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EgMLP1 Gene Expression in Oil Palm Ramet Infected with *Ganoderma boninense*

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ABSTRACT

Oil palm (*Elaeis guineensis*) is an important crop commodity in Indonesia. However, the effort to increase its production is hindered by basal stem rot (BSR), a devastating disease caused by *Ganoderma boninense*. The early symptoms of *G. boninense* infection cannot be observed with naked eyes. It can only be observed at the later stage of the infection. By the time the symptoms appear, the palm is beyond recovery. Hence an early detection technique is essential for effective management of BSR in oil palm. The aim of this research is to analyze and compare the gene expression profile of a defense-related gene encoding early methionine-labeled polypeptide (EgEMLP1) by using quantitative reverse-transcription PCR between the leaves of untreated and treated oil palm cultures with *G. boninense*. First, *G. boninense* was isolated and inoculation on oil palm cultures, then RNA isolation was conducted on both oil palm cultures from 3 to 7 weeks post infection (wpi) and purified RNA was used for cDNA synthesis. Finally, EgEMLP1 gene expression was measured using qRT-PCR. The result showed that the expression of EgEMLP1 gene had increased 5.65, 15.66 and 17.96 fold at 3, 5 and 7 wpi compared to those of controls. Furthermore, the result also showed that direct infection method on in vitro oil palm cultures shorten the time needed to infect *G. boninense* to oil palm compared to rubber wood block method. With less process, direct inoculation method can be an alternative in gene expression in oil palm ramets infected with *G. boninense* research and up-regulated EgEMLP1 gene expressed has the potential to be developed as a biomarker for early detection of *G. boninense* infection.

Keywords: basal stem rot, biomarker, direct inoculation, oil palm culture, qRT-PCR

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INTRODUCTION

Oil palm plantations have big prospects in Indonesia and some countries in South East Asia. In 2010, Indonesia had been the greatest exporter of crude palm oil (CPO) in the world with total area of 8 million hectares and had been expected to increase to 12 million hectares in 2020 (Teoh 2010). One of the problems in oil palm cultivation is a disease called basal stem rot (BSR) which is caused *Ganoderma boninense* infection. The loss of this disease alone is estimated to 40 trillion rupiah. *Ganoderma* is a wood rot fungi that can decompose lignin into carbon dioxide and water. Based on Purnamasari *et al*. (2012), *Ganoderma* sp. is the specific species to oil palm. There are at least three species of *Ganoderma* that are associated with this disease, *G. boninense, G. zonatum,* and *G. miniatocinctum* (Utomo *et al*. 2005).

Several ways have been done to control BSR disease, either mechanically, chemically, or biologically. Nevertheless, those ways do not provide satisfactory results and not very economical. BSR disease is a slow-progressing disease and fairly difficult for detection at early stages of infection. Therefore, this disease is often detected at advanced stages and by then it was too late to address the disease (Sapak *et al*. 2008).

There are few oil palms known to be genetically tolerant or resistant to BSR disease, but those genes are not much explored. There are differences of gene expression due to *Ganoderma* sp. infection on oil palm, but the profile of these differentially expressed genes is still limited. It is important to identify the differentially expressed genes to study the molecular interaction between host and pathogen which play a role in overcoming BSR disease (Tan *et al*. 2013).

In previous study by Tee (2008), microarray analysis on mycorrhiza, *Ganoderma*, and mycorrhiza-*Ganoderma* infected oil palm shows differentially expressed genes related to plant defense mechanisms and stress responses, such as putative beta 1.3-glucanase, early methionine labeled polypeptides (MLP1), and methallothionein-like protein (MT). Early methionine labeled polypeptides (MLP1) is a transcription factor that is part of group 1 late embryogenesis abundant (LEA) protein. LEA protein is found on higher plant species and animals, it is associated with tolerance to drought and cold (Goyal *et al*. 2005).

Thereby, the aim of this study was to analyze and compare the gene expression profile of a defense-related gene encoding *Elaeis guineensis* early methionine-labeled polypeptide (EgEMLP1) by using quantitative reverse-transcription PCR between the leaves of untreated and treated oil palm cultures with *G. boninense.*

MATERIALS AND METHODS

Plant Materials

One-year-old oil palm tissue-culture plantlets (ramets) obtained from PT Wilmar Benih Indonesia was used in this study. All ramets must have healthy dark-green leaves and shoots; similar in size, leaves numbers, and height; active and healthy roots without any contaminations on the media, and from the same cultivar. Three plantlets were used as healthy control and the other three planlets were directly infected with *Ganoderma*. Leaves material from each treatment were harvested at 3, 5, 7 weeks post infection (wpi) respectively.

This study is conducted in Plant Tissue Culture Laboratory, Biotechnology Faculty, Atma Jaya Catholic University of Indonesia, Jakarta.

Inoculation of *Ganoderma boninense*

Artificial inoculation of *G. boninense* was performed using mycelia inoculation technique (Chong *et al*. 2012) with some modifications. Inoculum of *G.*

boninense was prepared by cutting mycelia of 6-day-old mycelia on potato dextrose agar (PDA) into 1cm x 1cm plugs using a sterile scalpel. *Ganoderma* isolate that used in this study was one of the culture collection of PT Wilmar Benih Indonesia and performed rapid infection in oil palm seedlings in previous experiment (Purnamasari *et al*. 2018).

The mycelial plugs were inoculated in the root area of one-year-old ramets and incubated for 3, 5 and 7 weeks. After inserting mycelial plugs, the plantlets should be planted to previous position in their flask tubes. All inoculation process must be done under sterile environment in laminar airflow cabinet. Control and infected plantlets were placed in tissue culture laboratory at 25 °C prior to leaves sampling for RNA extraction.

RNA Isolation and Purification

The leaves from control and fungal treatment were harvested at 3, 5 and 7 wpi, respectively. Total RNA of the plantlets' leaves was extracted with GeneJET Plant RNA Purification Mini Kit according to manufacturer's protocol (ThermoScientific, US). Prior to cDNA synthesis, RNA must be free from contaminants such as genomic DNA.

Tubes were incubated at 37 °C for 30 minutes. Then in 1 mL of 50 mM EDTA was inserted into the tube and incubated at 65 °C for 10 minutes. Pure RNA can be used directly for cDNA synthesis.

cDNA Synthesis

The DNAse-treated RNA was reverse-transcribed with RevertAid First Strand cDNA Synthesis Kit (ThermoScientific, US). Positive controls of kit (GAPDH) was used.

The tube was centrifuged briefly and then incubated at 42 °C for 60 minutes. Then the tube incubated at 70 °C for 5 minutes to stop the reaction. The reaction product can be directly used for PCR or can be stored at -20 °C for less than one week.

Quantitative Reverse Transcription (qRT)-PCR

PCR micro plate was prepared and each PCR reaction contained components in Table 1. Primers used for this experiment were the target gene, EgEMLP1, and internal control gene (cyclophillin), EgCYC (Tan *et al*. 2013). Sequence of primers are listed in Table 2.

The qRT-PCR performed in a Real Time PCR Bio-Rad iQ™5 (BioRad, US). The PCR condition was 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 1 min, and 72 °C for 1 min. A negative control without template also included. All PCR reactions performed in triplicates.

Data Analysis

Comparative ΔΔCt method with reference gene applied for the quantification of gene

Table 1 qRT-PCR components

Component name	Amount	Unit
First strand cDNA	1.0	µL
Forward primer	0.4	µL
Reverse primer	0.4	µL
2x KAPA SYBR® FAST qRT-PCR Master Mix	10.0	µL
Nuclease Free Water (NFW)	8.2	µL
Volume	20.0	µL

Table 2 Primer sequences for qRT-PCR (Tan *et al.* 2013)

Nama Primer	Accession Number	
Primer *forward-reverse* EgEMLP1	EL695076 EL695347	GCGTGAAGGGGTCGATAT CAACACCAAACAGAGGAAACC
Primer *forward-reverse* EgCYC	EL684686	GGGAAGCACGTCGTCTTTGG TTCTACCGGCACGATGAGCA

expression, where Ct is the number of PCR cycles required for the fluorescence to reach the threshold level. Normalization performed with endogenous genes encoding oil palm cyclophillin (EgCYC). The relative fold change (R) computed by a formula, R = $2–^{\Delta\Delta}$Ct, where 2 is the amplification efficiency of the respective genes. The relative expression fold change of treated sample calculated by comparing the abundance of transcripts in treated sample with that of control sample at respective time points. A gene is considered to be differentially expressed if the relative transcript abundance increased ≥ 2-fold or decreased ≥ 50% compared to controls.

RESULTS AND DISCUSSION

One-year-old oil palm ramets in a state of 3 healthy cultures and 3 cultures that had been infected by *Ganoderma*. On the seventh wpi, healthy ramets (untreated with *G. boninense*) showed continuous growing of green leaves and healthy root performance. Whereas in the *Ganoderma* infected ramets, the necrosis at leaf tips were obvious and brownish white *Ganoderma* myceliums colonized the palm roots (Figure 1).

Based on the visualization result of RNA isolation by agarose gel electrophoresis, samples performed two strong bands which form the bands of 28S rRNA and 18S rRNA (Figure 2). The presence of both bands indicates that the RNA is intact and can be used for cDNA synthesis. The concentration and purity of RNA were measured by NanoDrop 2000 UV-Vis at OD 260/280 and listed in Table 3.

CDNA synthesis is conducted by reverse transcriptase and oligo (dT) primer. oligo (dT) primer is used because the desired product is a reverse transcript of mRNA, which has a poly-A tail. Then verify the cDNA synthesis by performing GAPDH, rbcL, and EgEMLP1 gene amplification. GAPDH gene amplification as an internal control of kit, made by reverse transcription of RNA GAPDH results that have been provided kit and a forward-reverse primer GAPDH. RbcL gene amplification as an internal control of plant RNA, carried out with the results of

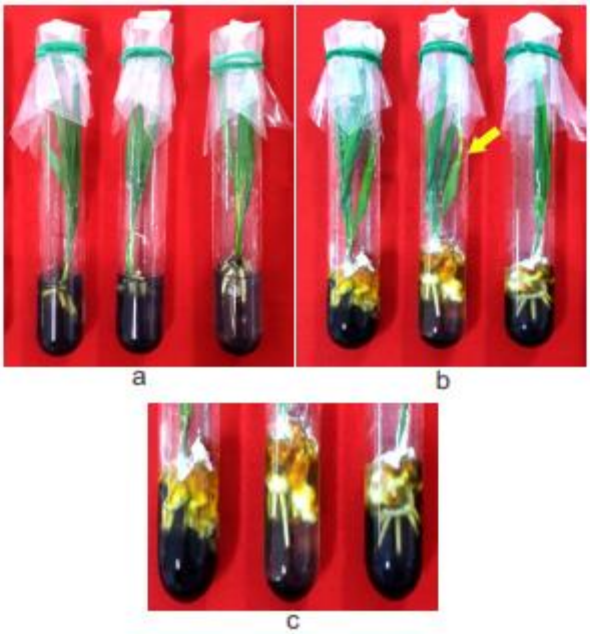

Figure 1 Oil palm culture: a Healty ramets; b *Ganoderma* infected ramets; c Roots with *Ganoderma* mycelium.Yellow arrow shows leaf necrosis.

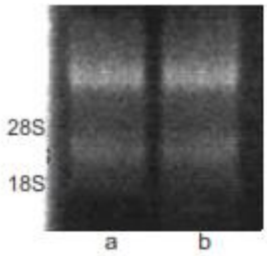

Figure 2 RNA isolated from oil palm leaves: a Healthy oil palm; b Infected oil palm.

Table 3 RNA quantity and purity measurements

Treatment	WPI	[RNA] ng µL^{-1}	A260/280
N	3	124.1	2.01
	5	190.6	2.03
	7	161.2	2.02
S	3	148.1	2.01
	5	172.8	2.01
	7	221.0	2.02

N:Healthy oil palm; S:Infected oil palm; WPI:Week post infection.

reverse transcription of oil palm RNA and rbcL forward-reverse primer. RbcL gene is found in all plants and organisms that carry out photosynthesis. This gene encodes a large subunit of the enzyme ribulose-1.5-bisphosphate carboxylase/oxygenase (Rubisco) for the fixation of CO_2 during photosynthesis of C4 plants (Bowman *et al*. 2013). EgEMLP1 amplification as a target gene, are carried out with the results of reverse transcription of oil palm RNA and EgEMLP1 forward-reverse primer. EgEMLP1 gene plays a role in adult plants defense mechanism, especially against abiotic stress such as drought. Verification of cDNA synthesis was performed for each RNA isolation (Figure 3).

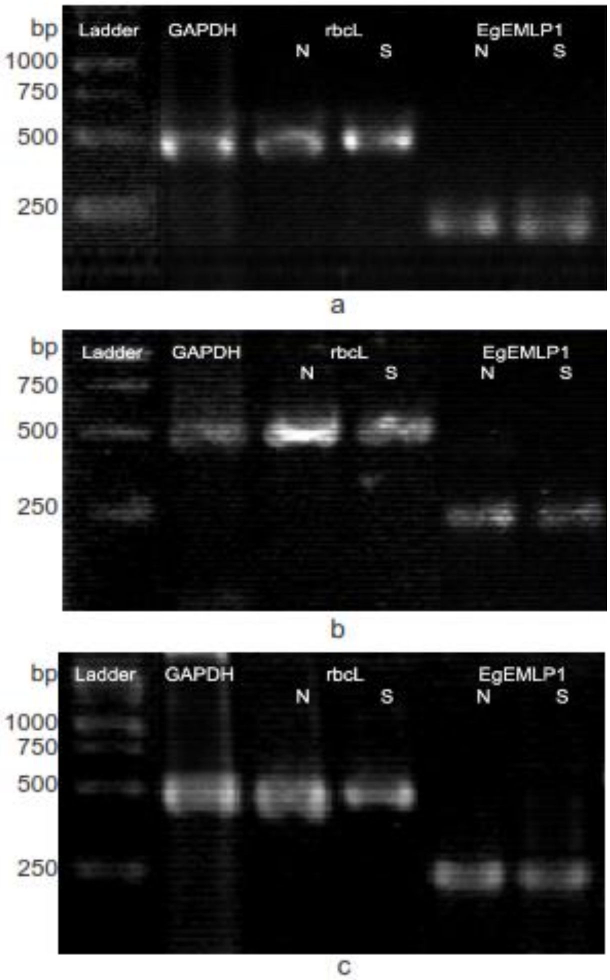

Figure 3 cDNA synthesis verification: a 3 WPI; b 5 WPI; c 7 WPI. N:Healthy oil palm; S:Infected oil palm; WPI:Weeks post infection.

cDNA then used to compare the gene expression EgEMLP1 between healthy and infected plants using qRT-PCR. All reactions performed in triplicates and normalize with EgCYC as endogenous control. EgCYC encodes cyclophillin, a house-keeping gene that is continuously expressed and has many important functions, one of them is acting as chaperones in protein folding (Kumari *et al*. 2013). Quantification of gene expression using comparative Ct method (ΔΔ Ct), relative fold change calculated by the formula $R= 2^{–\Delta\Delta\ Ct}$. The calculations showed that the gene EgEMLP1 in infected oil palm cultures is excessively expressed compared to healthy oil palm seedlings. In 3 wpi, EgEMLP1 gene is expressed 5.65 fold higher in infected cultures than in healthy cultures. In 5 weeks post infection, this gene is expressed 15.66 fold and in 7 wpi expressed 17.96 fold. Increased expression of gene that encodes early polypeptides labeled methionine indicates plant response to drought stress caused by *Ganoderma*. Due to decayed trunk, the distribution of water and nutrients in oil palm is disrupted. Furthermore, this causes a drought condition in the leaves of oil palm which provide signals to express more EgEMLP1 gene. The increasing profile of EgEMLP1 gene expression from the third week until the seventh week showed a consistent performance. Moreover, the EgEMLP1 gene might be up-regulated by infected oil palm ramets (Figure 4).

The previous research had been done by Tee *et al*. (2013), using oil palm seedlings inoculated by *Ganoderma* using rubber wood block (RWB). Target genes encoding Em proteins H2 (early-methionine-labeled-polypeptides) was excessively expressed in *Ganoderma* infected oil palm root samples. The analysis was performed using microarray that be able to can analyze multiple genes in a single experiment. However, the quality of gene expression

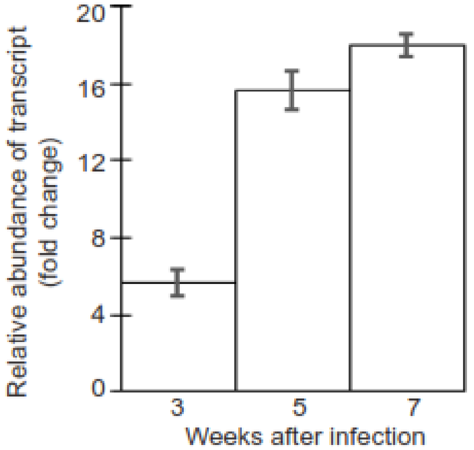

Figure 4 EgEMLP1 gene expression profile.

results can differ greatly between procedures performed, so real-time quantitative PCR (qPCR) is more commonly used to validate gene expression profiles obtained from microarray (Morey *et al*. 2006). The microarray results from Tee *et al*. (2013) is consistent with this study although the methods and techniques are different.

According to Tan *et al*. (2013), oil palm seedlings used as plant materials and was inoculated by *Ganoderma* using RWB. EgEMLP1 gene expression in root samples of oil palm seedlings is excessively expressed when compared to healthy oil palm seedlings, but the expression of these genes in leaf sample of oil palm seedling is decreased compared to healthy oil palm seedlings. It might be caused by the different ways and time of *Ganoderma* infection, and also associated with different tissue samples. The expression of early methionine labeled polypeptides on the roots of *Ganoderma* infected oil palm with of rubber wood block method is up-regulated. However, the gene expression is down-regulated in the roots of *Trichoderma* and mycorrhiza infected oil palm. This difference expression pattern may be caused by different species of fungi that infects the palm, *Trichoderma* and mycorrhiza are beneficial to oil palm while *Ganoderma* does not (Tan *et al*. 2015).

Besides using the roots for gene expression analysis, leaves of the *Ganoderma* infected oil palm is also used because asier to obtain. However, unlike the expression of early methionine labeled polypeptides on the roots, the expression of this gene is down-regulated on the leaves. Generally, genes associated with plant defense can be up-regulated to increase plant immune systems against invading pathogens and down-regulated by the pathogens to withstand the plant defense mechanism, but the specific role of the gene that encodes early methionine labeled polypeptides in response to stress biotic or BSR disease is unknown (Tan *et al*. 2013).

There are various ways to inoculate oil palm seedlings with *Ganoderma* artificially. Lim *et al*. (1992) succeeded in inoculating the injured roots of oil palm seedlings with wheat-oat medium containing *Ganoderma*. Idris *et al*. (2004) inoculate the primary root seedlings of oil palm by dipping it into a test tube containing *Ganoderma* in POPW medium (a mixture of rice, oil palm wood powder, supplemented with sucrose, ammonium sulfate, calcium sulfate, and bacto peptone). But the most effective way to inoculate palm oil is RWB method. According to Khairuddin *et al*. (1991), infection with *Ganoderma* inoculated RWB method give 100% success rate.

Although the RWB method provide a high success rate, it needs considerable time compared with direct inoculation of *Ganoderma* to the root of oil palm tissue culture. Then it can be assumed that the level of *Ganoderma* infection in oil palm germinated seedlings had not yet reached the same level as oil palm from tissue culture materials (ramets).

According to Purnamasari *et al*. (2018), a rapid inoculation technique, in planta disease assays showed that the artificial *Ganoderma* infestation using this method was reproducible and faster incubation time. Furthermore, this method provided less preparation effort compared with the existing RWB method.

CONCLUSION

Oil palm culture infected with *Ganoderma boninense* have different EgEMLP1 gene expression profile on the leaves of infected oil palm ramets compared to healthy plants. The result showed that the expression of EgEMLP1 gene increased 5, 16 and 18 folds at 3, 5 and 7 wpi, respectively, compared to controls. This study also shows that up-regulated EgEMLP1 gene expression in oil palm provide a candidate gene that has the potential to be developed as a biomarker for early detection of BSR disease caused by *G. boninense*. The increasing expression of gene that encodes early polypeptides labeled methionine may indicate plant response to drought stress caused by *Ganoderma*.

Direct inoculation method on in vitro oil palm ramets shorten the time needed to infect *Ganoderma boninense* compared to rubber wood block method.

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