Indonesian Journal of Biotechnology

VOLUME 29(3), 2024, 129-138 | RESEARCH ARTICLE

Genes expression analysis of *EgUnk1*, *EgZFP2*, and *EgIPK2b* in oil palm using Ct value correction and two relative quantification approaches

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SUBMITTED 3 January 2024 **REVISED** 25 March 2024 **ACCEPTED** 28 April 2024

ABSTRACT The determination of transcript accumulation values significantly affects gene expression in oil palm. Various genes are involved in pathogen infection, including probable 2-oxoglutarate-dependent dioxygenase *At5g05600* (*EgUnk3*), zinc finger protein 2-like (*EgZFP2*), and inositol polyphosphate multikinase beta-like (*EgIPK2b*). Gene expression is typically measured using relative quantitative methods to calculate differences in quantitative values in the expression levels of targeted genes compared to a reference gene. However, the effectiveness of these methods in assessing the expression of *EgUnk3*, *EgZFP2*, and *EgIPK2b*, which are involved in *Ganoderma boninense* infection in oil palm seedlings, requires evaluation. This study aimed to establish an effective and straightforward method for analyzing the expression of *EgUnk1*, *EgZFP2*, and *EgIPK2b* genes in oil palm seedlings infected with *G. boninense*, utilizing Ct value correction through regression coefficients on the $2^{-\Delta\Delta Ct}$ and $E^{-\Delta\Delta Ct}$ approaches. A correlation regression revealed values of 0.28, -0.32, and 0.29 for delta Ct of *EgUnk1*, *EgZFP2*, and *EgIPK2b*, respectively. However, a negative correlation in the Ct mean was corrected by linear regression for the targeted genes: -0.55, -0.81, and -0.29 for *EgUnk1*, *EgZFP2*, and *EgIPK2b*, respectively. The amplification factor (E) and efficiency value (R) using the *EgActin* gene were 1.95 and 94.92%, respectively. Normalization of log10 on the fold change value $2^{-\Delta\Delta Ct}$ and 1.95-$\Delta\Delta Ct$ approaches using the regression coefficient yielded consistent results for the *EgUnk1*, *EgZFP2*, and *EgIPK2b* genes. Overall, *EgUnk3* and *EgIPK2b* genes exhibited downregulated expression in susceptible oil palm seedlings (-0.60 for $2^{(-\Delta\Delta Ct)}$ and -0.58 for $1.95^{(-\Delta\Delta Ct)}$), whereas *EgIPK2b* gene showed up-regulated and the highest value in inoculated resistant seedlings (1.39 for $2^{(-\Delta\Delta Ct)}$ and 1.34 for $1.95^{(-\Delta\Delta Ct)}$). Basal stem rot disease (BSR) in oil palm decreased *EgUnk1* and *EgIPK2b* expression in susceptible seedlings but increased *EgZFP2* gene expression in resistant ones. The results of this research provide valuable corrections to Ct values obtained directly from RT-qPCR machines using simple linear regression. Consequently, the Ct values of target genes and reference genes exhibit smaller bias values, rendering gene expression levels more reliable.

KEYWORDS *Ganoderma boninense*; Livak method; Pfaffl method; Regression method; Relative expression gene; RT-qPCR

1. Introduction

Several factors can contribute to bias in Ct values, including samples quality, sampling methods, the RNA samples extraction process, and experimental conditions (Sanders et al. 2014). These aspects may possibly will bias and affect the interpretation of results and data analysis of RT-qPCR. The cycle threshold (Ct) value obtained from the Real-Time (RT) quantitative Polymerase Chain Reaction (RT-qPCR) machine is often directly used as primary experimental data in gene expression analysis. This is considered data that has high validity and accuracy since obtained from a machine. However, by using simple regression analysis, a regression coefficient can be found from the delta Ct (ΔCt) value of the targeted genes to reduce bias in the raw results from the machine (Cui et al. 2015). This coefficient value is a reference for determining the level of bias of the Ct value (Cui et al. 2015). After the bias value has been corrected, the Ct value of the target gene can be used for further analysis calculations. With corrected primary data, the results of the expression levels of target genes involved in basal stem rot (BSR) disease in oil palm are more accurate and have less bias. Previous study, there are several reports on PCR-based method in

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oil palm (Josephin et al. 2024; Polosoro et al. 2024).

RT-qPCR technology has recently for plants disease detection (Chow et al. 2019; Paul et al. 2020). The RT-qPCR represents the most powerful technology to amplify and detect accumulated mRNA transcription (Kralik and Ricchi 2017). The RT-qPCR advantages include low mRNA concentration, limited tissue samples, high sensitivity, high specificity, good reproducibility, and a wide dynamic quantification range (Ma et al. 2021). The analysis method for the expression level of the RT-qPCR results has developed into two methods, such as absolute quantification and relative quantification (Boulter et al. 2016; Sun et al. 2014). The similarity of the methods used the genes as internal control, such as *Cyp2*, *GRAS*, *PD00380*, *PD00569*, *ACTIN*, *GAPDH*, and *GvHK* genes in the oil palm planting material (Chan et al. 2014; Zuhar et al. 2021). These genes were identified as the most stably expressed genes across all samples (Chan et al. 2014), with the *ACTIN* gene recognized as the best reference gene for oil palm seedlings (Zuhar et al. 2019). The relative quantification method is used to analyse the transcription of the target gene transcription with the relative value of the reference (Regier and Frey 2010).

Two relatively quantification approaches are widely used to obtain the value of the fold change are the $2^{-\Delta\Delta Ct}$ of the Ct Mean (Livak and Schmittgen 2001; Schmittgen and Livak 2008) dan kinetic PCR efficiency correction or Pfaffl method $E^{-\Delta\Delta Ct}$ averages from Ct Mean (Pfaffl 2004, 2007). The advantages of the $2^{-\Delta\Delta Ct}$ of the Ct Mean approach based on the assumption that transcript amplification of mRNA efficiencies are 100% (Adamski et al. 2014). However, second approach with kinetic PCR efficiency has an accurate knowledge of its concentration of cDNA templates (Svec et al. 2015). Approaches used in determine transcript accumulation will affect gene expression analysis results.

The Unk3 involved in glucosinolates biosynthesis in *Arabidopsis thaliana* (Kakizaki et al. 2017). Glucosinolates are a secondary metabolites whose involved plant disease responses (Sánchez-Pujante et al. 2017). *ZFPs* gene involved in regulators of biotic and abiotic stress signalling in several plants (Han et al. 2020; Wu et al. 2022; Zhang et al. 2016). *IPK2b* gene involved in transcription factor in biological process, such as seed germination and seedling development (Yang et al. 2017). There is limited information regarding the role of these genes in oil palm's response to *G. boninense* infection. Therefore, the expression level of *EgUnk3*, *EgZFP2*, and *EgIPK2b* genes involved in the *G. boninense* infection process is need to be tested through relative quantitative approaches. The aim of this study was to establish an effective and easy calculation method for the analysis of *EgUnk3*, *EgZFP2*, and *EgIPK2b* gene expression based on the Ct value correction using regression coefficients and on the $2^{-\Delta\Delta Ct}$ and $E^{-\Delta\Delta Ct}$ approaches in oil palm seedlings infected with *G. boninense*. The results will provide an overview of the effectiveness of these approaches in the relative genes expression, especially in reducing ΔCt bias.

2. Materials and Methods

2.1. Plant genetic materials of oil palm seedlings

Genetic materials from oil palm seedlings aged 6 months after *G. boninense* infection. Distribution of seedlings from germinated seeds consisted of resistant (C03, C07, and C08 accessions), and susceptible (C01 accesion) with control and inoculated treatments. The genetic materials derived from Indonesian Oil Palm Research Institute (IOPRI), North Sumatera. The research activities were conducted at the Molecular Biology Laboratory, IOPRI, Bogor-West Java.

2.2. Ribonucleic acid (RNA) isolation and cDNA synthesis of oil palm seedlings

Fifty mg RNA from oil palm roots were isolated using the Plant RNA Mini Kit (RP050) from Geneaid, Taiwan. Synthesis of first-strand cDNA was conducted using $Oligo(dT)_{20}$ primer with reverse transcriptase hybridizes to the poly(A) tail of mRNA. Quality of RNA and cDNA visualize on 1% agarose gel and TBE 0,5x with SYBR Safe DNA gel stain. The concentration of RNA and cDNA were measured using NanoDropTM 2000/2000c Spectrophotometers from United State of America.

2.3. Quantification of RT-qPCR analysis

The three targeted genes, which are consist of *EgUnk3*, *EgZFP2*, and *EgIPK2b*, used in this quantification analysis. The RT-qPCR reaction mixtures consisted of 5 µL SYBER Green, 0.625 µL each of forward and reverse primers (Table 1), 3.75 µL NFW buffer, and one µL cDNA. The cDNA samples from three biological replicates and three technical replicates of each oil palms seedlings. The expression values of Ct mean from the Actin gene and targeted genes were calculated using the StepOnePlus Real-Time PCR System and StepOne Software v2.3 (Applied Biosystems, UK). The actin gene was chosen as the reference gene due to its stable expression observed in earlier analyses (Zuhar et al. 2019).

Based on the description of previous research team has designed the primers (Table 1) based on 3 transcriptomic datasets from leaves and roots of oil palm plants resistant and susceptible to *Ganoderma* disease (Faizah et al. 2022). Second, the primer designed was obtained from literature studies on potential genes involved in biotic and abiotic stresses. Third, the selection of primers that have been designed is based on initial evaluation of oil palm leaf and root samples infected with *Ganoderma* disease (Faizah et al. 2022).

2.4. The best cDNA concentrations and PCR efficiency of Actin gene

Dilution of the Actin gene was performed at concentrations of 5x (100 ng/mL), 10× (50 ng/mL), 25× (20 ng/mL), dan 50× (10 ng/mL) to obtain the best cDNA concentration and as internal reference to assay the samples on these three targeted genes. The optimal cDNA concentration

TABLE 1 Primer sequences used for the *EgUnk1*, *EgZFP2*, *EgIPK2b*, and *EgActin* expression analysis in oil palm.

No.	Gene	Abbrev.	Locus	Primer sequence of cDNA	
				Forward	Reverse
1	*probable 2-oxoglutarate-dependent dioxygenase At5g05600*	*EgUnk3*	LOC105045834	TAGTGTGGAGCATCGTGTGA	CATGCAGACCCAACTTCCT
2	*zinc finger protein 2-like*	*EgZFP2*	LOC105050368	GGTAGTGAGAGGGGCAACAA	ACCTCCATTCAACGCAATCT
3	*inositol polyphosphate multikinase beta-like*	*EgIPK2b*	LOC105050983	GGGGTTAGGGTTAAGCTCGT	TGTTGTTGCCGTTCCATTAG
4	*Actin-101*	*EgActin*	LOC105032827	CCCACCTGAACGGAAATACA-	CGGATGGCACCTCAGTCTTA

was 50 ng/µL. The three targeted genes were not diluted, however based on a melt curve with one peak for each genes. The purpose of dilution is to reduce bias caused by technical during analysis and human error.

The amplification efficiency of Actin gene in oil palm were determined by Ct value and log concentration of cDNA using primers specificity of oil palm Actin. The PCR efficiency was calculated using the formula: E= ($10^{-1/slope}$-1) × 100; in which E= amplification factor; Slope= the slope of the linear regression (a value at Y=ax+b) between the mean Ct value and the normalized log dilution of cDNA concentrations (Svec et al. 2015). Based on the formula, the amplification factor (E) and efficiency value (R) of *EgActin* of 1.95 and 94.92%, respectively. Correlation coefficients (R2) of the *EgActin* was greater than 0.99 that indicate the primers selected are suitable for further RT-qPCR analysis.

2.5. Linear regression to minimalize bias Ct value

Linear regression was performed on the Ct values in the targeted genes *EgUnk1*, *EgZFP2*, and *EgIPK2b*. The distribution of Ct values in each target gene was carried out by linear regression to determine the value of the regression coefficient (Cui et al. 2015). Furthermore, the Ct value of the target gene was normalized with Ct Actin to determine the quantity of the bias based on its linear regression value. The normalized values were used to analyze the relative gene expression using two approaches, such as amplification factor $1.95^{-\Delta\Delta Ct}$ and a constant value of 2 at $2^{-\Delta\Delta Ct}$ (Regier and Frey 2010).

2.6. Comparative quantitative approach of gene expression analysis

Two approaches of analysis were used to determine expression value in the targeted genes of *EgUnk1*, *EgZFP2*, and *EgIPK2b*. First approach was revered as PCR efficiency with amplification factor (Kralik and Ricchi 2017; Pfaffl 2007). In this approach, the relative gene expression quantity (R) was determined using the formula as follows. $R = [E]^{-\Delta (Cp\ target-CpEgActin)}$; in which R: relative gene expression quantity; Cp = Crossing points deviation of Ct (threshold cycle values) (Pfaffl et al. 2002). Second approach which revered as $2^{-\Delta\Delta Ct}$ from mean of Ct (Schmittgen and Livak 2008). The transcript accumulation of the *EgUnk1*, *EgZFP2*, and *EgIPK2b* genes was normalized using the accumulated transcript of the endogenous Actin gene of oil palm.

The step by step to acquire the fold change value with a regression coefficient to minimize bias are as follows. First, comparing the value of fold change from approaches $2^{-\Delta\Delta Ct}$ and $1.95^{-\Delta\Delta Ct}$. Second, identify causes of data bias and eliminate point outlier data before normalization. Third, linear regression based on targeted genes of regression coefficients. Additional, calculation of delta Ct and the value of fold change as in approaches $2^{-\Delta\Delta Ct}$ and $1.95^{-\Delta\Delta Ct}$. Relative gene expression was normalized using Log10 fold change. Furthermore, the ANOVA analysis followed by the Newman-Keuls Student test was performed in the XLSTAT (Addinsoft, USA) and RStudio version 1.4.1717 program data analysis. The potential for bias is possible, however a simple linear regression reduces the bias of the two approaches being compared.

3. Results and Discussion

3.1. PCR Efficiency of Actin gene

In the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines, the efficiency of PCR is one of the components of the stepwise optimization of RT-qPCR on gene expression to reach R=0.99 and efficiency of 100 ± 5% (Zhao et al. 2021). The closer the E value is to 100, the more efficient the primer and RT-qPCR analysis is to obtain the Ct value (Kralik and Ricchi 2017). In this study, the efficiency value closed to 100%, which affects the amplification factor less than the constant value 2 (Figure 1). PCR efficiency is a very important factor in RT-qPCR analysis to determine optimal cDNA quantification. The exponential process of the number of cDNA molecules in each cycle will be maximized if it is reflected in the PCR efficiency which is close to the value of R = 100% (Kralik and Ricchi 2017).

Based on the comparative Ct method, it assumes that the efficiency value is constant equal to two from the standard curve of Ct value and log cDNA concentration (Livak and Schmittgen 2001; Schmittgen and Livak 2008). However, the efficiency value can be varied and it is possible to know the error rate in different fold change. These shows that the Ct value method is strongly influenced by variations in PCR efficiency (Ramakers et al. 2003). Thus, the difference in the value of this constant influences the

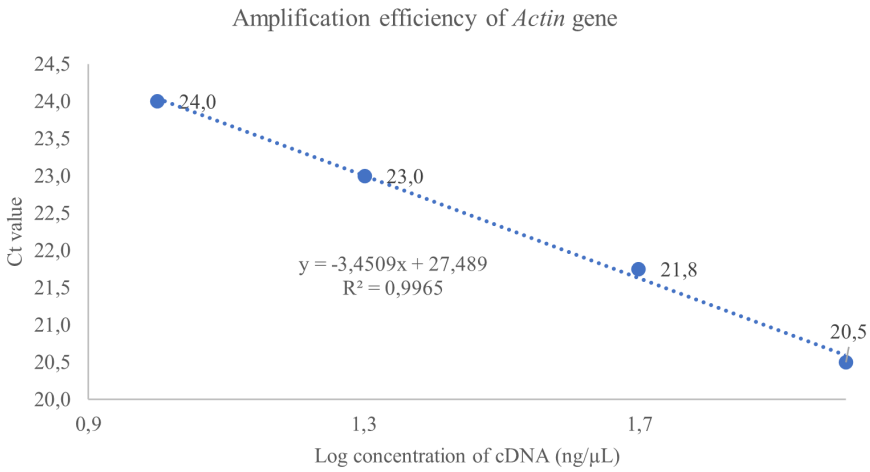


FIGURE 1 The amplification efficiencies of *Actin* gene was determined by means of a standard curve using a four-fold dilution of cDNA (Faizah et al. 2022).

calculation of the formula to get the fold change (Ruiz-Villalba et al. 2021).

3.2. Correction of Ct value of Actin and targeted genes EgUnk1, EgZFP2, and EgIPK2b using correlation coefficient

The Pearson correlation coefficient among delta Ct of *EgUnk1*, *EgZFP2*, and *EgIPK2b* targeted genes with Ct of Actin gene showed the positive and negative correlations, 0.28, -0.32, and 0.29, respectively (Figure 2). However, after decreasing the Ct of the Actin gene, negative correlation were obtained -0.55, -0.81, and -0.29, respectively (Figure 3). The correction in the correlation coefficient indicates there is has a systematic bias or over-estimation of the Ct value. Linear regression is a simple and effective way to estimate normalization coefficients and minimize bias (Belitz and Stackelberg 2021). For coefficients close to 1, ΔCt does not generate much bias, but for the coefficients far from 1, the bias can be substantial (Cui et al. 2015). The normalized Ct value of the reference gene using the regression coefficient method was also used to reduce the effect of overall homeostasis and housekeeping gene expression of β-actin in bird species (Cantarero et al. 2020).

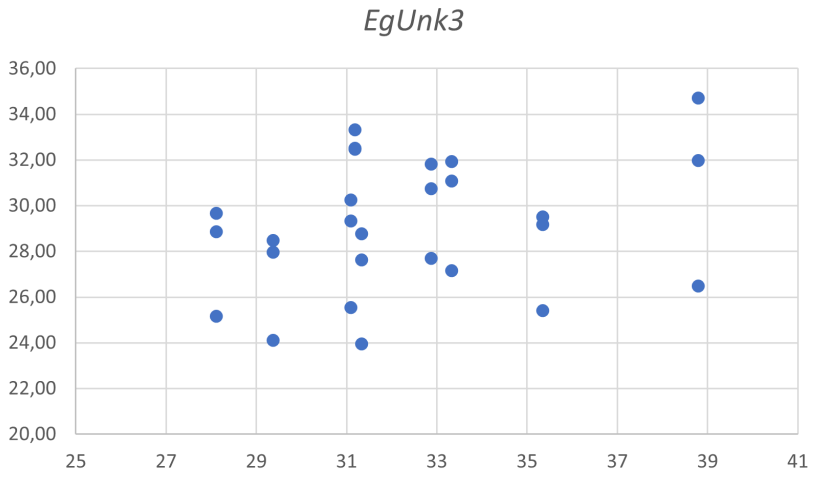
Standardization needs to be done by correcting the bias value with linear regression. The finding of this study is that there is a bias in the Ct value generated by the qPCR machine. However, this bias can be minimized with the regression correlation coefficient. The values obtained from normalization of the target gene's Ct with the corrected reference gene's Ct resulted in a negative correlation. Correction by linear regression resulted a better mean of Ct value for gene expression analysis.

An important point in the analysis of comparative data on gene expression has been shown to have cDNA concentrations at the time of analysis, stable and unregulated internal control of genes, PCR dilution and primers efficiency, the slope ranges from 3.1-3.3, outlier data points, analysis of regression coefficients on target and accumulation of Ct reference genes, P-value and regression coefficients. If the Ct value of the target gene is greater than Ct Actin, then the value of $1.95^{-\Delta\Delta Ct}$ <1 implies a decrease in expression caused by the treatment of *G. boninense* infection.

3.3. The best approach of $1.95^{(-\Delta\Delta Ct)}$ to calculate relative expression of EgUnk1, EgZFP2, and EgIPK2b genes

The amplification factor of 1.95 in the $1.95^{-\Delta\Delta Ct}$ has an impact on the expression value of the targeted genes compared to the constant value of 2 used in the $2^{-\Delta\Delta Ct}$ approach. Additionally, correcting the mean Ct value of model genes is essential to minimize bias (Ruiz-Villalba et al. 2021). Based on Figure 4, approach of $2^{(-\Delta\Delta Ct)}$ and $1.95^{(-\Delta\Delta Ct)}$ showed a no significant difference in the log10 relative expression genes of *EgUnk1*, *EgZFP2*, and *EgIPK2b*. Log10 transforms the fold change to minimize bias and error, and this modification does no effect on the data analysis. The *EgUnk3* gene showed that the susceptible inoculated seedling has down-regulated of the lowest FC value of -0.60 and -0.58 for $2^{(-\Delta\Delta Ct)}$ and $1.95^{(-\Delta\Delta Ct)}$, respectively (Figure 4). The *EgZFP2* gene has a different trend, the inoculated resistant seedling shows up-regulated and the highest FC value 1.39 and 1.34 for $2^{(-\Delta\Delta Ct)}$ and $1.95^{(-\Delta\Delta Ct)}$, respectively (Figure 4). The *EgIPK2b* gene shows relatively down-regulated gene expression in susceptible and resistant *Ganoderma* (Figure 4).

Approaches of $2^{-\Delta\Delta Ct}$ and $1.95^{-\Delta\Delta Ct}$ on log10 relative expression genes showed not significantly different after minimizing the bias with regression coefficients. On the other hand, this contradicts the opinion that the values in both methods are not based on the mean value, but the bias value of the normalization calculation also affects the transcriptional mRNA and gene expression value. Based on the constant value of 1.95 on the $1.95^{-\Delta\Delta Ct}$ which was the PCR efficiency value of 94.92%, it is no significant



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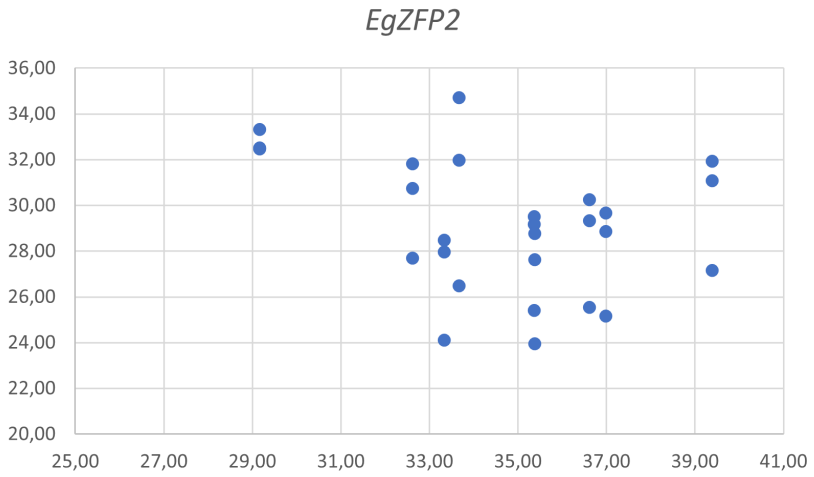

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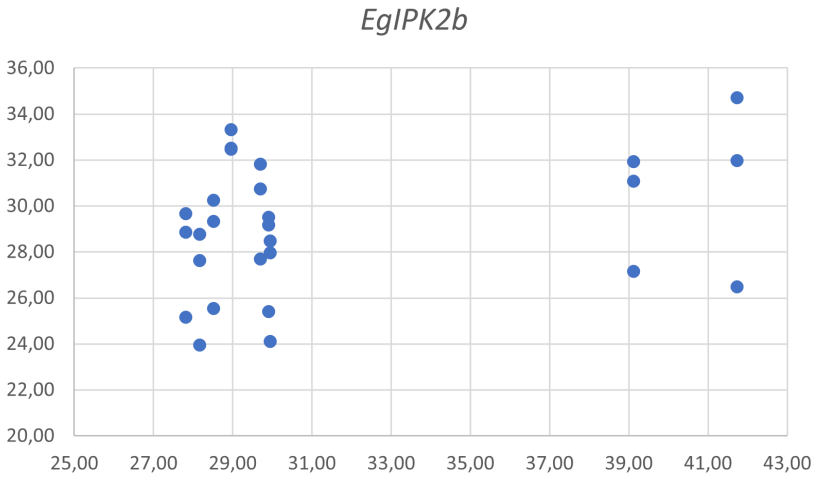
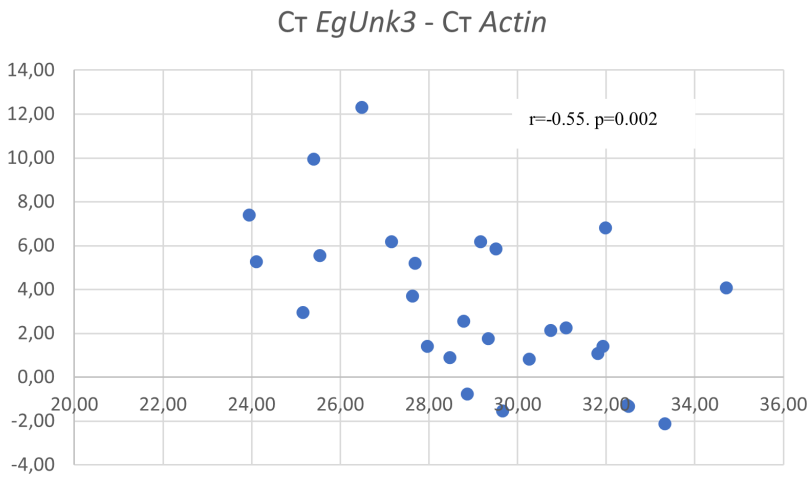


(c)

FIGURE 2 Correlated of the mean Ct values of the *EgUnk1*, *EgZFP2*, and *EgIPK2b* with the Ct values of the Actin gen. r = Pearson correlation coefficient; *p* = P-value from testing the correlation.



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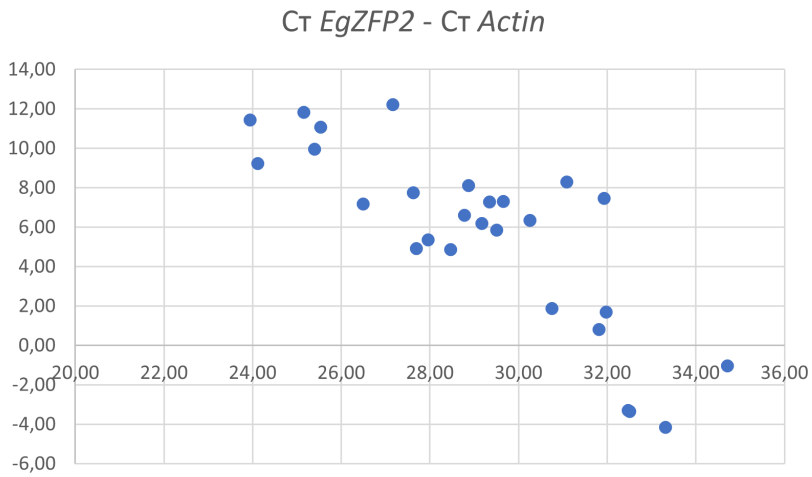

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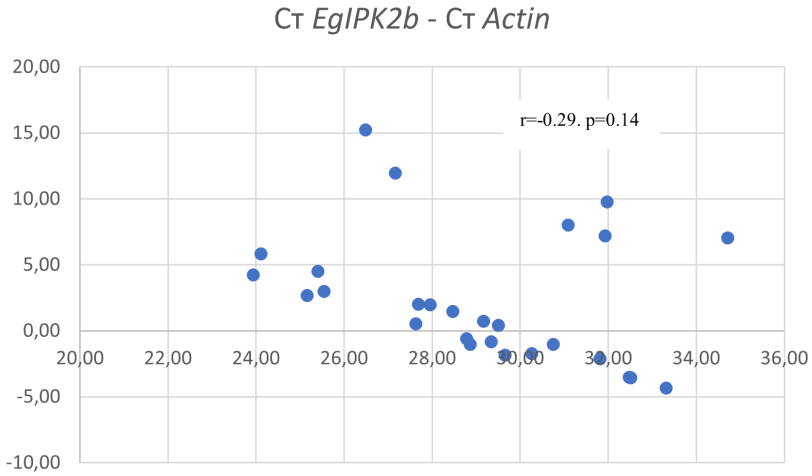
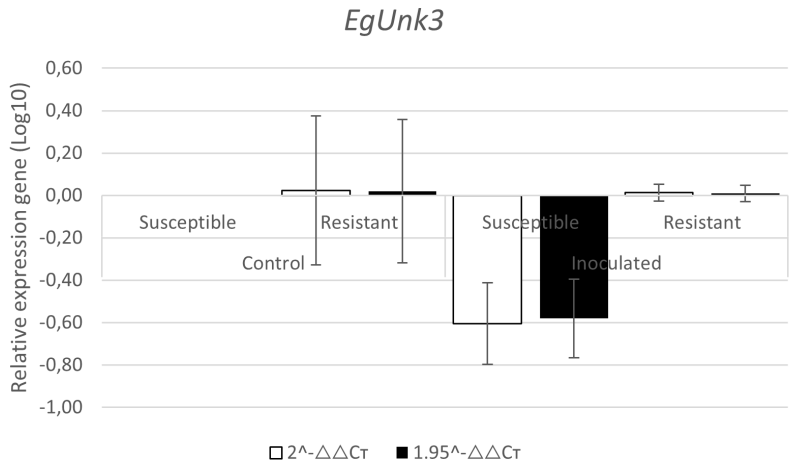


(c)

FIGURE 3 Negatively correlated between the mean of the ΔCt values from the targeted genes *EgUnk1*, *EgZFP2*, and *EgIPK2b* with *EgActin* after normalization via conventional subtraction. dCt = ΔCt; r = Pearson correlation coefficient; *p* = P-value from testing the correlation coefficient against 0.



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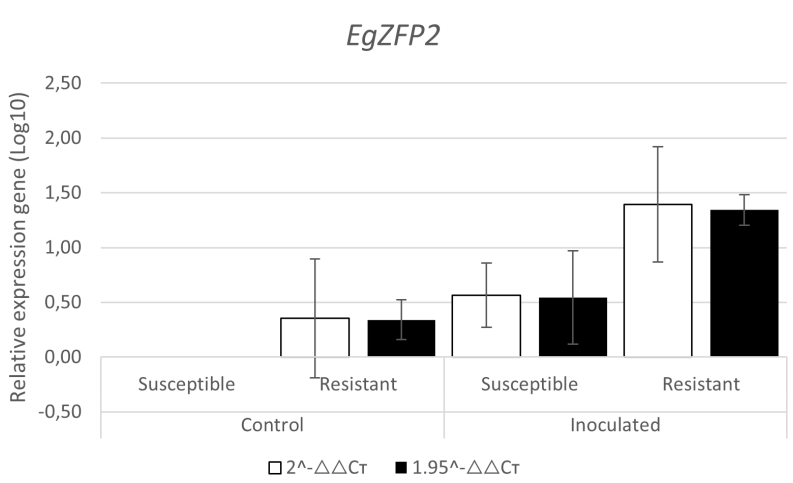

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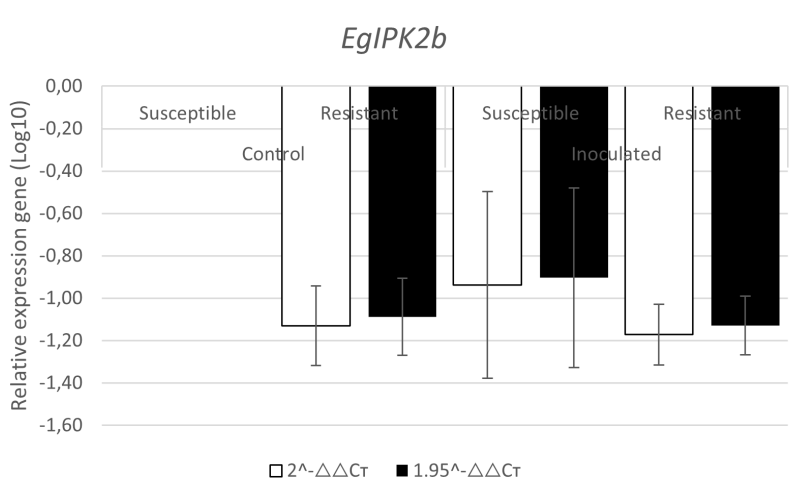

(c)

FIGURE 4 Comparison of Log10 fold change relative expressed genes of *EgUnk1*, *EgZFP2*, and *EgIPK2b* based on $2^{(-\Delta\Delta Ct)}$ and $1.95^{(-\Delta\Delta Ct)}$ approaches.

with the $2^{-\Delta\Delta Ct}$ approach. The two analytical calculation methods are not significantly different, meaning that both approaches can be used to calculate the FC value. Based on this, the value of the constants in the formulas of the two calculation models is important to determine gene expression patterns (Rao et al. 2013). On the other hand, the calculation shows that there is a bias that needs to be minimized (Ruiz-Villalba et al. 2021).

Although not significant in the two approaches above, the approach with PCR efficiency with amplification factor is better than $2^{-\Delta\Delta Ct}$ approach since it is able to control the effectiveness of the process of RT-qPCR analysis technique. It showed that the range of 95–105% of PCR efficiency value was close to constant value 2. The optimum PCR effectiveness is 100% (Ruiz-Villalba et al. 2021). The PCR efficiency value was close to the optimum is one of the components of the stepwise optimization of qPCR on gene expression to reach R=0.99 and efficiency of 100 ± 5% (Zhao et al. 2021).

3.4. Expression analysis for resistant and susceptible oil palm seedlings towards G. boninense

The mRNA expression of the *EgUnk3* gene was reduced by 0.58 times in susceptible plants infected with *G. boninense* (Figure 4). The same trend of *EgIPK2b* also decreasing gene expression occurred in susceptible seedlings that were inoculated by 0.90 times. In general, *EgUnk3* and *EgIPK2b* showed downregulated expression in the susceptible of oil palm seedlings. On the other hand, *EgZFP2* gene expression increased in oil palm seedlings infected with BSR disease (Figure 4). The increased gene expression occurred in both inoculated and control seedlings, ranging from 0.57 to 1.00 times folded change for susceptible and resistant seedlings. The highest increase occurred in resistant seedlings that were inoculated 1.34), while the lowest (0.00) was in susceptible seedlings without inoculation. Generally, BSR infection increased and up-regulated of *EgZFP2* gene expression in the oil palm seedlings.

The value of *EgUnk3* and *EgIPK2b* downregulated expression in susceptible oil palm seedlings indicated that the gene was not directly involved in the presence of *G. boninense* infection. Based on previous study, the *AtIPK2*β gene plays a role in the biological function of response to glucose during seed germination (Yang et al. 2017). In addition, the inositol signaling was indirectly involved in signal transduction in abiotic stress responses (Jia et al. 2019). Otherwise, the *EgZFP2* gene showed upregulated expression in *G. boninense* inoculated seedlings. The gene expression was very responsive in inoculated seedlings and the presence of the *G. boninense* pathogen triggered an increase the transcription factor of the *EgZFP2* gene in oil palm. This result is in line with previous studies that the zinc finger proteins is involved in plant disease resistant (Gupta et al. 2012). In addition, ZFPs also involved in plant growth regulator (PGR) regulation in abiotic stress, such as abscisic acid, gibberellic acid, jasmonate/ethylene, brassinosteroids, salicylic acid, and auxin signal response (Wang et al. 2022).

This research has limitations and requires further development. These limitations include using only one susceptible seedlings accession, compared to 3 resistant accessions for oil palm seedlings. Second, it is necessary to calculate the PCR efficiency value before using the constant value formula 2 in the $2^{-\Delta\Delta Ct}$ method. In this study, the $E^{-\Delta\Delta Ct}$ and $2^{-\Delta\Delta Ct}$ approaches produced undifferent values, however the $E^{-\Delta\Delta Ct}$ method was better than $2^{-\Delta\Delta Ct}$. Third, analysis of the expression of the *EgUnk3*, *EgZFP2*, and *EgIPK2b* genes was not followed by further analysis using DNA-based markers. These genes need to have their DNA sequenced and the primer sequence designed. These candidate markers can be applied and validated in a widespread and varied oil palm population for early detection of BSR disease in oil palm plantations.

4. Conclusions

The relative quantitative analysis of the *EgUnk3*, *EgZFP2*, and *EgIPK2b* genes, using Actin as a reference, successfully determined the accumulation of mRNA transcription in oil palm inoculated with *G. boninense*. PCR efficiency is important to calculate the amplification factor that used to relative expression gene analysis. Approach of $1.95^{-\Delta\Delta Ct}$ is better than $2^{-\Delta\Delta Ct}$ of the mean targeted gene approach, however the optimum value of gene expression is relatively the same if the PCR efficiency value up 100% and amplification factor between 1.95–2.05. Normalization of delta Ct, simple linear regression, and log10 transformation, effectively minimized bias in determining the value of relative expressed genes in the *EgUnk3*, *EgZFP2*, and *EgIPK2b* genes involved in *Ganoderma infection*. BSR disease involve the *EgUnk3* and *EgIPK2b* genes in the susceptible seedlings, otherwise the *EgZFP2* gene increased expression gene in resistant oil palm seedlings inoculated with *G. boninense* pathogen.

Acknowledgments

The authors would like to thank the team of research collaboration between the Indonesian Oil Palm Research Institute (IOPRI) Medan and Bogor, for the technical research assistance. The Indonesian Oil Palm Estate Fund, grant number PRJ-31/DPKS/2018, funded part of this research.

Authors' contributions

RF, RAP, SW designed the study. RF, AB, SW carried out the laboratory work. RF, RAP, SS analyzed the data. RF, SS, AB, DS wrote the manuscript. All authors read and approved the final version of the manuscript.

Competing interests

The author declare that they have no competing interest.

References

Adamski MG, Gumann P, Baird AE. 2014. A method for quantitative analysis of standard and high-throughput qPCR expression data based on input sample quantity. PLoS One 9(8):e103917. doi:10.1371/journal.pone.0103917.

Belitz K, Stackelberg PE. 2021. Evaluation of six methods for correcting bias in estimates from ensemble tree machine learning regression models. Environ. Model. Softw. 139:105006. doi:10.1016/j.envsoft.2021.105006.

Boulter N, Suarez FG, Schibeci S, Sunderland T, Tolhurst O, Hunter T, Hodge G, Handelsman D, Simanainen U, Hendriks E, Duggan K. 2016. A simple, accurate and universal method for quantification of PCR. BMC Biotechnol. 16:27. doi:10.1186/s12896-016-0256-y.

Cantarero A, Andrade P, Carneiro M, Moreno-Borrallo A, Alonso-Alvarez C. 2020. Testing the carotenoid-based sexual signalling mechanism by altering CYP2J19 gene expression and colour in a bird species: Red ketocarotenoid signalling mechanism. Proc. R. Soc. B Biol. Sci. 287(1938):20201067. doi:10.1098/rspb.2020.1067.

Chan PL, Rose RJ, Abdul Murad AM, Zainal Z, Leslie Low ET, Ooi LCL, Ooi SE, Yahya S, Singh R. 2014. Evaluation of reference genes for quantitative real-time PCR in oil palm elite planting materials propagated by tissue culture. PLoS One 9(6):e99774. doi:10.1371/journal.pone.0099774.

Chow YY, Rahman S, Ting ASY. 2019. Evaluating the host defense responses in oil palm to complex biocontrol endophyte–pathogen–host plant interaction via Fluidigm® real-time polymerase chain reaction (RT-PCR). Biol. Control 129:148–157. doi:10.1016/j.biocontrol.2018.10.011.

Cui X, Yu S, Tamhane A, Causey ZL, Steg A, Danila MI, Reynolds RJ, Wang J, Wanzeck KC, Tang Q, Ledbetter SS, Redden DT, Johnson MR, Bridges SL. 2015. Simple regression for correcting δCt bias in RT-qPCR low-density array data normalization. BMC Genomics 16(1):82. doi:10.1186/s12864-015-1274-1.

Faizah R, Putranto RA, Raharti VR, Supena N, Sukma D, Budiani A, Wening S, Sudarsono S. 2022. Defense response changes in roots of oil palm (*Elaeis guineensis* Jacq.) seedlings after internal symptoms of *Ganoderma boninense* Pat. infection. BMC Plant Biol. 22:139. doi:10.1186/s12870-022-03493-0.

Gupta SK, Rai AK, Kanwar SS, Sharma TR. 2012. Comparative analysis of zinc finger proteins involved in plant disease resistance. PLoS One 7(8):e42578. doi:10.1371/journal.pone.0042578.

Han G, Lu C, Guo J, Qiao Z, Sui N, Qiu N, Wang B. 2020. Corrigendum: C_2H_2 zinc finger proteins: Master regulators of abiotic stress responses in plants. Front. Plant Sci. 11:115. doi:10.3389/fpls.2020.00298.

Jia Q, Kong D, Li Q, Sun S, Song J, Zhu Y, Liang K, Ke Q, Lin W, Huang J. 2019. The function of inositol phosphatases in plant tolerance to abiotic stress. Int. J. Mol. Sci. 20(16):399. doi:10.3390/ijms20163999.

Josephin A, Whulanza Y, Rahman SF, Lischer K, Surya MI, Martiansyah I, Rahman W, Hashim U. 2024. Easy extraction of *Ganoderma boninense* liquid sample using portable on-chip device. Indones. J. Biotechnol. 29(1):33–39. doi:10.22146/ijbiotech.83645.

Kakizaki T, Kitashiba H, Zou Z, Li F, Fukino N, Ohara T, Nishio T, Ishida M. 2017. A 2-oxoglutarate-dependent dioxygenase mediates the biosynthesis of glucoraphasatin in radish. Plant Physiol. 173(3):1583–1593. doi:10.1104/pp.16.01814.

Kralik P, Ricchi M. 2017. A basic guide to real time PCR in microbial diagnostics: Definitions, parameters, and everything. Front. Microbiol. 8:108. doi:10.3389/fmicb.2017.00108.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25(4):402–408. doi:10.1006/meth.2001.1262.

Ma H, Bell KN, Loker RN. 2021. qPCR and qRT-PCR analysis: Regulatory points to consider when conducting biodistribution and vector shedding studies. Mol. Ther. Methods Clin. Dev. 20:152–168. doi:10.1016/j.omtm.2020.11.007.

Paul S, Singh S, Chakrabarti A, Rudramurthy SM, Ghosh AK. 2020. Selection and evaluation of appropriate reference genes for RT-qPCR based expression analysis in *Candida tropicalis* following azole treatment. Sci. Rep. 10:1972. doi:10.1038/s41598-020-58744-7.

Pfaffl M. 2004. Quantification strategies in real-time PCR. La Jolla: International University Line (IUL). p. 87–112.

Pfaffl M. 2007. Relative quantification. England: Taylor & Francis Group.

Pfaffl MW, Horgan GW, Dempfle L. 2002. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res. 30(9):e36. doi:10.1093/nar/30.9.e36.

Polosoro A, Enggarini W, Kusumanegara K, Hadiarto T, Miftahudin M, Supena EDJ. 2024. Detection and quantification of splicing variants of *Hd3a* gene in oil palm. Indones. J. Biotechnol. 29(1):40–47. doi:10.22146/ijbiotech.88327.

Ramakers C, Ruijter JM, Lekanne Deprez RH, Moorman AF. 2003. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neurosci. Lett. 339(1):62–66. doi:10.1016/S0304-3940(02)01423-4.

Rao X, Huang X, Zhou Z, Lin X. 2013. An improvement of the 2ˆ(-delta delta CT) method for quantitative real-time polymerase chain reaction data analysis. Biostat. Bioinforma. Biomath. 3(3):71–85.

Regier N, Frey B. 2010. Experimental comparison of relative RT-qPCR quantification approaches for gene expression studies in poplar. BMC Mol. Biol. 11:57.

doi:10.1186/1471-2199-11-57.

Ruiz-Villalba A, Ruijter JM, van den Hoff MJ. 2021. Use and misuse of Cq in qPCR data analysis and reporting. Life 11(6):496. doi:10.3390/life11060496.

Sánchez-Pujante PJ, Borja-Martínez M, Pedreño MÁ, Almagro L. 2017. Biosynthesis and bioactivity of glucosinolates and their production in plant *in vitro* cultures. Planta 246(1):19–32. doi:10.1007/s00425-017-2705-9.

Sanders R, Mason DJ, Foy CA, Huggett JF. 2014. Considerations for accurate gene expression measurement by reverse transcription quantitative PCR when analysing clinical samples. Anal. Bioanal. Chem. 406(26):6471–6483. doi:10.1007/s00216-014-7857-x.

Schmittgen TD, Livak KJ. 2008. Analyzing real-time PCR data by the comparative CT method. Nat. Protoc. 3(6):1101–1108. doi:10.1038/nprot.2008.73.

Sun Z, Kuczek T, Zhu Y. 2014. Statistical calibration of QRT-PCR, microarray and RNA-Seq gene expression data with measurement error models. Ann. Appl. Stat. 8(2):1022–1044. doi:10.1214/14-AOAS721.

Svec D, Tichopad A, Novosadova V, Pfaffl MW, Kubista M. 2015. How good is a PCR efficiency estimate: Recommendations for precise and robust qPCR efficiency assessments. Biomol. Detect. Quantif. 3:9–16. doi:10.1016/j.bdq.2015.01.005.

Wang Q, Song S, Lu X, Wang Y, Chen Y, Wu X, Tan L, Chai G. 2022. Hormone regulation of CCCH zinc finger proteins in plants. Int. J. Mol. Sci. 23(22):14288. doi:10.3390/ijms232214288.

Wu Z, Shen S, Wang Y, Tao W, Zhao Z, Hu X, Yu P. 2022. Genome-wide identification and expression analysis of the zinc finger protein gene subfamilies under drought stress in *Triticum aestivum*. Plants 11(19):2511. doi:10.3390/plants11192511.

Yang Q, Sang S, Chen Y, Wei Z, Wang P. 2017. The role of *Arabidopsis* inositol polyphosphate kinase AtIPK2β in glucose suppression of seed germination and seedling development. Plant Cell Physiol. 59(2):343–354. doi:10.1093/pcp/pcx186.

Zhang H, Zhao T, Zhuang P, Song Z, Du H, Tang Z, Gao Z. 2016. NbCZF1, a novel C_2H_2-type zinc finger protein, as a new regulator of SsCut-induced plant immunity in *Nicotiana benthamiana*. Plant Cell Physiol. 57(12):2472–2484. doi:10.1093/pcp/pcw160.

Zhao F, Maren NA, Kosentka PZ, Liao YY, Lu H, Duduit JR, Huang D, Ashrafi H, Zhao T, Huerta AI, Ranney TG, Liu W. 2021. An optimized protocol for stepwise optimization of real-time RT-PCR analysis. Hortic. Res. 8:179. doi:10.1038/s41438-021-00616-w.

Zuhar LM, Madihah AZ, Ahmad SA, Zainal Z, Idris AS, Shaharuddin NA. 2021. Identification of oil palm's consistently upregulated genes during early infections of *Ganoderma boninense* via RNA-seq technology and real-time quantitative PCR. Plants 10(10):2026. doi:10.3390/plants10102026.

Zuhar LM, Madinah AZ, Ahmad SA, Zainal Z, Idris AS, Shaharuddin NA. 2019. Determination of reference genes for normalisation of gene expression study of *Ganoderma*-infected oil palms. J. Oil Palm Res. 31(4):550–560. doi:10.21894/jopr.2019.0051.