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EXPRESSION OF SELECTED GROUPS OF PATHOGENESIS-RELATED (PR) PROTEINS AT EARLY STAGE OF *Ganoderma boninense* INFECTION OF OIL PALM (*Elaeis guineensis* JACQ.) SEEDLINGS

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***Abstract*:** Basal stem rot (BSR) is a major fungal disease of the oil palm (*Elaeis guineensis* Jacq.) caused by the pathogenic fungi of the Ganoderma spp. which degrade plant cell wall components. The information on molecular response of oil palm to these pathogens are still limited, even though it is crucial to develop a better strategy in handling and eliminating BSR. The most prevalent and virulent species associated with BSR is *Ganoderma boninense*. However, poor understanding of the defence response in oil palm towards this necrotrophic fungus has complicated the resolving measures. Early detection of Ganoderma attacks in oil palm where physical symptoms have not yet appeared can offer opportunities to prevent the spread of this necrotrophic fungi. Hence, characterization of defence-related molecular changes and production of anti-fungal agents during early interaction with *G. boninense* is of utmost important. In order to have a better understanding of the defence mechanisms deployed by oil palm against *G. boninense*, we compared the pathogenesis related proteins production of uninoculated with *G. boninense* inoculated oil palm seedlings. Oil palm (*Elaeis guineensis*) seedlings were artificially infected with *G. boninense* inoculums and RNA samples were taken from root tissues at different stages within less than 2 weeks post infection (0, 3, 7, 11 days after infection) and used for RNA-Seq analysis. Differential gene expression (DEGs) analyses displayed induced expression of genes encoding various types of PR proteins including a few classes of PR proteins that have not been previously reported, which can potentially be an important component of the complex defence machinery deployed by oil palm against *G. boninense*.

***Keywords*:** *Elaeis guineensis*, *Ganoderma boninense*, pathogenesis-related protein (PR), differentially-expressed genes (DEGs)

INTRODUCTION

Oil palm (*Elaeis guineensis* Jacq.), a perennial crop that produces oil rich fresh fruit bunches (FFB), is a plantation crop of economic importance. The oil palm industry is an important industry in Indonesia and Malaysia and these two countries are the main palm oil producer that contribute about 85% of the world's palm oil production. In order to obtain a sustainable production of palm oil, breeding and selection of palms with high yield capacity and disease resistance are essential without focusing on expansion of plantation area. Therefore, a better understanding of oil palm disease infection process and host defence mechanisms at the molecular level is essential. Oil palm is vulnerable to fungal diseases, including Fusarium wilt, bud rot caused by Phytophthora, upper stem rot (USR) and basal stem rot (BSR). Among these, BSR is considered to be the most serious and prevalent fungal disease, resulting in huge economic

losses of up to 500 million USD a year (Arif et al., 2011). BSR encompasses decaying of lower stem and root system, which lead to critical symptoms at the foliar part of oil palm such as unopened spear leaves, senescence and yellowing of upper fronds, reduced and "one-sided mottling" of canopy and crown become flattened and blooming of basidiocarps on the bottom stem. In recent years, there have been several reports on transcript sequence which encodes pathogenesis related (PR) proteins such as glucanases, chitinases, protein inhibitors, defensins, and proteins which have potential in oil palm defense mechanism (Ho et al., 2016). However, due to the limited number of transcripts and proteins which are analysed, the data remained partial and inadequate to provide a complete picture of oil palm defense in response to Ganoderma spp. especially *Ganoderma boninense* at the early stages of infection. The main objective of this study was to analyse the expression profile of PR proteins in oil palm roots artificially inoculated with *G. boninense* at early stages of infection. Furthermore, we also compared expression levels of selected PR proteins in all treatments (seedlings inoculated with fully *G. boninense* colonised rubber wood block, seedlings inoculated with empty rubber wood blocks and uninoculated seedlings). A comprehensive understanding of oil palm defence mechanisms will provide more opportunities for developing strategies to eliminate the disease and improving the resistance of oil palm to the disease, considering that it is one of the primary producers of edible oil and oil related products in the world.

METHODS

Experimental Design

The experiment consisted of a single fungus, *Ganoderma boninense* PER 71 each at two levels (uninoculated and inoculated) arranged in a complete randomized design with six replicates per treatment. Untreated plants served as negative controls (absolute control).

Host Plant and Fungal Inoculums Preparation

Four-month old, oil palm seedlings were used for treatment/artificial inoculation in this study. These seedlings (DxP *Elaeis guineensis* Jacq.) was acquired from Sime Darby Research Station Banting, Selangor. The seedlings were planted in inert clay pot/vase and irrigated twice a day. Freshly made potato dextrose agar (PDA, Difco) was added onto a double-autoclaved rubber wood block of 5 x 5 x 5 cm as carrier, placed in heat resistant polypropylene bags and autoclaved at 121 ^{o}C for 30 min, before being cultured with *Ganoderma boninense.* The culture was then incubated at room temperature in darkness for 4 weeks. Uninoculated RBW was used as negative control.

Inoculation of *G. boninense* on Oil Palm Seedlings (Artificial Infection)

Artificial infection of *G. boninense* on oil palm seedlings was carried out. The colonised RWB was put in direct contact with the entire roots of the seedlings and placed in clay pots quarter-filled with soil. Then additional soil was added to fully cover the RWB until reaching the bole of the seedlings. Seedlings that were treated with uninoculated RWB were used as negative control. While untreated seedlings were used as absolute control.

Experimental Layout and Sampling

All seedlings were placed and arranged in complete randomised design in the greenhouse and was watered twice daily using distilled water. All treated seedlings was harvested at 0, 1, 3, 5, 7, 9, 11, and 14 d after treatment, while absolute control was harvested at time 0. The roots and young leaves samples

were flash-frozen in liquid nitrogen and kept at −80 ◦C for gene expression analysis. The infected plants were left to grow until visible symptoms of *G. boninense* infection was identified.

Total RNA Extraction & Determining Quality of Isolated Total RNA via Gel Electrophoresis.
Extraction of total RNA was done from oil palm roots from uninoculated and inoculated as well as the absolute control based on the modified method of Prescott and Martin (1987). Approximately, 0.5 g of frozen root tissues was used.

Determining Quality of Isolated Total RNA Quality and Quantity
The extracted total RNAs were visualized via gel electrophoresis to test the integrity and presence of distinct bands of 18S and 28S ribosomal RNA. However, the presence of minor degradation on the gel was also detected. Apart from that, contamination of genomic DNA can also be identified based on the presence of the high molecular weight fragment. Therefore, it was necessary to eliminate the genomic DNA contaminant because it may disrupt the other analysis later on. The gDNA was eliminated via DNase I treatment and other contaminants eliminated via RNA clean-up. The quality of the RNA was analysed using a bioanalyser before being used for RNA-Seq analysis and determining differentially expressed genes (DEG).

RESULTS AND DISCUSSION

Table 1 provides examples of the expression profiles of the genes encoding PR proteins in *Ganoderma*-inoculated oil palm tissues, as well as their possible functions. It was based on changes in gene expression level that are equal or more than 2-fold within *Ganoderma*-treated oil palm tissues compared to un-inoculated oil palm tissues. The genes that were up-regulated displays the possibility of taking part as a component of plant defence against *Ganoderma* spp. whereas the genes that were down-regulated are believed to be suppressed by the pathogen. The description on these genes and their individual gene expression profiles and references are included in the table.

Pathogenesis-related 1 (PR-1) proteins are ubiquitous defence mechanisms synthesized by plants due to pathogens attack or production of salicylic acid (SA), and also frequently used as markers for systemic acquired resistance. They are synthesized in response to pathogen components by host plant cells and initiate the defense transduction pathways together with other defence proteins. PR-1 may support, directly or indirectly, to the resistance to pathogen infections in plants.

Table 1: A summary of gene expression profiles of individual defence related genes in inoculated oil palm seedlings compared with un-inoculated oil palm seedlings.

Transcripts	**Putative functions**	**Abbreviation**	**Gene expression profiles in inoculated oil palm seedlings compared with un-inoculated oil palm seedlings**
Pathogenesis-related 1 Protein Pathogensis-related protein 1-like	PR-1; antifungal with unknown modes of action, cellular and molecular targets	*EgPRP1-like*	Up-regulated in the roots of inoculated oil palm seedlings at 3 and 7 dpi and; down-regulated in the roots of inoculated oil palm seedlings at 11 dpi

		EgPRP1 *EgPRB1-2-like*	Up-regulated in the roots of inoculated oil palm seedlings at 3 and 7 dpi and; down-regulated at 11 dpi Up-regulated in the roots of inoculated oil palm seedlings
Glucanases, β-D-glucan *exohydrolase, Glucan endo-1,3-glucosidase*	PR-2; degrade fungal cell wall component by hydrolyzing β-1,3-glucosidic linkages and promote the release of cell-wall derived fungal elicitors	*EgGlc12-like* *EgGlc3-X1* *EgGlc3-X2* *EgGlc6* *EgGlc3*	Up-regulated in the roots of inoculated oil palm seedlings at 3, and 7 dpi; and down-regulated at 11 dpi Up-regulated in the roots of inoculated oil palm seedlings at 3 dpi, and down-regulated at 7 dpi Up-regulated in the roots of inoculated oil palm seedlings at 3 dpi, and down-regulated at 7 dpi Down-regulated in the roots of inoculated oil palm seedlings at 3 dpi and up-regulated at 11 dpi Up-regulated only at 11 dpi in the roots of inoculated oil palm seedlings
Chitinases/chitinase-like Class I chitinase Class II chitinase Class III chitinase Class V chitinase	PR-3, PR-4, PR-8 and PR-11; cleave the β-1,4-glycosidic linkages between N-acetylglucosamine residues in fungal chitin to chitin oligosaccharides	*EgCHI1* *EgCHI1-l* *EgCHI1-l X3* *EgCHI1B* *EgCHI2* *EgChit3-1* *EgChit4* *EgPRP4-l*	Up-regulated in the roots of inoculated oil palm seedlings at 3 dpi and suppressed at 7 dpi Up-regulated in the roots of inoculated oil palm seedlings at 3 dpi; suppressed at 7 and 11 dpi Up-regulated in the roots of inoculated oil palm seedlings at 3 dpi; suppressed at 7 dpi Up-regulated in the roots at 5 dpi and suppressed at 7 and 11 dpi Up-regulated in the roots of inoculated oil palm seedlings at 11 dpi Up-regulated in the roots of inoculated oil palm seedlings at 3 dpi

		EgOSM-l	and down-regulated in the roots of inoculated oil palm seedlings at 7 and 11 dpi Up-regulated in the roots of inoculated oil palm seedlings at 3 dpi, downregulated in the roots at 7 dpi and 11 dpi Up-regulated in the roots of inoculated oil palm seedlings at 3 dpi, down-regulated at 7 and 11 dpi Down-regulated in the roots of inoculated oil palm seedlings at 3 dpi, and up-regulated at 7 and 11 dpi
Lipid Transfer Protein	LTPs; bind lipids and induce permeability changes in membranes of fungal pathogens	*NsLTP-GPI2* *NsLTP-GPI-2L*	Up-regulated in the roots at 7 dpi and increases again at 11 dpi Up-regulated in the roots at 0 dpi, then down-regulated at 3 and 7 dpi, up-regulated again at 11 dpi.

The components of fungal cell wall or cell membrane such as chitin, glucans and ergosterol have been reported as pathogen-associated molecular patterns (PAMPs) in many pathosystems. It was revealed that various oil palm chitinases and glucanases were indeed up-regulated in oil palm roots in response to Ganoderma-treatment and may play role in degrading the fungal cell wall resulting in the release of PAMPs (Ho *et al.,* 2016).

Based on previous studies, plant non-specific lipid transfer proteins (NsLTPs) are ubiquitous and low molecular mass active proteins that are essential for plant cytology, such as cell wall organization, stabilization of membranes and signal transduction as well as playing vital roles in plants for catalysing the transport of phospholipids, glycolipids, fatty acids and steroids between cell membranes and more importantly in resistance to biotic and biotic stress and plant growth and development (Chen et al., 2016; F. Liu et al., 2015). One of the putative key defence mechanism reported in the Fusarium head blight-resistant cultivar was based on several defence genes encoding LTPs and other specific PR proteins which are probably induced by ethylene and jasmonate signalling (Gottwald et al., 2012).

Several *NsLTPs* are disclosed in plants, *DIR1* encoded a NsLTP in *Arabidopsis*, which played a vital role in plant defense against pathogens. *NsLTPs* are sensitive to many plant hormones, including SA, methyl jasmonate (MeJA) and abscisic acid (ABA). *NsLTP* was slightly down-regulated in response to exogenous application of SA, greatly up-regulated by MeJA at 24 h which are the final stage of stress, while down-regulation were detected at initial stage which are 6 and 12 h (Chen et al., 2016).

CONCLUSIONS

In conclusion, this study reports on several classes of PR proteins expressed at the early stages of Ganoderma-oil palm interaction. Detection of early defense response mechanism may provide valuable markers for monitoring infected palms for early measures to protect the palms for prolonging their productivity period which is critical for the sustainability of the oil palm industry which is experiencing major yield losses due to BSR.

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