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Characterization and isolation of oil palm lipid transfer protein (EgLTP) gene putatively responsible for defense against fungal infection (*Ganoderma boninense)* during basal stem rot infection

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Lipid transfer protein (LTP) is essential for the activation of plant defence responses. One of the LTP roles is to involve in the defence against fungal infection in plant cell. In this study, our aim was to isolate the LTP gene from oil palm based on LTP gene sequence from other monocot gene databases and to get insight to the function of this gene during fungal-plant infection. Four model plants were selected which are *Oryza sativa, Zea mays*, *Triticum aestivum* and *Lilium longiflorum*. Five degenerate primer pair combinations for conserved amino acid based on LTP gene sequence using these three model plants were designed. Polymerase chain reaction (PCR) amplification using these degenerate primers has resulted in amplification of various sizes of fragments, which are currently being cloned and sequenced. All products are subsequently characterized and compared using BLAST in NCBI. The result showed similarity about 100% with *O. sativa*, 68% with Sorghum *bicolor*, 66% with *Z. mays*, and 64% with *Zea diploperennis*,. Interestingly, we found that the expression of *Elaies guineensis* Lipid transfer protein (EgLTP) gene measured using Real-Time PCR showed that the expression level of EgLTP in infected oil palm was temporally down regulated. The results suggest that, down regulation of the EgLTP is related to the establishment of infection by *Ganoderma boninense*.

Key words: Lipid transfer protein, basal stem root, *Ganoderma* infection, oil palm.

INTRODUCTION

The plant defence response is related with a number of early and late actions with revere to the beginning of the stress in plant cells Plant lipid transfer proteins (LTPs) are a homogeneous class of small (9 to 10 kDa), abundant, ubiquitous and mostly basic proteins containing eight cysteine residues with four conserved disulfide bridges (Lee et al., 1998). LTPs Membranes biogenesis involves lipid movement from its synthesis site, specially the endoplasmic reticulum, to other organelles, like chloroplasts and mitochondria. The search for lipid carrier proteins in the cell led to the innovation of lipid transfer proteins (LTPs) in various plant species. Initially, their

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suggested major role was the transport of phospholipids molecules synthesizes in the endoplasmic reticulum either to other cell membrane compartment or between membranes. However, that functioning no longer established *in vivo* (Arondel et al., 2000). Instead LTPs now have been shown as being involved in many other different biological functions. Because of LTP have signal peptide indicative of secretary, protein and observed mostly in the cell walls and cuticle, the primary role of plant LTP could be the assembly of cutin and wax in the surface layer (Segura et al., 1993; Pyee et al., 1994; Cameron et al., 2006). LTPs may be also enhance to the environmental stress, as well as being protective against fungal and viral infections (Garcia-Olmeda et al., 1995; Kristensen et al., 2000; Guiderdoni et al., 2002; Park et al., 2002).The surprising antibiotic properties of LTP were discovered by screening plant proteins for their ability to inhibit the growth of fungal and bacterial pathogens (Terras et al., 1992).

Many LTP-like proteins purified from barley leaves or an LTP isolated from maize leaves were shown to inhibit the growth of a bacterial pathogen and a fungus. This was confirmed by the isolation of LTP-like proteins from cell-wall preparations from the leaves of *Arabidopsis thaliana* or spinach. A synergistic effect against the fungus occurred when the LTPs were combined with thionins. The cloning of three barley anti-pathogen proteins allowed the study of the induction of the corresponding genes (Molina et al., 1993).

Interest in the study of the changes in gene expression patterns of *Ganoderma boninense* infected oil palm has increased since basal stem rot caused by this fungus is the most serious disease of oil palm in Malaysia, that's lead to more attention to investigate about that genes might be involve in plant-pathogen interactions. Plants respond to fungal attack by eliciting various mechanisms, the main one among them is the plant defence response. LTP is involved in long-distance signalling during systematically acquired resistance in A. *thaliana* (Maldonado et al., 2002). Moreover, a wheat LTP competes with elicitin for a receptor site on plasma membrane that participate in controlling plant defence response, thereby suggesting a role for LTP as signal mediator (Buhot et al., 2001; Blein et al., 2002). LTP may encode proteins that play a critical role in establishing the defence mechanism in palms. This may assist in the development of diagnostic tools and the identification of resistant or less susceptible oil palm varieties. Furthermore, Yingfan et al. (2009) state that the mechanism of LTP in resistance of pathogen speed up the inter-membrane transfer of glycolipids *in vitro*. Quantitative methods for global and simultaneous analysis of expression profiles, such as Real-Time PCR would be powerful in giving overall understanding of the molecular and biochemical basis of the response to infection by *Ganoderma* (Ravigadevi etal., 2005).

In the continued search for a self-sustaining method of managing *Ganoderma* infection, it has become increasingly clear that basic information on the biochemical and molecular events that accompany *Ganoderma* infection is missing. It is important to know as many possible differences in gene expression between two types of cell or between two conditions. In this study, we have analyzed the expression pattern of LTP gene in uninfected oil palm and infected one during two months of infection with the fungus to determine the expression level of the gene in oil palm.

MATERIALS AND METHODS

Plants materials and fungal infection experiment

All the seedlings used in this study were from the normal seedlings D x P (Dura x pisifera seedlings) obtained from Sime Darby plantations following the normal nursery practices. The root tissue washed soil-free under tap water shortly dried subsequently soaked in liquid nitrogen ready for direct DNA and RNA extraction. The seedlings used in this study were germinated for 1 months then artificially inoculated according to the Protocol of *Ganoderma* Artificial Infection (Nur and Abdullah, 2008),the poly-bag were cut and the seedlings removed , the heat resistant polypropylene bag were removed from *Ganoderma* inoculums block (Al-Obaidi et al., 2010). Then the root of each seedling exposed directly to the block, the poly-bag refilled with soil, the seedlings were irrigated twice a day, the inoculated oil palm seeds was monitored for 2 months and at every 2 weeks, destructive sampling will be done to assess *Ganoderma* lesion.

DNA analysis and manipulation

Genomic DNA extraction has been performed using modified CTAB method as described by Doyle and Doyle (1990) with some modifications (2% CTAB,100 mM tris-HCl, pH 8, 2 mM NaCl, 20 mM EDTA, 2% PVP 40,2% ß-mercaptoethanol), RNA was removed using RNase A (20 µg/µl) followed by 30 min incubation at 37°C. The DNA pellet was finally dissolved in 30 µl of TE solution, pH 8.0, or ultra pure d H_2O. Primers used in this work were designed based on the known sequences of LTP gene from other plants available at the GenBank. The published sequences for the desired genes were retrieved from the GenBank database. DNA and amino acid sequence alignment was performed using CLUSTALX (http://align.jp). Their amino acid sequences were aligned to allow the identification of possible conserved regions within the gene. The degenerate primers were then designed based on the sequence of the conserved regions. The specificity of each primer was verified by sequences manipulation suite (http://www.bioinformatics.org/sms2/pcr_products.htm) using DNA sequences. Degenerate primers were designed and synthesized only using the sequences that were very unlikely to bind to genes other than the genes of interest. *Oryza sativa, Zea mays*, *Lilium longiflorum* and *Triticum aestivum* used to design the primer LTPf (5′ cym kmc tsg ymc ysw gac rg 3′) and LTPr (5′ ckc gta hgy kwk sgm ctg s 3′).

PCR reaction to confirm the presence of LTP in oil palm genome using that degenerate primers. PCR reactions were done using MJ

Table 1. Primers used for RT-PCR analyses of LTP putative gene in oil palm.

Primer	Sequence	Length (bp)
*LTP*rtf	5´CCCGACTGGCCCGTGACAG3´	19
*LTP*rtr	5´ CGCGTATGCGAGCGACTGC3´	19
*LTP2*rtf	5´GGGCTGCACGCTGCGGCGCAAG3´	22
*LTP2*rtr	5´GGTGCGCCACGCAGTCGCACGC3´	20

BIO Rade, THERMAL CYCLER PCR conditions were: 1 cycle at 95°C for 2 min, 30 cycles at 95°C for 30 s, 60°C for 1 min, 72°C for 1 min, and a final step at 72°C for 5 min. Gel extraction of the genomic DNA using Qaigen kit according to the manufacture then cloning using pGEMT easy Promega cloning vector system I (cat# A1360) as described in the protocol. Plasmid purified from recombinant bacterial clones (*E. coli* JM109) using the protocol mention by Sambrook (Sambrook et al., 1989).Sequencing reactions were performed using the"ABI PRISM dye terminator cycle sequencing ready reaction" kit and DNA sequences were determined with the 3130 xl genetic analyzer (Applied Biosystems). Sequence analyses were performed using Chromas software (http://www.flu.org.cn/en/download-49.html) and ClustalW algorithm for multiple alignment (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Blast searches (McGinnis and Madden, 2004) at TIGR (http://www.tigr.org/tdb/tgi/plant.shtml) and at the International Nucleotide Sequence (http://www.ncbi.nlm.nih.gov/) databases were performed with programs Blastx with some changing in the default parameters.

RNA extraction, cDNA synthesis and RT-PCR

Total RNA was extracted using conventional modified CTAB method (Al-Obaidi et al., 2010), using CTAB extraction buffer (2% CTAB, 100 mM tris pH 8, 20 mM EDTA,1.4 M NaCl, 0.2 of 2-mercaptoethanol), Chloroform two times washing rather than chloroform :isoamylalcohol (24:1), absolute ethanol precipitation for 4 to 7 days, then nucleic acid precipitation ,70% ethanol washing, draying the pellet followed by dissolving using 20 µl DEPC treated water. Contaminating DNA was removed using Invitrogen amplification grade I Dnase treatment kit according to the provided protocol. High capacity cDNA Reverse Transcription Kit with RNAse inhibitor (Applied Biosystems, USA) cDNA were used for cDNA synthesis. RT–PCR experiments were performed by using Power SYBR® Green PCR Master. Each RT–PCR was performed in a total volume of 12 µl using 100 ng/reaction of total RNA and 50 ng of each of the two primers. Two sets of primers for RT-PCR were designed using Primer Express Software provided with Applies Biosystems 7500 real time are listed in Table 1.

While the expression level (RQ or Ct values) of actin does not vary significantly between the control and treated samples when the same amount of template was used for real time PCR, actin was used as an endogenous control in this experiment (Kok-Ang et al., 2010). The gene-specific primer set (forward, 5'- ctc cac ccg aac gga agt att c -3' and reverse, 5'- ccc ggc aac cct aca tga ctt g -3') for the actin gene was designed from the nucleotide sequence information for oil palm actin cDNA (GenBank accession no. AY550991.1).The specificity of the primers was assessed in separate PCR experiments using, as a template, recombinant plasmid DNA containing the appropriate LTP clone. PCR assays using up to 200 ng of plasmid DNA confirmed that each oligonucleotide pair amplified specifically the correct LTP gene. RT-PCR analysis was performed for RNA samples from uninfected root (control) and infected roots from samples 0, 2, 4, 6 and 8 weeks after infection.

RESULTS

Successfully the EgLTP (oil palm lipid transfer protein gene) amplified from the genomic DNA (1.94 mg/ml and purity 1.87 A260/A280) by PCR. The putative oil palm EgLTP fragments were gel purified and cloned into pGEMT easy vectors in *E. coli* (JM109). Figure 1 shows the result from colony PCR amplification with the putatively cloned EgLTP fragment (~500 bp). Sequence alignment of the deduced amino acid of lipid transfer gene from the putative clone of EgLTP of the cloned fragment using BLAST at NCBI (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) indicated similarities to LTP amino acid sequences of other monocotyledons. The LTP sequences were highly conserved in all the clones (up to 100% identity with rice) and showed very high homology to the sequences of other LTP genes in GenBank (Table 2 and Figure 2).

Pure, high quality RNA obtained using the modified CTAB method as shown in Figure 3 after DNAse digestion was used to produce cDNA, ready for quantitative RT-PCR. Normal PCR used to verify the specificity of the RT-PCR using the cDNA as a template. Amplification of the EgLTP by RT-PCR with primers, LTPrtf and LTPrtr resulted in the expected 120 bp amplicon (Figure 4). The results confirmed that the primers used were effective and the target gene was expressed in plants under the conditions studied as shown by the dissociation curves for amplifications using both primers for actin as well as for the target gene EgLTP (Figure 5). Figure 6 shows the amplification plot for the real time analysis of expression of EgLTP post infection.

DISCUSSION

In order to understand the plant defense mechanism, the characterization and the identification of those genes involved in the interaction with basal stem rot infection

Table 2. Similarity between oil palm LTP gene and other monocot (Actual deduced amino acid alignment showed in Figure 2).

Monocots	Positives	scores	Bits	Gapes	Identities	Homolgy (%)
Oryza sativa	44/44	55.5	132	0/44	44/44	100
Zea diploperennis	35/45	37.0	84	1/45	29/45	64
Sorghum bicolor	37/45	37/45	92	1/45	31/45	68
Zea mays	37/45	37.0	84	1/45	30/45	66

PCR cloning

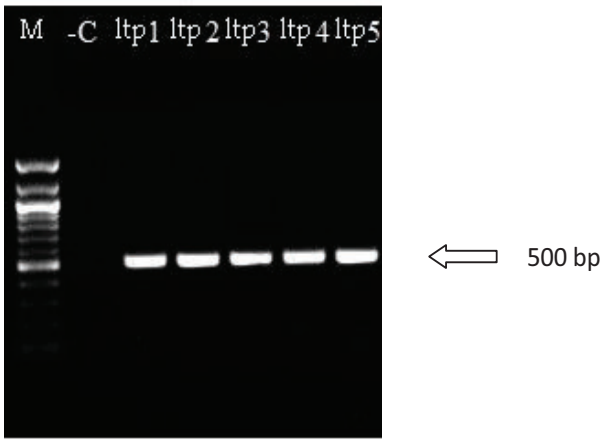

Figure 1. Ethidium bromide-stained 1.0% (w/v) agarose gel of PCR cloning. Positive amplification for lane 1 to 5 shows that the presence of LTP fragment (~500bp) from the DNA samples. –C lane is the negative control (without DNA sample). Lane M: 100bp DNA ladder (Promega).

will be useful (Al-Obaidi et al., 2010), EgLTP successfully partially identified and isolated in oil palm based on the similarity with other monocots. The results was supported by bioinformatics analysis where the EgLTP gene fragment showed interestingly homology with the LTP gene in rice and more than 64% similarity in other monocots. Their expression is regulated during development and in response to several stimuli. The most destructive disease for crop plant is caused by fungal infection (Strange and Scott, 2005). The costs of control are high and approximately 22% of global agrochemicals used in plant production are fungicides and these have a net annual worth of about US$7 billion (Dinham, 2005). In oil palm, the basal and upper stem rot diseases caused by *G. boninense*, and vascular wilt disease caused by *Fusarium oxysporum* f.sp. elaeidis, can cause substantial losses of palms (Durand-Gasselin et al., 2005). Providing the information for development of strategies to prevent the infection of the fungus especially in the early stages of the development of the plant can be only made by the identification of those genes involved in resistance to the *G. boninense* in oil palm roots. Profile of changes in the expression of these genes may give insights into the stage of the infection and the behaviour of the fungus in the root cells, since *G. boninense* rapidly degrades starch, lignin and cellulose and causes extensive breakdown of root cortical cell walls during infection. In this is study, the Q-PCR results showed that the EgLTP level of expression were obviously reduced after infection in weeks time in comparison to the control (Figure 7), these findings in oil palm come with some of the previous reports mention about the down-regulation of the LTP gene after time course microbial infection in wheat (Dinham, 2005), also some previous reports support the idea that root LTP was down-regulated in deferent stress conations in root (Fatemeh et al., 2009).

Nevertheless, many other studies showed that the LTP level of expression will increase and induce after fungal infection. The lipid signal is essential for the activation of plant defence responses, but downstream components of the signalling pathway are still poorly defined. Plant nonspecific LTPs (ns-LTPs) are basic proteins, 9 to 10 kDa in size, that are known for their ability to enhance *in vitro* the inter-membrane exchange and (or) transfer of various polar lipids, such as phospholipids and glycolipids (Dinham, 2005).

Plant lipid transfer proteins (LTPs) are a class of small, soluble and basic proteins which are synthesized as prepeptides with putative signal sequences and are targeted to epidermal cell walls in plants. LTPs suggested to be involved in the secretion and deposition of extracellular lipophilic materials and in transport of cutin monomers required for the biosynthesis of surface wax (Thoma et al., 1994). These proteins were so named because of their ability to stimulate the transfer of a broad range of lipids between membranes *in vitro* (Yamada, 1992). They exhibit broad antimicrobial activity *in vitro* (Garcia-Olmeda et al., 1995) and because of their high isoelectric point, they may act as membrane permeabilizing agents.

LTPs are induced in response to pathogen infection and they are included in the PR-14 family of PR-proteins (Van Loon and Van Strien, 1999). Purified LTPs have

Sequence organization

A

Oryza sativa	82	ITCGQVNSAVGPCLTYARGGAGPSAACCSGVRSLFAAASTTATR	213
		ITCGQVNSAVGPCLTYARGGAGPSAACCSGVRSLFAAASTTATR	
Subject	26	ITCGQVNSAVGPCLTYARGGAGPSAACCSGVRSLFAAASTTATR	69

B

Sorghum bicolor	82	ITCGQVNSAVGPCLTYARGGAGPSAACCSGVRSLFAAASTTA	213
		I CGOV SA PCL Y G PSA CCSGVRSL AA TTA	
Subject	26	ITCGQVNSAVGPCLTYARGGAGPSAACCSGVRSLFAAASTTATR	69

C

Zea diploperennis	82	ITCGQVNSAVGPCLTYARGGAGPSAACCSGVRSLFAAASTTATR	213
		ITCGQV SA PCL Y G PSA CCSGVR LF AAST A R	
Subject	26	ITCGQVNSAVGPCLTYARGGAGPSAACCSGVRSLFAAASTTATR	69

D

Zea mays	82	ITCGQVNSAVGPCLTY-ARGGAGPSAACCSQVRSLFAAASTTATR	213
		ITCGQV SA PCL Y G GPSA CCSGVR L AAST A R	
Subject	30	ITCGQVNSAVGPCLTY-ARGGAGPSAACCSQVRSLFAAASTTATR	69

Figure 2. Amino acid sequence organization and variability of EgPGIP. (A) region show similarity with *Oryza sativa, (*B) show it with *Sorghum Bicolor*, (C) shows with *Zea diploperennis*, and (D) for the similarity between the oil palm and *Zea mays.*

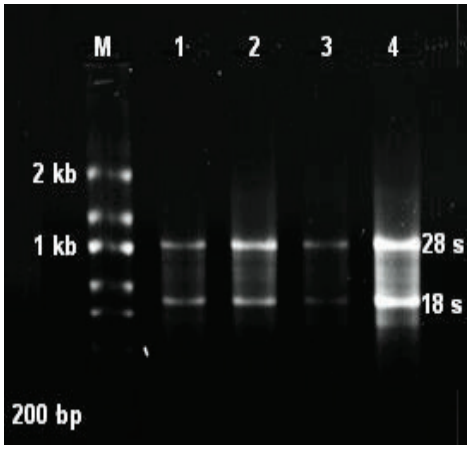

Figure 3. Ethidium bromide-stained 1.0% (w/v) agarose gel of successful RNA extraction after DNase treatment for infected samples by *G. boninense* infection. Well 1-4 (2µ sample+1µ RNA dye each) show the RNA samples 28 and 18 s. Lane M: Low Range RNA marker (Fermentas).

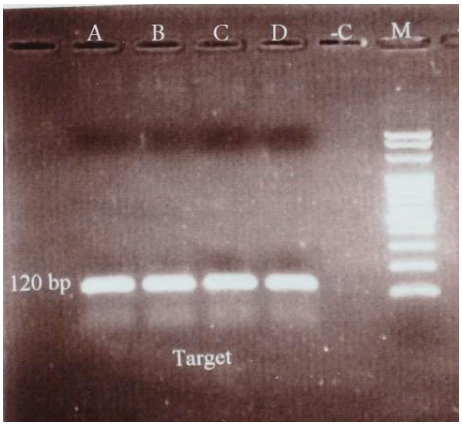

Figure 4. Ethidium bromide-stained 1.0% (w/v) agarose gel of PCR (real time condition), using oil palm cDNA as template and real time specific primers (Pgiprtf and Pgiprtr). Positive amplification for lane 1 to 3 shows the presence of PGIP cDNA fragment (~ 120bp). -C lane is the negative control (without cDNA sample). Lane M: 100 bp DNA ladder (Promega).

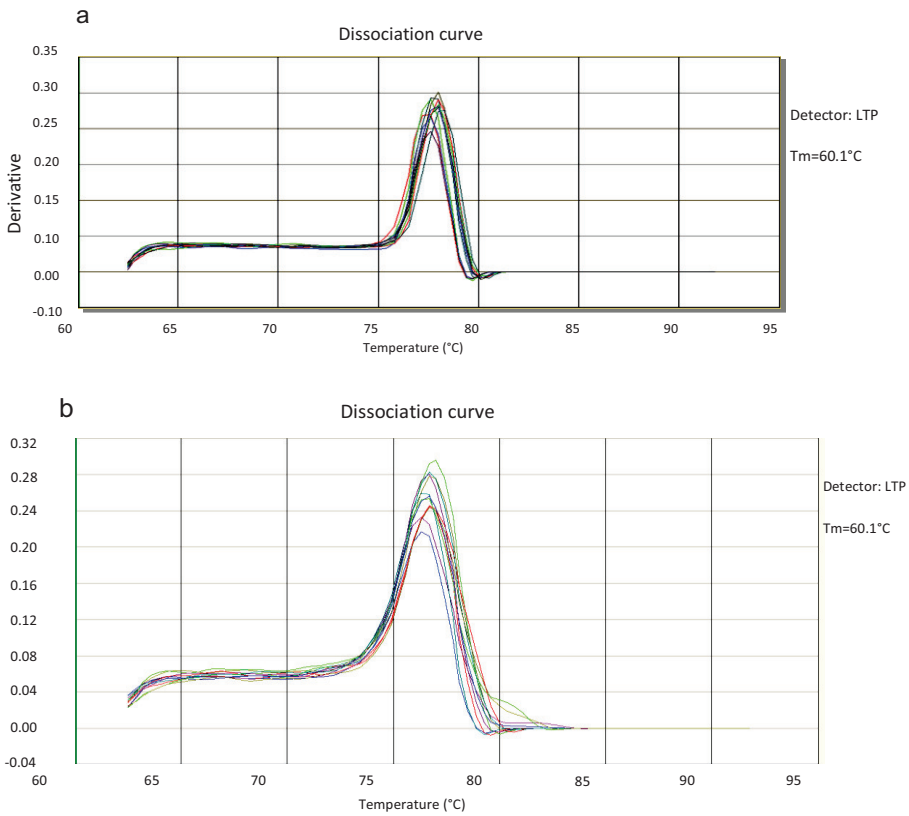

Figure 5. Dissociation curve for (a) target gene (EgLTP), showing the primer efficacy for specific product, amplicon size (120 bp) and (b) endogenous control (*Actin*), showing the primer efficacy for specific product, amplicon size (80 bp). The single peak at 77.5°C for target gene and 77°C for actin gene verify that the primers used were efficient and effective.

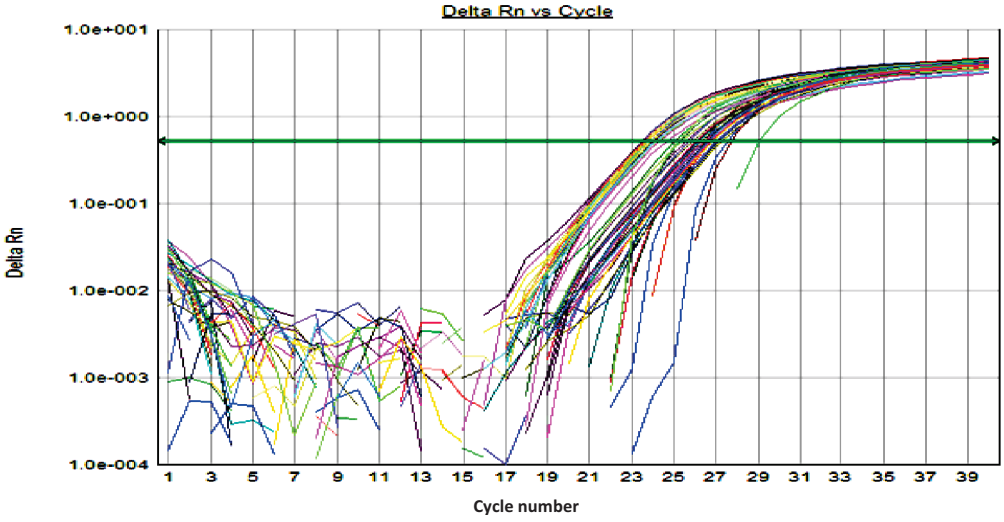

Figure 6. Amplification plot for Real Time-PCR reaction for samples 2, 4, 6 and 8 weeks with its control after *G. boninense* infection showing the fluorescence signal versus cycle number for both target (*EgLTP* gene) and endogenus control (*actin*) using oil palm cDNA as a template.

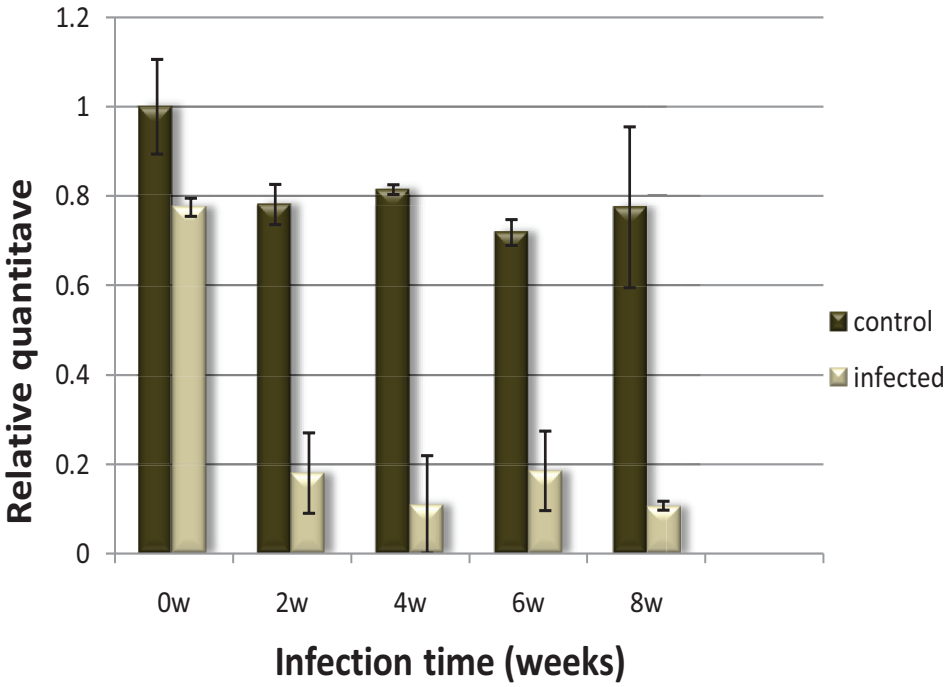

Figure 7. RT-PCR analysis of PGIP gene expression in oil palm inoculated with *Ganoderma boninense*. The size of the amplicon of EgLTP gene transcripts was 120 bp and for the actin gene transcript was 80 bp. This latter was used as the constitutive control. Bars in the graph shows RQ values for expression of the gene at 0,2, 4, 6 and 8 weeks of the infection and the error bar shows the standard deviation of the mean of four biological replicates, all (p Value) for the five infection stages < 0.01(highly significant).

strong antifungal activities although the underlying mechanism of action is still unclear (Ge et al., 2003). One of the suggested mechanisms for LTP antifungal activity that LTP Possibly could form pores when inserted into the fungal cell membrane, allowing low molecular weight compounds, such as nucleotides and coenzymes, to leak but the results show that these activity is inhibited by unknown mechanism and cause LTP reduction on LTP starting from 2 weeks time after inoculation, The LTPs can interact with receptors located on plant plasma membrane that have previously been identified as elicitin receptors (Buhot et al., 2001).

These elicitins induce disease resistance response in some hosts. DIR1, a putative apoplastic lipid transfer protein has been shown to interact with a lipid-derived molecule to promote long distance signalling in defence (Maldonado et al., 2002). Therefore, it was possible that *Ganoderma* affect that receptors and then no elicitin will be produce and that will reduce the LTP transcription level and the infection occur. While the down regulation of the gens that might be responsible for defence against the fungal infection might help the pathogen to escape from host recognition (Schlink, 2010) and this might help the pathogen to complete its life cycle and enter the necrotrophic growth stage which causes extensive root lose, this will make the roots not able to support the water demand of the roots especially considering that the young plant have small rotting system (Flores et al., 2002; Liu et al., 2006). Since LTP antifungal mode of action is not well known yet, though they may insert themselves in fungal membranes and form a pore resulting in an efflux of intracellular ions culminating in cell death, this lead us to conclude that *Ganoderma* might deactivate this function for the EgLTP and survive to be able to cause that sever infection.

Conclusion

EgLTP gene from oil palm was successfully identified. Expression analysis during the *G. boninense* infection of the oil palm was shown to down-regulate EgLTP expression in roots similar to some other plants reports and contrary to many reports of LTP induction after infection. This gene may have the potential to be developed as biomarkers of BSR disease and identification of *G. boninense* tolerant oil palms. It is suggested that, this would involve the selection of oil palm showing up

regulation of the gene during challenge with the fungus. Our results suggest that *G. Boninense* deactivate the LTP antifungal activity in such way that lead to the reduction of LTP expression level. Future studies on oil palm LTP will involve recombinant approaches to help progress in the knowledge of the binding process of lipids on LTP and of the interactions of LTP with membranes. Focusing more on our current and planned research for the protein profile comparison between the BSR infection and the control oil palm, at a longer term, a research on *Ganoderma* mode of action will also be helpful in understanding the fungal mechanisms that lead to BSR infection.

ABBREVIATIONS

BSR, Basal stem rot; **EgLTP**, *Elaies guineensis* Lipid transfer protein; **LTP**, Lipid transfer protein; **CTAB**, cetyl trimethylammonium bromide; **BLAST**, basic local alignment search tool; **EDTA**, ethylenediaminetetraacetic acid; **TE**, tris EDTA; **DEPC**, diethylpyrocarbonate; **PC**R, polymerase chain reaction; **RT-PCR**, real time-PCR; **PVP**, polyvinyl pyrrolidone; **Q-PCR**, Quantitative PCR.

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