Differential expression of root specific genes of oil palm seedlings at early stage of Ganoderma boninense infection

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Abstract. The Ganoderma boninense is the pathogen causing basal stem rot (BSR) disease in oil palm, degrades oil palm root cell walls and modify plant metabolisms. Research groups have studied molecular mechanisms responses to G. boninense infections. A few have indicated the importance of studying specific gene expressions in relation to resistance mechanisms. This study aims to identify differentially expressed genes in oil palm roots showing susceptible or resistance responses and visualize their accumulated transcript in the heatmap form. The susceptible and resistant seedlings showing symptoms of BSR infection and healthy ones were sampled. Sixteen genes associated with G. boninense infection were identified and their expression were analyzed. Total RNAs were extracted from root samples and cDNA synthesized. Quantitative real time PCR were performed using cDNA, and differential gene expressions were evaluated. Two genes showed the highest transcript accumulation in susceptible and resistant seedlings treated with or without G. boninense. Ten out of the 16 genes belonged to Group I and the other six to Group II. Seven out of the 16 genes were differentially expressed in the samples either with or without G. boninense inoculation. The seven genes may be associated with early infection responses to G. boninense.

Keywords: DEGs, basal stem rot, resistance mechanisms, qPCR, heatmap analysis.

1. Introduction

Infections of the basal stem rot disease both directly and indirectly will affect some responses of genes associated with plant resistance mechanism systems. [1] observed there was an increased of 17.96-fold change of the Early Methionine-labeled Polypeptide (EgEMLP1) gene in G. boninense inoculated oil palm ramets during the period of 3 – 7 weeks after inoculation (WAI). This fold change indicated EgEMLP1 gene is an up-regulated gene associated with G. boninense infection in oil palm. [2] states there are four Ganoderma resistance loci identified in 14 full-sib families derived from nine parent populations (five Dura Deli and four La Mé-Yangambi populations). The two loci were
associated with responses at the first observed symptom (T1S) and the other two loci were associated with time of death because of infection of the fungi (TD). The T1S and TD associated loci were not linked and they were segregated independently [2].

Ganoderma infection resulted in cell wall degradation [3]. Tan et al. [4] have identified the presence of 33 laccase genes based on de novo assembly of G. boninense genome. The laccase genes have four protein domains, including cysteine and histidine residues and seven out of the 33 identified genes were like the known expressed transcripts [5]. However, [4] and [5] did not mention the name of the identified genes. [6] stated based on differentially expressed gene (DEG) identification that the diverse genes associated with defence against disease infection (such as: EgPR-1 [PR-protein], EgBGIA [protease inhibitors], EgLYK3 [PRR protein], EgCht [chitinase] and EgEXPB18 [expansin] genes) were increased their expression at 3 and 7 days after inoculation (DAI) and decreased at 11 DAI. In contrast, transcription factors [EgERF113 and EgMYC2] expression were dramatically increased at 11 DAI. The two transcription factors have the potential to regulate plant defence systems. Moreover, [6] also indicated expression of genes eliciting ROS (reactive oxygen species), such as EgPER (peroxidase) and EgRBOH (NADPH oxidase) were also increased during the treatment periods.

Based on results of the previous studies, it is very important to elucidate the gene expression during infection of G. boninense in oil palm and evaluate their possible role in resistance mechanisms. However, identity of genes associated with the onset of early internal symptoms and late symptoms in oil palm seedling nurseries is not yet fully known. Therefore, this study aims to identify differentially expressed oil palm root genes from seedlings showing susceptible or resistance responses to basal stem rot and visualize their accumulated transcript expression levels in the heatmap form.

2. Materials and methods

2.1. Plant Materials

The oil palm materials evaluated consisted of seedlings from two crosses of Dumpy-derived material from IOPRI (Indonesian Oil Palm Research Institute), North Sumatera, Indonesia and one cross of Yangambi population of commercial variety (Table 1). The seedlings were treated with and without G. boninense. Sampling of root tissues were carried out at early internal symptoms expression of G. boninense infection during the five and six months after inoculation (Figure 1).

Table 1. Genetic materials used to differential expression of root specific genes of oil palm at early Ganoderma boninense infection stage.

| Sample number | Treatments     | Response to G. boninense | Crossing number | Genetic materials |
|---------------|----------------|--------------------------|-----------------|-------------------|
| 03            | Inoculation    | Susceptible              | 01              | DxP Dumpy         |
| 55            | Inoculation    | Resistant                | 07              | DxP Yangambi      |
| 07            | Non-inoculation| Susceptible              | 01              | DxP Dumpy         |
| 31            | Non-inoculation| Resistant                | 03              | DxP Dumpy         |

The G. boninense isolate SSU008 from collection of the Plant Protection Laboratory of IOPRI, having high virulence character to oil palm was used as inoculum. In the PCR processes, the RNAs isolated from G. lucidum were used as the Ganoderma origin and as positive control for comparison to the results of oil palm RNA isolation and analysis. In addition, amplification of specific primer namely β-Actin [7] for reference gene, Gan1-2 [8][9] for Ganoderma species and Gan-b for G. boninense.
2.2. Sample preparation and total RNA extraction

A total of 50 ng fresh oil palm roots was cut into small pieces and put into the Tissue Lyser II homogenized the tissue and simplify the RNA isolation processes. Total RNAs were extracted from either the infected root or healthy root samples following the procedure as described in the Qiagen RNeasy® Plant Mini Kit. Into the maximum of 100 mg homogenized tissues, 450 µl of the RLT buffer or RLC buffer was added. The mixtures were vortexed vigorously and the lysate was transferred onto the QIA shredder spin column (pink), placed in 2 ml of the collection tube and centrifuged at full speed for 2 minutes. The supernatant was transferred from a flow-through to the new microcentrifuge tube without contacting the pellets. As much as 0.5 vol. of ethanol (96%) are added to clean the lysate and mix at once using a pipette. The sample (ca. 50 µl) was transferred, with various precipitation to RNeasy Mini spin column (pink) in a 2 ml collection tube, centrifuged for 15 seconds at >8000 x g, and disposed of the flow-through. The RW1 buffer (700 µl) was added to RNeasy spin column, centrifuged for 15 seconds at >8000 x g, and discarded the flow-through. The next step is to add 500 µl of RPE buffer to the RNeasy spin column, centrifuged for 15 seconds at >8000 x g, and discard flow-through. Add 500 µl of RPE buffer to the RNeasy spin column and centrifuged for 2 minutes at >8000 x g. Finally, the dried membrane was centrifuged at full speed for 1 minute, the RNeasy spin column is placed in 1.5 ml collection tube, and 30-50 µl RNase-free water added at once. The RNAs were eluted from the RNeasy spin column by centrifugation for 1 minute at >8000 x g. If the expected RNA results are more than 30 µg, the last step was repeated once using 30-50 µl of RNase-free water. The collected RNAs were stored and ready for later processes.

2.3. Total RNA quality and quantity

The quality and the quantity of isolated total RNAs were checked using a 1% agarose gel. The concentration was measured using Nanodrop. Subsequently, the total RNAs were purified using the [10] method. RNase free water was added into the isolated RNA samples up to 100 µL and into each 100 µL sample was added 600 µL of RA1-ethanol-premix buffer (300 µL RA1 + 300 µL ethanol 96-100%). The 700 µL bind of RNA was added into the collection tube and centrifuged at 800 x g for 30 seconds. The lower collection tube was removed and replaced with a new one. RNA washing was conducted twice, first by adding 700 µL RA3 and centrifuging at 8000 x g for 30 seconds. After removing the liquid in the collection tube removed, and the collection tube can be used again. The second by adding 350 µL RA3 and centrifuging at 8000 x g for 2 minutes. To collect the pure RNA, 30 µL of RNase free water was added to bin of RNA, centrifuged 8000 x g for 1 minute and liquid
descended into the 1.5 mL tube was purified RNA. The final RNA concentration was measured using Nanodrop (Thermo Scientific).

2.4. DNase treatment and cDNA synthesis

The DNase treatment procedures followed the methods as described by [11]. Into a 1.5 ml tube was added 8 µL pure RNA, 1 µL 10x Reaction Buffer, and 1 µL DNase I. For samples with RNA concentrations of less than 100 ng/µL, it needed two tube reactions. The mixtures were incubated at room temperature for 15 minutes, 1 µL Stop Solution was added and the mixtures were heated at 70°C for 10 minutes. After heating, the mixtures were cooled at once into a bucket containing ice water. After DNase treatment, the RNA concentration was again measured using Nanodrop.

Based on the determined RNA stock concentration after DNase treatment, 1000 ng RNAs were pipetted out from the stock and transferred into a tube having Oligo(dT)20 primer kit [12]. These kits used as a primer for first-strand cDNA synthesis with reverse transcriptase. A conventional PCR amplification was carried out as follow: one cycle of pre-denaturation stage at 37 °C for 30 seconds, followed by 12 cycles of denaturation at 48 °C for 4 minutes, annealing at 55°C for 30 seconds, 95°C extension amplification for 5 minutes, and 10°C long extension with 12 cycles. Finally, the concentration of the PCR products was measured using Nanodrop.

2.5. Real-Time PCR (RT PCR)

To validate whether the synthesized cDNA in previous step (step 2.2) was from oil palm RNAs, specific primers were used to PCR amplify Actin gene of oil palm and they were used for internal reference gene for early cDNA quality analysis. The PCR validation for cDNA quality and reference gene followed procedures developed by [7]. The PCR mix consisted of 5 µL PCR Ready mix, 0.4 µL each of forward and reverse primers, 4.2 µL NFW buffer, and 1 µL (50 ng) cDNA. The PCR amplification steps were one cycle of pre-denaturation at 95°C for 1 minute, followed by 35 cycles of denaturation at 95 °C for 15 seconds, primer annealing at 54 °C for 15 seconds, primer extension at 72 °C for 10 seconds, one cycle of final extension at 72 °C for 1 minute, and cooling at 10 °C.

Before cDNA samples was used for quantitative real-time PCR analysis, serial dilution (100, 50, 20, and 10 ng/µL) was conducted to determine the optimum cDNA template concentrations. The quantitative real-time PCR analysis was conducted using StepOne Plus Real Time PCR machine (Applied Biosystem). The quantitative real-time PCR mix consisted of 5 µL SYBER Green, 0.625 µL each forward and reverse primers, 3.75 µL NFW buffer, and 1 µL cDNA. The mixtures were briefly centrifuged to remove bubbles which negatively affect the quantitative real-time PCR detection. In additional, an NTC (non-template control) was also used as the primers negative control. To find out the level of primers effectiveness used and efficient analytical techniques, the results of RT-qPCR amplification can be determined by the value of the melt curve observed, the peak pattern of the graph appears and both of mean value of the threshold cycle ($C_T$) in the targeted and reference genes.

2.6. Differentially expressed genes analysis in resistant and susceptible oil palm seedlings

The relative quantity of gene expression was evaluated by real time PCR with a comparative threshold method and calculated Actin gene as internal reference [7]. The relative transcript abundance for each gene is quantified by normalization to the Actin gene expression. The mean value of the threshold cycle ($C_T$) was the relative expression value for all genes obtained by the method $2^{\Delta\Delta C_T}$ [13][14]. The $C_T$ is number of amplification cycles when it reaches a certain threshold value in real time PCR, while the $\Delta C_T$ is the $C_T$ values differences between Actin gene and the targets. Based on the mean of $2^{\Delta\Delta C_T}$ method of quantitative data were obtained fold change value from mRNA expression. The fold change value from the targeted genes and the reference gene were analyzed by comparing ratios and differential expressions of specific genes in the tested sample [15]. Final data of
the accumulated transcripts were visualized as heatmap clusters and ratios with modification categories [15][16][17] (Figure 2).

| E+03 | E+02 | E+01 | E+00 | E-01 | E-02 | E-03 | n ≤ E-04 |
|------|------|------|------|------|------|------|---------|
| 1 > n > 5 | 1 < n < 5 | ND | 0.2 < n < 1 | n ≤ 0.2 |

**Figure 2.** Heatmap ratio and evaluated gene differential expression classifications [15][16] with modification.

### 3. Results and Discussion

#### 3.1 Amplification of cDNA

Results of the PCR amplification using using the β-Actin, Gan1-2 and Gan-b primer pairs showed that the synthesized cDNA was good, the extracted RNA was from the tested oil palm RNAs, and there is no RNA from *Ganoderma* sp. The presence of amplicon in the PCR amplification using Actin gene specific primers (Figure 3.A) showed that the RNA quality used to synthesize cDNA was good and resulted in good quality cDNA. The Actin gene specific primers amplify exon1-exon2 regions of the Actin cDNA (300 bp). PCR amplification using either Gan1-2 or Gan-b primers and the oil palm cDNA did not yield any amplicon. On the other hand, positive results were obtained on the PCR amplification using either Gan1-2 or Gan-b primers and the *Ganoderma* sp. cDNA. PCR amplified product of the Gan1-2 primers was a 200 bp amplicon (Figure 3.B), while that of Gan-b was a 2000 bp (Figure 3.C). The absence of PCR amplified product when using either the Gan1-2 or the Gan-b primers proved there is no contaminating *Ganoderma* sp. associated RNAs in the tested cDNA samples. Therefore, the isolated cDNAs can be used for differential expression analysis of the genes using quantitative real time PCR.

**Figure 3.** Visualization of PCR amplification to detect the cDNA quality using three primer pairs (A) oil palm β-Actin gene specific primers, (B) Gan 1-2 primers and (C) Gan-b primers. The cDNA was isolated from roots of susceptible and resistant accession, inoculated with and without *Ganoderma* sp. (SI); resistant accession, inoculated with *Ganoderma* sp. (RI); susceptible accession, without inoculation (SNI); and resistant accession, without inoculation (RNI). C (+) = cDNA from *Ganoderma* sp.; C (-) = No template control. M = 1 kb DNA size control.
3.2. Melt curve analysis

Based on results of the melt curve analysis, the Actin reference and the 16 target genes resulted in one peak with a range of temperature between 79.6 °C to 88.7 °C and a mean of 84.1 °C (Figure 4). The one peak melt curve indicated only one amplicon was obtained in the quantitative PCR analysis (Figure 5). However, target gene no. 4 and no. 11 resulted in two or more peaks. These were probably because of G/C ratio differences in the target sequences and were not because of a non-specific amplification [18].

![Melt curve of targeted genes (°C)](image)

**Figure 4.** Melt curve pattern of 16 targeted genes analysis and Actin as reference gene.

![Melt curve analysis](image)

**Figure 5.** Results of melt curve analysis from quantitative PCR of the internal reference Actin gene and the 16 target genes associated with G. boninense infection.

3.3. Targeted gene expression analysis

Accumulated transcripts of the 16 genes were probably associated with the resistance response of tested oil palm to G. boninense. Results of the analysis for the susceptible and resistant of oil palm seedlings were presented in Table 2. Either inoculated or non-inoculated susceptible seedlings showed different transcript accumulations for the 12 genes (Gene #1, #2, #4, #5, #6, #7, #8, #11, #12, #13, #15, and #16). The other four genes accumulate the same amount of transcript in the two treatments evaluated.

Either inoculated or non-inoculated resistant seedlings showed different transcript accumulations for the 8 genes (Gene #1, #2, #3, #5, #6, #10, #11, and #14). Among the eight others differentially expressed genes in the resistant seedlings, the gene #4 and #11 showed the highest expression level. The more susceptible oil palm seedlings, the more number of genes accumulating the highest transcripts (7 out of the 16 genes).
Table 2. Transcript accumulation in the susceptible and resistant oil palm seedlings either inoculated with or without *Ganoderma boninense*.

| Targeted genes | Susceptible | Resistant |
|----------------|-------------|-----------|
| Inoculation treatment | With | Without | With | Without |
| SI | | | RI | |
| SNI | | | RNI | |

Five (gene #1, #4, #7, #11, and #12) out of 16 genes in inoculated - susceptible oil palm seedlings (SI) showed higher fold change than that of without inoculation treatment (Table 2). On the hand, the resistant oil palm seedlings without inoculation treatment showed more genes having higher fold change values than that of with inoculation treatment (Table 2). For example, the gene #1 in inoculated susceptible seedlings shown up-regulated expression higher than un-inoculated resistant seedlings (Table 2). Likewise, in gene #6 also represented up-regulated gene expression, except un-inoculated resistant seedlings. However, genes of #4, #7, and #11 have consistency level of up-regulated gene expression in both resistant and susceptible seedlings even though with various categories (Table 2).

This expression phenomenon was also reported by Putranto et al. [15], which analyzed the gene expression in oil palm roots and leaves of susceptible and resistant seedling. Putranto et al [15] have showed various trends in transcripts accumulation of the evaluated genes. Fourteen out of 21 genes analysed were differentially expressed both in root and leaf tissue [15]. On the other hand, 3 out of 14 differentially expressed genes showed very strong expressions in specific conditions. One gene showed a high transcript accumulation in roots and leaves, other genes showed leaf specific, and the others showed roots specific expressions (15).

The interesting results from this study there were 2 genes (gene #4 and #11) that have a very high accumulation of transcripts in susceptible seedlings. These results were also reported by [15] which mentions the *EgEMLP-1* gene shown very high gene expression in roots in the Yangambi population. According to [15] the gene has a very important role in secondary metabolism at the basal of oil palm roots.

3.4. Gene expression heatmap
The differences in gene expression between susceptible with or without inoculation treatment are more striking when presented in the form of heatmap display (Figure 6). Most of the genes in the inoculated - susceptible oil palm seedling having a Z-score of 1.0 were exactly one standard deviation above the mean (Figure 6.A). This was supported by Figure 6.B which showed different expression of the two genes in the form of highest histogram fold change values, 911.56 (11) for resistant and 1398.46 (4) for susceptible oil palm seedlings, respectively.
On the other hand, controlled susceptibility traits. It means these are not the target genes associated with oil palm resistance responses to BSR disease. High expression in susceptible oil palm seedlings and resistant accession, with inoculation with and without G. boninense. (SI); susceptible accession, without inoculation (SNI); and resistant accession, inoculated with G. boninense. (RI); susceptible accession, without inoculation (RNI).

Based on the fold change values of transcript accumulation, the evaluated genes were divided into two groups (Figure 6.B). The target gene #4 and #11 were both on the same group (Group I), while the other 14 genes belonged to the second group (Group II). These group differences were associated with the high values of fold changes in the inoculated susceptible oil palm seedlings. High expression in four target genes (genes #1, #4, #11, and #12 based on Figure 5.A and 5.B) at early symptoms of G. boninense represented the responses of oil palm against infection of basal stem rot disease.

3.5. Differentially expressed gene ratios
Most of the tested genes showed high expression ratios in susceptible oil palm seedlings, except for target gene #6 and #15 (Figure 7). However, some of the target genes showed interesting gene expression ratios. They were divided into three groups, such as: (1) target genes having a high expression ratios in non-inoculated resistant oil palm seedlings, but high in susceptible inoculated palm (target gene #5, #13, and #16), (2) target genes having a high expression in resistant oil palm seedlings (target gene #6 and #15), and (3) target genes having high expression ratios in susceptible oil palm seedlings (target gene #1, #9, #12, and #14), respectively.

The up-regulated expression ratio in susceptible plants showed the genes are associated with the level of susceptibility to G. boninense infection (Figure 7). Therefore, those genes may play an active role in the early internal symptoms. On the other hand, down-regulated genes in resistant plants are shown in target genes #6 and #15 (Figure 7). However, target genes #5, #13 and #16 were up-regulated in inoculated susceptible oil palm seedlings and in resistant oil palm seedlings without inoculation (Figure 7).

Based on the trend of differential expression of genes (Table 2, Figure 6) and the ratio of the 16 targeted genes (Figure 7), it is assumed that there are 2 key genes (genes #4 and #11) and 4 other candidate genes (genes #1, #6, #7, and #12) capable of controlling susceptibility traits of oil palm to G. boninense. There were characterized by genes consistently high up-regulated fold change values in inoculated susceptible seedling compared to other treatments. If studied in more detail, different crosses samples in resistant seedlings with different treatments (inoculation and uninoculated) evidenced relatively fold change values in the genes tested, despite having up-regulated gene expression of ratios. In addition, these genes have a much lower value than the 6 genes have been controlled susceptibility traits. It means these are not the target genes associated with oil palm resistance responses to BSR disease.

![Figure 6](image-url)
Table 7. Ratio of differential expression of 16 genes associated with *Ganoderma boninense* infection at early internal symptoms in the oil palm nursery.

The results of this study are important for the molecular marker development for early detection of oil palm seedlings responses to BSR disease infection, especially for selecting out susceptible seedlings. Although the remaining seedlings that are not selected out by the markers may not be BSR resistance, the use of molecular markers at least reduce the number of susceptible oil palm seedlings be deployed to the field.

4. Conclusion

The 16 genes in oil palm roots probably important in susceptibility responses to *G. boninense* infection. There are two groups of genes based on their expression level, Group I consisted of target gene #4 and #11 while Group II consisted of 14 target genes. Based on the expression ratio in susceptible and resistant oil palm seedlings, the target genes were grouped into three categories, such as (1) genes having high expression ratios in non-inoculated resistant oil palm seedlings, but high in inoculated susceptible one (target gene #5, #13, and #16), (2) genes having high expression in resistant oil palm seedlings (target gene #6 and #15), and (3) genes having high expression ratios in susceptible palm (target gene #1, #9, #12, and #14).

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