogen, *G. boninense*. Therefore, to understand the underlying mechanism of ncRNA across *E. guineensis* and *G. boninense* genomes, in this research, the distribution of ncRNAs across both genomes are characterized and analyzed. As our understanding expands, novel functional networks may be modulated for the precise solution of the basal stem rot disease in oil palm.

MATERIALS AND METHODS

Workflow

The oil palm and *G. boninense* raw genome sequences were downloaded from public databases and served as the main input files in this study. Prediction of different types of ncRNA was performed by implementing different algorithms via specific prediction software and biological databases as illustrated in Figure 1.

Genome sequence retrieval

The genome sequence of *E. guineensis* was obtained from the Genomsawit website [http://genomsawit.mpob.gov.my], comprising 16 chromosomes (genetic scaffold) and 40,056 P5 scaffold sequences (Singh *et al*., 2013). Meanwhile, the genome of *G. boninense* G3 was obtained from the National Center for Biotechnology (NCBI) database with the accession number GCA_002900995.3 (Bioproject: PRJNA421251) (Utomo *et al*., 2018). Both genome sequences were downloaded onto the local directory in FASTA format for ncRNA prediction analysis.

tRNA prediction

tRNAscan-SE v.2.0 (Chan & Lowe, 2019) tool was installed on a LINUX server to screen the genomic sequences of *E. guineensis* and *G. boninense* for tRNAs. The default search mode is used, in which Infernal (Nawrocki & Eddy, 2013) is employed using newly built covariance models (CM) to identify and classify tRNA (Chan *et al*., 2021), while universal genetic code for tRNA isotype prediction is applied. The analysis allows to check for pseudogene and the score cut-off is set to be an overall score of fewer than 55 bits, with one of two conditions met: a primary sequence score of fewer than 10 bits or a secondary structure score of fewer than 5 bits (Chan *et al*., 2021). The text-based output is in BED format that allows the storage of genomic regions as coordinates and associated annotations.

rRNA, snRNA, and snoRNA predictions

rRNAs, snRNAs, and snoRNAs were detected using Infernal Software v1.1.4 which was installed on a local LINUX server. First, the Rfam CM file was downloaded from the Rfam database. By using the *cmpress* program, the CM database was formatted into binary and searched against the genome sequences using the *cmscan* program. While running the *cmscan* program, several command line options as explained by Nawrocki *et al*. (2015) were enabled. A series of data mining

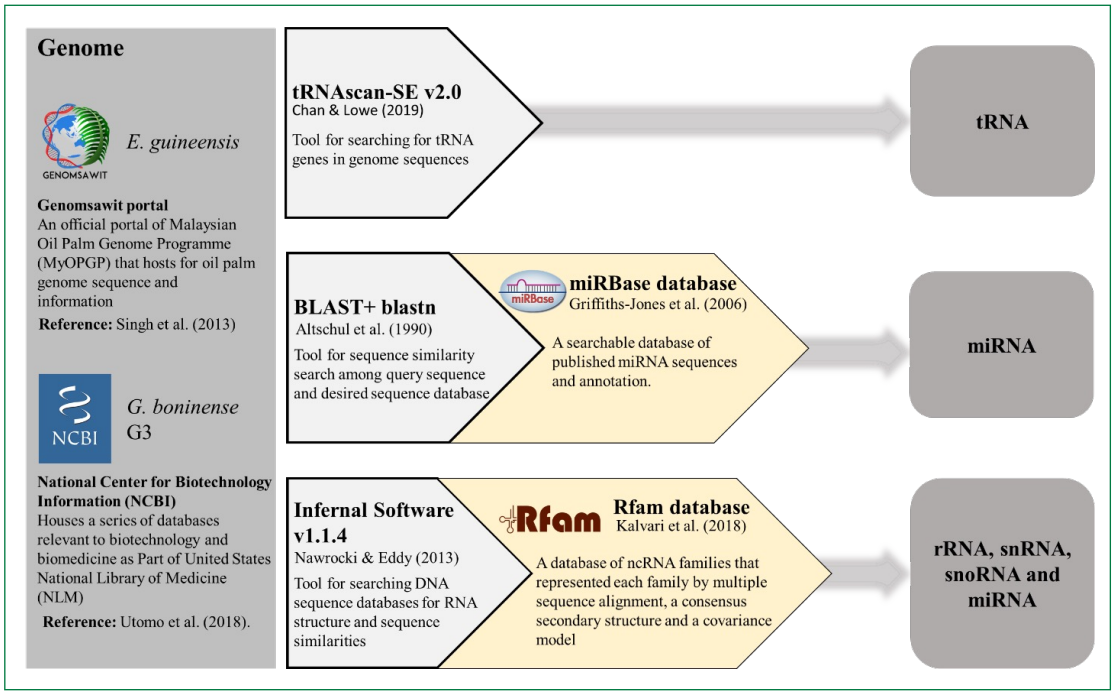

Fig.1. A schematic overview of the pipeline employed for the identification of ncRNAs. The genome could belong to *E. guineensis* or *G. boninense*.

processes were done to extract the predicted ncRNAs from the text-based output file that was generated from the analysis

miRNA prediction

In addition to Infernal prediction, miRNAs were detected by aligning assembled genome of *E. guineensis* and *G. boninense* to mature miRNA sequence from miRbase using BLASTn, with the *e-value* cut-off $1e^{-3}$ and word size 19. The resulting predicted miRNA sequences from both Infernal and BLASTn were overlapped to obtain the possible number of miRNA sequence regions in the genome.

RESULTS AND DISCUSSION

The different classes of predicted ncRNAs are shown in Table 1**.** After a series of searches via different bioinformatics tools, there are in total 2,233 and 369 ncRNAs discovered in *E. guineensis* and *G. boninense*, respectively.

Structural ncRNA

Structural ncRNA is a type of ncRNA that is found in the structure of a biomolecule. tRNA, rRNA, snoRNA, and snRNA are example of structural ncRNAs. Transfer RNA (tRNA) is an ncRNA with a length of 70 to 90 nucleotides (OpenStax, 2022). The basic and well-known function of tRNA was established in the 1960s as an important component of messenger RNA (mRNA) translation in delivering amino acid to the ribosome during protein synthesis after being coupled with the target amino acid by specific aminoacyl-tRNA synthetase (Crick, 1966; OpenStax, 2022). In the *E. guineensis* genome, a total of 543 tRNAs with an average length of 75 nucleotides were detected. 271 tRNAs with an average length of 79 nucleotides were detected in the *G. boninense* genome. 62 out of 543 tRNAs and 16 out of 271 tRNAs were predicted as pseudogenes in *E. guineensis* and *G. boninense* genomes, respectively.

Apart from tRNA, another class of structural RNA is rRNA, known to be the cell component that composes up to about 60% of a synthesizing organelle protein called ribosome (OpenStax, 2022). It catalyzes the formation of peptide bonds between two aligned amino acids and ensures that mRNA and ribosomes are properly aligned during protein synthesis. Based on the result by Infernal, a total of 626 and 55 rRNAs were predicted in the *E. guineensis* and *G. boninense* genomes, respectively.

Another structural ncRNA, snoRNA has a size ranging from 60 to 300 nucleotides and is primarily in charge of rRNA maturation and posttranscriptional modification (Liang *et al*., 2019). From Infernal analysis, *E. guineensis* is predicted to have 719 snoRNAs, while in the *G. boninense* genome, a total of 4 snoRNAs were predicted.

Besides snoRNA, Infernal predicted 128 and 13 snRNAs in the *E. guineensis* and *G. boninense* genomes, respectively. With approximately 150 nucleotides long, snRNA is well-known for

its function in splicing introns from the primary transcript (Hari & Parthasarathy, 2018). This ncRNA typically exists in the form of ribonucleoproteins (snRNPs), an association between snRNAs and proteins (Hari & Parthasarathy, 2018). snRNPs can be classified into two groups: major and minor. The major (U2-type) consists of U1, U2, U4, U5 and U6 snRNPs, while the minor (U12-type) consists of U11, U12, U4atac, U5 and U6atac snRNPs. The major type typically catalyzes the majority of the intron removal (more than 99%), whereby the minor catalyzes less than 1% of the intron removal (Alioto, 2007). In the *G. boninense* genome, the U1 sequence was not detected by Infernal. Therefore, the U1 sequences from Rfam were downloaded for alignment by BLAST+ blastn against the *G. boninense* genome, and interestingly, BLAST returned 0 output. The same issue was reported in *Pleurotus ostreatus* (Jacq.: Fr.) Kumm. genome by Qu *et al*. (2016), a study that also highlighted that even if snRNA U1 of basidiomycetes exists, the sequence might significantly vary from other organisms.

Regulatory ncRNA

Regulatory ncRNA is a type of ncRNA that plays a vital role in the regulation of downstream genes. miRNA is one example of regulatory non-coding RNA. miRNA is a class of ncRNA having a length of approximately 19 to 24 nucleotides. The majority of miRNA is transcribed into primary miRNA from DNA. Following that, primary miRNA is processed into precursor miRNA, and finally mature miRNA. (Ketting, 2011; O'Brien *et al*., 2018). miRNA has been extensively studied due to its crucial role in regulating gene expression by targeting messenger RNAs for their degradation (Trang *et al*., 2008; Li *et al*., 2009; Hydbring & Badalian-Very, 2013). In the *E. guineensis* genome, 174 precursor miRNAs were detected by Infernal. As for the *G. boninense* genome, there was no precursor miRNA detected by Infernal. Therefore, both genomes are BLAST against mature miRNA sequences from miRBase, and the search analysis has resulted in 43 and 26 predicted mature miRNA sequences, respectively.

ncRNAs involvement in plant resistance

A viable approach for accurate and rapid ncRNA identification in the genome is now possible with the emergence of today's technologies together with publicly accessible genome data and databases. While not all their functions are known, identification of ncRNAs in the genome is essential to investigate the mechanisms that promote plant resistance to *G. boninense* infection. As reviewed by Song *et al*. (2021), ncRNA with known involvement in plant-pathogen mechanism is primarily small interfering RNA (siRNA) and miRNA. Several miRNAs predicted in the *E. guineensis* and *G. boninense* genome from Infernal and BLAST analysis were identified as possible targets to enhance *E. guineensis* resistance to *G. boninense*. The identified miRNAs were based on previously known miRNAs that play important roles in diverse plant species by either down-regulating or up-regulating the expression of genes involved in conferring resistance to infection (Table 2).

In *E. guineensis*, up-regulation of the predicted miR160, miR162, miR166, miR167, and miR399 could be potentially exploited to enhance *E. guineensis* resistance against *G. boninense*. In a study by Campo *et al*. (2013), overexpression of miR160 in *Oryza sativa* enhances disease resistance by targeting the *Auxin Response Factor* (*ARF*) gene, which suppresses the auxin signaling pathway, thereby preventing disease onset (Navarro *et al*., 2006). However, Natarajan *et al*. (2018) showed that overexpression and knockdown of miR160 in potatoes increased plant susceptibility to infection, indicating that a moderate level of miR160 is necessary to sustain the antagonistic cross-talk between salicylic acid (SA)-mediated defense and auxin-mediated growth pathways. The predicted miR162 as observed by Li *et al*. (2020) targets dicer-like 1 (*DCL1*) transcript in *O. sativa* that consequently increases the induction of defense-related genes and the accumulation of reactive oxygen species (ROS), which are important signal molecules during plant-pathogen interactions (Camejo *et al*., 2016).

Increased ROS levels are revealed to induce SA-induced pathogenesis-related (PR) expression and trigger plant resistance to pathogen invasion (Chaouch *et al*., 2010). Such a procedure, however, may result in a minor loss in yield per plant. In cotton plants, the predicted miR166 was revealed by Zhang *et al*. (2016) to participate in the plant defense system against *Verticillium dahlia* by targeting Ca^{2+} dependent cysteine protease (*Clp-1*) and isotrichodermin C-15 hydroxylase (*Hic-15*) gene, which is needed for microsclerotium formation and hyphal growth upon infection. As a result, overexpression of miR166 induces fungal silencing. Other than that, up-regulation of the predicted miR167 that targets *Auxin Response Factor* 6 (*ARF6*) and *ARF8* as observed by Caruana *et al*. (2020) in *Arabidopsis thaliana* could result in positive plant-defense regulation by keeping the stomata small, preventing pathogenic cells from accessing leaf interior. Overexpression of miR167, however, may severely impair systemic acquired resistance (SAR) response. For this reason, striking a balance between short- and long-term protection, as well as the role of stomata as an early barrier to pathogen entrance, is crucial. Lastly, overexpression of the predicted miR399

Table 1. Summary of ncRNA prediction in *E. guineensis* and *G. boninense* genomes

Type of ncRNA			Method/ tool	Number of predicted ncRNA	
				E. guineensis	*G. boninense*
tRNA		tRNA decoding Standard 20 AA	tRNAscan-SE	475	253
		Selenocysteine tRNA (TCA)		0	0
		Possible suppressor tRNAs (CTA, TTA, TCA)		1	2
		tRNAs with undetermined/ unknown isotypes		5	0
		Predicted pseudogenes		62	16
		Total tRNA		**543**	**271**
rRNA		5.8S rRNA	Infernal	24	12
		5S rRNA		434	8
		Small subunit rRNA		72	14
		Large subunit rRNA		96	21
		Total rRNA		**626**	**55**
snoRNA		Total family	Infernal	95	3
		Total snoRNA		**719**	**4**
snRNA		Total family	Infernal	11	4
		Total snRNA		**128**	**13**
miRNA	Precursor	Total family	Infernal	27	0
		Total precursor miRNA		174	0
	Mature	Total family	BLASTn	36	24
		Total mature miRNA		43	26
		Total miRNA		**217**	**26**
		Total ncRNA		**2,233**	**369**

Table 2. List of miRNAs from *E. guineensis* and their targets involved in plant-pathogen interactions

miRNA	Organism	Pathogen	Target gene(s)	Reference
miR156	*Arabidopsis thaliana*	*Pseudomonas syringae* pv. Tomato (Pst) DC3000	*SPL9*	Yin *et al*. (2019)
miR160	*Oryza sativa*	*Magnaporthe oryzae*	*ARF*	Campo *et al*. (2013)
miR162	*Oryza sativa*	*Magnaporthe oryzae*	*DCL1*	Li *et al*. (2020)
miR166	*Gossypium*	*Verticillium dahliae*	*Clp-1* and *HiC-15*	Zhang *et al*. (2016)
miR167	*Arabidopsis thaliana*	*Pseudomonas syringae*	*ARF6* and *ARF8*	Caruana *et al*. (2020)
miR169	*Oryza sativa*	*Magnaporthe oryzae*	*NF-YA*	Li *et al*. (2017)
miR396	*Arabidopsis thaliana*	Necrotrophic and hemibiotrophic fungal pathogens	*GRF*	Soto-Suárez *et al*. (2017)
miR397	*Gossypium*	*Verticillium dahliae*	*GhLAC4*	Wei *et al*. (2021)
miR398	*Triticum aestivum*	*Fusarium culmorum*	*CSD*	Salamon *et al*. (2021)
miR399	*Arabidopsis thaliana*	Necrotrophic and hemibiotrophic fungal pathogens	*PHO2*	Val-Torregrosa *et al*. (2022)
miR2118	*Gossypium*	*Verticillium dahliae*	*TIR-NBS-LRR*	Yin *et al*. (2012)

could be potentially exploited by resulting in loss-of-function of *PHOSPHATE2* (*PHO2*), coupled with an increase in inorganic phosphate (Pi) content and accumulation of ROS to promote resistance toward infection in *A. thaliana*., as observed by Val-Torregrosa *et al*. (2022)

Downregulation of miRNAs has previously been shown to confer resistance towards its pathogen in several plant species. This means, there could be a similar exploitation of the 6 predicted miRNAs (miR156, miR169, miR396, miR397, miR398, and miR2118) found in *E. guineensis* genome for disease resistance. According to a study by Yin *et al*. (2019), downregulation of miR156 expression in *A. thaliana* that targets *Squamosa Promoted Binding Protein-Like 9* (*SPL9*) promotes the production of ROS to confer resistance. Other than that, disease resistance could also be promoted through the suppression of miR169 that targets the nuclear factor (NF-YA) family as reported in rice plants in response to *Magnaporthe oryzae* infection by elevating the formation of the flexible and complex transcription factory system that is needed for plant defense response (Li *et al*., 2017). The predicted miR396 as observed by Soto-Suárez *et al*. (2017) acts as a negative regulator in disease resistance

by targeting the *Growth-Regulating Factor* (*GRF*) in *A. thaliana.* Downregulation of miR369 triggers hosts reprogramming for defense.

Plant protection is also demonstrated by strengthening the first line of defense, the cell wall. For instance, the suppression of the predicted miR397 as observed by Wei *et al*. (2021) could significantly increase basal lignin content by increasing the expression of the target gene, *GhLAC4*. Lignin protects plant cell walls from degradation mediated by cellulase or fungi secretion. Therefore, upregulating the target gene involved in basal lignin content production could be useful. In rice plants, downregulation of the predicted miR398 enhances plant resistance toward *M. oryzae* by promoting the expression of two Cu/Zn super oxidase dismutase genes (*CSD1* and *CSD2*) and a copper chaperone for superoxide dismutase (Sunkar & Zhu, 2004). These target enzymes have been reported to act as scavengers of harmful reactive oxygen species (ROS), thereby preventing further damage to plants through protection against oxidative stress associated with pathogen infection (Sunkar & Zhu, 2004). Another miRNA study in the cotton plant (Yin *et al*., 2012) by downregulation of the predicted miR2118 results in the accumulation of a resistance protein for regulating pathogenesis (TIR-NBS-LRR), which consequently results in a high defense response against *V. dahlia*, a fungal plant pathogen.

In *G. boninense*, two predicted miRNAs (miR-4968-3p & miR5653) have known target genes that can be exploited to prevent the infection of *G. boninense*. One of the miRNAs in *G. boninense* that could be potentially fine-tuned is miR-4968-3p, which was reported to target caspase/metacaspase, an enzyme that induces apoptosis (programmed cell death) in the fungal population (Rahul & Rajesh, 2016). However, further study is needed to investigate whether the same effect of miR-4968-3p reduction activity could promote apoptosis in *G. boninense* too. Besides, miR5653 was reported to target diverse proteins including ubiquitin carriers and Serine/threonine protein kinase (Pandey *et al*., 2013). The previous study reviewed that ubiquitin carrier and Serine/threonine protein kinase play a crucial role in the regulation of various essential cellular processing including regulation of metabolic regulation, control of cell cycle, activation of various transcription factors as well as recycling of abnormal proteins (Ciechanover, 1994). Therefore, overexpression of miR5653 could be fatal to *G. boninense* and this hypothesis needs further study and verification.

For the miRNA-mRNA interaction to be considered biologically significant, conditions, as outlined by Riolo *et al*. (2021) must be satisfied; miRNA and target mRNA must be co-expressed, the interaction between miRNA and specific miRNA responsive element (mRE) target site is proven, miRNA-mediated effects on target protein expression and biological function are demonstrated. Microarray profiling, Northern blotting, and quantitative real-time polymerase chain reaction (RT-qPCR) are techniques that can be used to validate co-expression (Nuovo, 2010; Sansom *et al*., 2010). The interaction between miRNA and mRE target sites can be proven using the current gold standard method, reporter gene assay (Nicolas, 2011). To evaluate protein expression from the effects of post-transfection of either miRNA mimics or inhibitors, conventional techniques including Western blotting, enzyme-linked immunosorbent assay (ELISA), and immuno-cytochemistry assays can be used. (Thomas *et al*., 2010). Finally, various *in vivo* and *in vitro* assays, including biochemical assays, can be carried out to show that protein regulations mediated by miRNAs correspond to changes in biological function.

Before identifying miRNA involved in plant-pathogen interaction, miRNA therapeutic strategies such as miRNA mimics, recombinant expression vectors carrying miRNA encoding sequences, and oligonucleotide-based miRNA inhibitors can be applied to alter and reverse pathological miRNA expression (Rooij & Kauppinen, 2014). However, single miRNA not only can regulate large subsets of mRNA targets but single mRNA is also frequently targeted by multiple miRNAs (Gebert & MacRae, 2019) and therefore this strategy needs to be carefully evaluated in plant model to prevent severe side effects miRNA therapeutics. Non-coding RNAs study may not be as popular as the coding RNAs. However, multiple studies have proven that these RNAs are capable of regulating gene expression through diverse mechanisms (Pauli *et al*., 2011; Yamaguchi & Abe, 2012; Palazzo & Lee 2015).

CONCLUSION

In total, 2,233 and 369 ncRNAs were predicted in the *E. guineensis* and *G. boninense* genomes, respectively. Most of the identified ncRNAs were limited to known ncRNAs. In comparison to other ncRNAs, miRNAs have been thoroughly investigated, providing insight into their specific biological roles. Other ncRNAs, on the other hand, have poorly understood biological roles, thus, limiting their potential to be used in genetic engineering. Despite limited current knowledge about ncRNA available to date, identifying these ncRNAs could be the key step toward understanding their biological role in depth, which later could be used in genetic engineering for enhancement of *E. guineensis* resistance toward *G. boninense.*

ACKNOWLEDGEMENTS

This work was funded by FGV Agri Services Sdn. Bhd. The authors wish to thank Nur Syamimi Yusoff and Nur Qistina Othman for their help and assistance. We would like to express our gratitude to the CEO of FGV R&D Sdn. Bhd. for the support given in this project.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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