Validation of Early Infection Markers for Ganoderma in Oil Palm at Three Endemic Ganoderma Locations

Validasi Marker Infeksi Dini Ganoderma pada Kelapa Sawit di Tiga Lokasi Endemik Ganoderma

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Abstrak Penyakit yang memengaruhi kelangsungan hidup industri kelapa sawit adalah busuk pangkal batang yang disebabkan oleh Ganoderma. Tingkat serangan Ganoderma saat ini tidak dapat dikendalikan, disebabkan oleh iklim yang tidak menentu bagi kelapa sawit. Saat ini, terdapat banyak pendekatan untuk mendiagnosis Ganoderma secara dini, salah satunya menggunakan metode molekuler. Tujuh gen marka infeksi dini pada kelapa sawit dihasilkan dari kajian transkriptomik sebagai referensi, yakni LEUCO, ETHYLENE, CHALCONE, ANTHOCYANIDIN, ETHYLENE, MANNOSE, dan SENESCENCE. Tujuan dari penelitian ini adalah memvalidasi dan mengkonfirmasi keberadaan infeksi Ganoderma di tiga lahan endemik kelapa sawit di Indonesia yaitu perkebunan Cisalak Baru, Rejosari, dan Bekri. RT-qPCR dilakukan dari RNA akar kelapa sawit dengan empat tingkat keparahan infeksi yang berbeda. Isolasi RNA dilakukan manual dan dilakukan sintesis cDNA, untuk menganalisis dan mengkuantifikasi tingkat penyandian gen. Selain itu, analisis ontologi gen (GO) juga dilakukan untuk menjelaskan peran masing-masing gen yang diuji. Hasil penelitian mengungkapkan bahwa CHALCONE adalah satu-satunya marka awal infeksi Ganoderma yang secara konsisten muncul di tiga lokasi. Perbedaan dari hasil analisis tiap kebun sangat erat kaitannya dengan umur kelapa sawit, tingkat keparahan infeksi, serta lokasi endemik. Hasil GO menyatakan bahwa peranan tujuh gen tersebut terkait dengan response infeksi. Studi ini berhasil mengkonfirmasi infeksi awal pada tiga kebun, menjelaskan variabel yang memengaruhi efikasi dan sensitifitas deteksi molekuler, dan menjelaskan fungsi dan pentingnya gen tertentu sebagai marka.

Kata kunci: deteksi dini, Ganoderma, marka, RT-qPCR, analisis GO

Abstract *A serious disease that affects the viability of the oil palm industry is basal stem rot, which is caused by Ganoderma. The current level of disease can be viewed as unmanageable, given that the palms were growing in an unfavorable or unsuitable climate. Today, there are numerous approaches to diagnose diseases early, and one of them using molecular methods. Seven genes for early infection markers were effectively generated by a reference's transcriptome study, including LEUCO, ETHYLENE, CHALCONE, ANTHOCYANIDIN, ETHYLENE, MANNOSE, and SENESCENCE. The purpose of this study is to validate and confirm the presence of Ganoderma infections in three endemic oil palm field in Indonesia i.e. Cisalak Baru, Rejosari, and Bekri plantation. This study conducted real time qPCR of RNA from oil palm roots with four different severities of infection. Manual processing of RNA isolation and cDNA synthesis were carried out, to provide quantification expression level. In addition, gene ontology (GO) analysis was also performed in order to explain the roles of each gene tested. The results*

Penulis yang tidak disertai dengan catatan kaki instansi adalah peneliti pada Pusat Penelitian Kelapa Sawit

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revealed that CHALCONE is the only marker that consistently elucidate the Ganoderma's early infection appear in three locations. The drawbacks of the analysis results are tightly correlating to the age of oil palm as well as endemic location. GO results declare that seven genes function related to the response of infection. This work was successful in confirming early infection in three places, elucidating the variables influencing the efficacy and sensitivity of molecular detection, and revealing the function and importance of particular genes for detection.

Keywords: *early detection, Ganoderma, marker, RT-qPCR, GO analysis*

INTRODUCTION

The basal stem rot disease of oil palm caused by *Ganoderma* sp. is the most destructive disease in both immature and mature plants in Indonesia (Priwiratama *et al*., 2014). The attack rate continues to increase in the second or third generation plants up to 40%. Ganoderma infection at a level of 1% nationally can cause losses of more than IDR 2.5 trillion per year. Ganoderma disease is now also starting to attack first generation plants in new oil palm development areas in Sulawesi and Papua. In Indonesia, this disease has caused enormous economic losses (Priwiratama & Susanto, 2020; Purnamasari *et al*., 2013), so that it has the potential to paralyze oil palm agribusiness. Even MPOB reported that losses due to Ganoderma attacks reached USD 933 million and USD 314 million respectively in Indonesia and Malaysia in 2010 (MPOC, 2010).

Various efforts to control stem rot disease caused by *Ganoderma* sp. have been widely practiced by business actors, but it does not seem to produce satisfactory results and in fact Ganoderma attacks are increasingly widespread with greater losses. Because early signs of *Ganoderma* sp. are difficult to identify, technology that can identify early outbreaks of *Ganoderma* sp. is essential. Therefore, it is very important to know the level of Ganoderma attack as early as possible so that it can be controlled effectively and efficiently. As one of the major oil palms producing countries in the world, Indonesia needs to develop these diagnostic techniques to be applied in the field so that they will make a real contribution to effective control of stem rot attacks.

Differentially expressed genes (DEGs) obtained from RNA-sequencing (RNA-seq) transcriptomic libraries of oil palm roots infected with *G. boninense* were examined in a study by (Zuhar et al., 2021). From the transcriptome libraries of root tissues infected with *G. boninense* at various phases of infection, a total of 126 DEGs were found. It was discovered through functional annotation using pathway enrichment studies that the DEGs were engaged in the pathogen defense response. Using independent oil palm seedling and mature palm samples, real-time quantitative PCR (qPCR) was used to further corroborate the expression of the chosen DEGs. During *G. boninense* infection, seedlings and mature plants consistently displayed elevation of seven putative defense-related DEGs. The development of these seven genes as biomarkers for the early detection of BSR in oil palm is possible. In this study the RNA from oil palm roots from three different sites were isolated in order to confirm and validate whether the seven markers derived through transcriptomic studies could be applied at every variety of oil palm in various sites in three oil palm generations.

METHODS

1. Sample retrieval

The secondary roots of oil palm were collected as much 5 grams per oil palm tree, from three different locations of Ganoderma endemic, namely Kebun Cisalak Baru, Banten, West Java (PTPN VIII), Kebun Rejosari, Lampung (PTPN VII), and Kebun Bekri, Lampung (PTPN VII) (Table 1). In every location, the level of Ganoderma severeness has been decided. Four levels of severeness including control (no Ganoderma infection), early infection, moderate infection, and severe infection have been observed based on several parameters (Table 2). Each of them consists of at least 2 to 3 oil palm trees as biological replicates. The fresh roots were immediately stored in the 4 C and processed in the laboratory.

Table 1. Detail information of oil palm location, variety and year of planting in three selected location
Tabel 1. Informasi rinci terkait lokasi, varietas, dan tahun tanam kelapa sawit pada tiga lokasi terpilih

Field location	Level of severity	Detail's location	Variety	Year of planting
Cisalak Baru, Banten, West Java (PTPN VIII)	Healty (control), early infection, moderate infection, and severe infection	Afdeling 1, block 11	DxP Rispa (PPKS)	1996/1997
Bekri, Lampung (PTPN VII)	Healthy (control)	Afdeling 3, block 703	DxP PPKS	2005
	Early, moderate, and severe infection	Afdeling 3, block 858	DxP PPKS	2005
Rejosari, Lampung (PTPN VII)	Healthy (control)	Afdeling 2, block 536	DxP Marihat	2001
	Early, moderate, and severe infection	Afdeling 2, block 616	DxP PPKS	2001

Table 2. The level of Ganoderma infection in oil palm from Kebun Cisalak Baru, Kebun Rejosari, and Kebun Bekri
Tabel 2. Tingkat infeksi Ganoderma pada Kebun Cisalak Baru, Kebun Rejosari, dan Kebun Bekri (Suharyanto *et al.*, 2012).

Level of severity	Parameters
Control	No Fruiting body of Ganoderma, no spear leaves observed, the position is far away from the infected plants, the fruit bunches look healthy.
Early infection	No Fruiting body of Ganoderma detected, there are at least 2 spear leaves, the position of the plant is one tree apart from severe plants, and plants still well reproducing (the fruit bunches are countable).
Moderate infection	No Fruiting body of Ganoderma detected, there are at least 3 spear leaves, the position of the plant is exactly close to the severe plant, plants are still reproducing (there are fruit bunches)
Severe infection	It was observed that the fruiting body of Ganoderma at the base of the stem (at least one fruiting body), there were at least 3 leaves at the tip of the spear that closed, the plant did not have fruit bunches.

Source: Suharyanto et al., (2012).

2. RNA isolation and Real Time PCR

A total of 0.25 grams root samples were isolated, then crushed with liquid nitrogen in a mortar and pestle. Next, the samples were incubated at 65°C for 1 hour, and flipped carefully every 15 minutes. RNA extraction was started by using 1 volume of Chloroform: Isoamyl alcohol (C:I), followed by centrifugation. The clear phase was taken and given a solution of Phenol: Chloroform: Isoamyl alcohol (P:C:I) 1:1:1 and re-extracted with a solution of C:I in the next step. The clear phase was again taken and given 10M LiCl to a final concentration of 2M, then incubated at 4°C overnight. The next day, the mixture were then centrifuged and the pellet was added with

SSTE buffer, 3M Na-acetate pH 5.2, absolute ethanol and incubated at -70°C for 3 hours. Afterwards, the supernatant was removed, the pellet was washed with 70% cold ethanol and air-dried. The RNA pellet was then dissolved with 30 µL of ddH_2O to check its concentration and purity with nanodrops. The resulting RNAs were then converted into cDNA using the ReverTra Ace™ qPCR RT Master Mix with gDNA Remover (Toyobo, Code No. FSQ-101), Japan) according to the procedure of ReverTra Ace™ qPCR RT Kit. Finally, the cDNA concentrations were checked using nanodrop.

Gene expression validation was carried out using RT-qPCR (Applied Biosystems Real-Time PCR Instruments) using primer for specific target genes (Table 3). RT-qPCR conditions carried out with the cycling conditions as follow: 95°C as initial denaturations for 2 minutes; 45 cycles of 95°C for 5 seconds, annealing 55 or 57°C for 30 seconds, and melting stage 95°C for 15 seconds, step and hold 60°C for 1 minute.

Table 3. The primer used for RT-qPCR seven markers for Ganoderma infection
Tabel 3. Primer yang digunakan untuk RT-qPCR tujuh gen marka infeksi Ganoderma

Primer	Sekuens (5' → 3')	Tm (°C)
ANTHO	F: ACAACATGGTCCCCGGTCT R: GGTGGAGGATGCTCTTGTAGGT	57
LEUCO	F: TCCGTTTTGGGCGGTTCT R: CGGCGGACTTTCCTCTTTTC	55
ETHYLENE	F: AAGAGCAAGGCAGGGAATGG R: CTTCTGCGCTGTCAAAGGTTC	57
MANNOSE	F: TCGGATGGGAACCTTGTGG R: CCGATCTCGTTGGAGGATACAG	57
CHALCONE	F: GAGCAGATCCAATGCAAGGTGT R: GGTTGAGGAGGTGGAAGGTGA	55
SENESCENCE	F: GGCACGGCCATCAGTAGAGTA R: AGCCAAGCGTTCATAGCGAC	55
THAUMATIN	F: ACGAGGGAGATGTCGATGAA R: GACTGCGGTGGTAAACTTGC	55

Source: Zuhar et al., (2021)

3. Networking analysis and Gene Ontology (GO) analysis

To validate the involvement of seven marker genes in early infection level of Ganoderma in oil palm, networking analysis using String-db webserver (https://string-db.org/) was performed. Seven proteins including Anthocyanidin synthase (ANTHO), Leucoanthocyanidin reductase-like (LEUCO), ETHYLENE-responsive transcription factor 1b-like (ETHYLENE), MANNOSE-specific lectin-like (MANNOSE), CHALCONE synthase (CHALCONE), SENESCENCE-associated partial (SENESCENCE), and THAUMATIN-like protein (THAUMATIN) were

inputted into the column, with Arabidopsis as model plants. The enrichment of networking interaction was performed three times until the networking is develop and enrich. The Gene Ontology (GO) including function, process, and KEGG pathway were generated automatically in the "analysis" tab. Visualization of GO was then carried out by Revigo webserver (http://revigo.irb.hr/).

RESULTS AND DISCUSSION

1. Validation expression of seven markers gene in early Ganoderma infection

The molecular approach was used to validate the Ganoderma infection rate category in the three oil palm plantations (*Cisalak Baru, Rejosari*, and *Bekri*). RNA isolation was followed by RT-qPCR expression level analysis. Transcriptome data from the roots of the 12-month-old Oil Palm var Tenera seedling plant served as the basis for the primers and target genes used (Zuhar *et al*., 2021). Seven key genes were generated as a result of the intersecting upregulated genes.

The obtained RNA concentration ranged from 50 ng/L to 337.1 ng/L. Following reverse transcription into cDNA, the RNA was diluted to 100 ng/L each. With the support of RNA isolation and RT-qPCR expression level analysis, the infection rate of Ganoderma in oil palm in the three locations (Kebun Cisalak Baru, Rejosari, and Bekri) was verified. The seven genes that regulate them were then used as a marker for early Ganoderma infection identification.

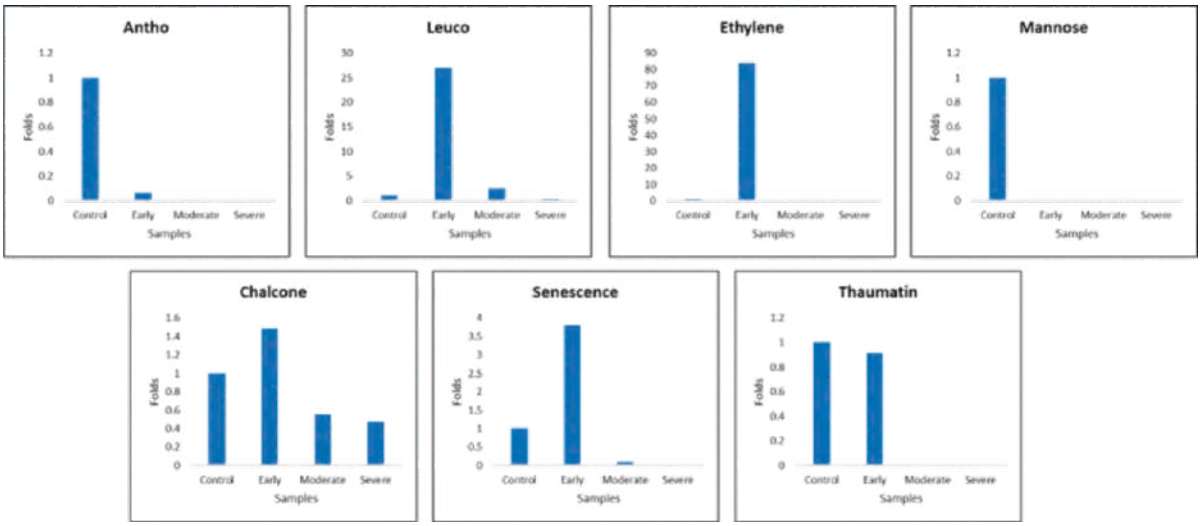

Figure 1. Expression level of seven gene markers to detect Ganoderma infection in control, early infection, moderate infection and severe infection in Kebun Cisalak baru

Gambar 1. Tingkat ekspresi tujuh marka gen untuk mendeteksi infeksi Ganoderma pada kontrol, infeksi dini, infeksi sedang dan infeksi berat di Kebun Cisalak baru

The data from *Kebun Cisalak Baru* revealed that the CHALCONE, LEUCO, ETHYLENE, and SENESCENCE genes were elevated 1.49, 26.98, 84.04, and 3.80 folds, respectively, compared to the control. However, the opposite was seen in the ANTHOCYANIDIN, THAUMATIN, and MANNOSE genes. As compared to the control, the expression of those three genes was downregulated in the early stages of infection, and then in mild and severe infections, it was even absent. This might be as a result of variations in the early-stage severity of Ganoderma infection or as a result of variations in the years of oil palm plantation. The palms employed in previous study were seedlings that were 7 days after infection and 1 year old. Meanwhile, the oil palm in the *Kebun Cisalak Baru* was planted in 1997 or around 22 years old.

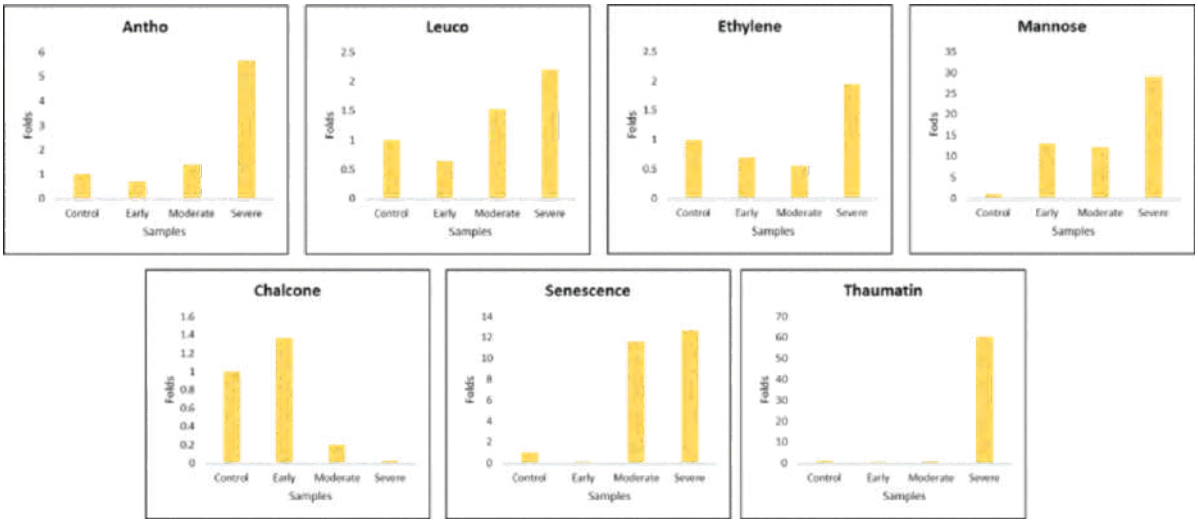

Figure 2. Expression level of seven gene markers to detect Ganoderma infection in control, early infection, moderate infection and severe infection in Kebun Bekri

Gambar 2. Tingkat ekspresi tujuh penanda gen untuk mendeteksi infeksi Ganoderma pada kontrol, infeksi dini, infeksi sedang dan infeksi berat di Kebun Bekri

In contrast, the gene expression trend was identical in the oil palm root samples from Kebun Bekri and Kebun Rejosari. Despite the fact that the folds are varied, the outcome of expression level calculation reveals that CHALCONE is the elevated marker in Ganoderma-infected oil palms in the early phase. ANTHO, LEUCO, ETHYLENE, MANNOSE, SENESCENCE, and THAUMATIN are the genes that are elevated in late phase of Ganoderma infection. The age of oil palms in both locations is practically identical, ranging from 6 to 12 years old, which may explain this tendency.

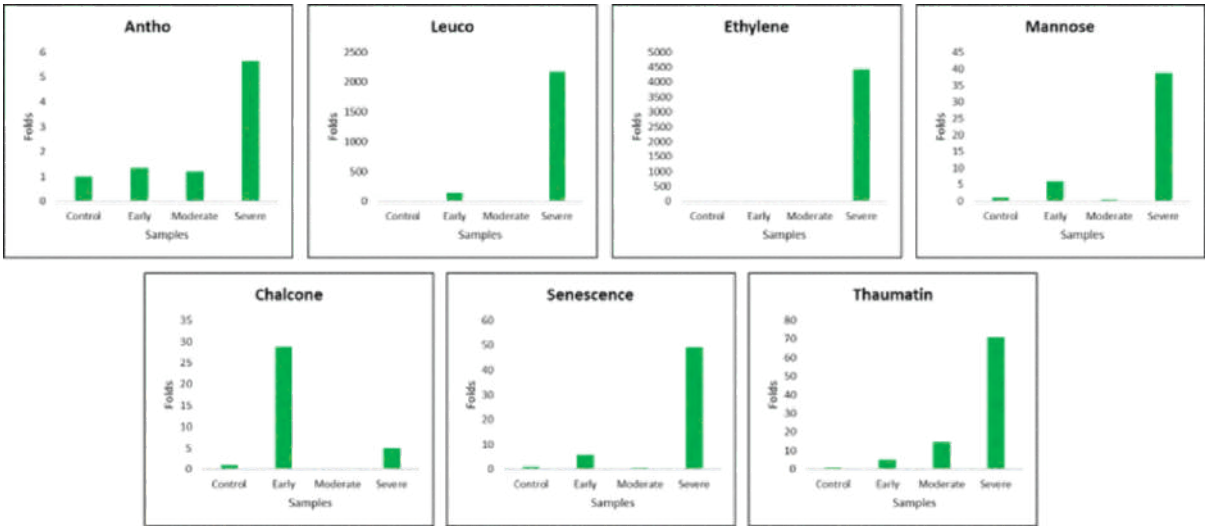

Figure 3. Expression level of seven gene markers to detect Ganoderma infection in control, early infection, moderate infection and severe infection in Kebun Rejosari

Gambar 3. Tingkat ekspresi tujuh penanda gen untuk mendeteksi infeksi Ganoderma pada kontrol, infeksi dini, infeksi sedang dan infeksi berat di Kebun Rejosari

Here, this study revealed that the validation of gene expression in the three locations showed varying results. The markers LEUCO, ETHYLENE, CHALCONE, and SENESCENCE are elevated at the early stages of the Ganoderma infection in the Kebun Cisalak Baru. As with the early stages of Kebun Bekri and Rejosari, CHALCONE synthase (CHALCONE) and MANNOSE-specific lectin-like were the elevated genes (MANNOSE).

According to those findings, CHALCONE is the constant marker gene that has been found in all locations at the same stage of infection. Age disparity is likely the main source of inconsistent expression from each region. Samples with oldest planting year were taken from Cisalak Baru, where the crops were planted in 1996–1997 (making them 23 years old as of 2022). These samples showed higher elevated genes indicative of defense mechanisms. Meanwhile, the samples from Rejosari (19 years old as of 2022) and Bekri (15 years old as of 2022), they exhibit a very similar pattern of elevated genes in early infection. Variation in plant origin or variety doesn't seem to have much impact because it comes from a genetically similar background.

These seven genes, develop into SAR (Systemic Acquired Resistance) or memory genes that plants will employ to carry out defense when the plant body is afflicted with Ganoderma infection (Banday & Nandi, 2015; Cheng et al., 2018). LEUCO is connected to ANTHO since it contributes to the production of anthocyanidins (dos Santos Silva et al., 2019; Liao et al., 2015). MANNOSE is part of the Lectin family, responsible for the action of resistance when infected with pathogens (Hwang & Hwang, 2011). When a plant is under stress, lectin expression is elevated. These lectins are hence known as "stress-inducible" plant lectins. Lectins will interact with many targets, either endogenous targets (i.e., the carbohydrate structures present inside the plant cell) or exogenous targets, depending on where they are located in the cell (i.e., the carbohydrates moieties on the surface of pathogens). Currently, it is thought that lectins play particular roles inside plant cells or in interactions with other organisms, as well as being involved in a wide range of biological processes connected to stress signal transduction and defense (Van Damme, 2022).

A critical component of plant defense against pathogens is transcriptional control (Gao et al., 2022; Zheng et al., 2019). Transcriptional factors attach to DNA during this process, controlling both the plant's regular growth and defense against pathogens. Since members of the conserved TF superfamily APETALA2/ethylene-responsive factor (AP2/ERF) are involved in growth, development, and reactions to a variety of biotic and abiotic stresses, such as drought, high salinity, extreme temperature, and pathogen infection, plants play a special role in the conservation of this family (Licausi *et al.*, 2013). Transcription factors, kinases, defense enzymes, and genes related to disease resistance were significantly differentially expressed among the 1226 ethylene-specific DEGs that were found by analysis of differentially expressed genes (DEGs). Numerous defense regulation-related genes and defense pathways were considerably enriched, according to KEGG metabolic pathway analysis and GO enrichment(Yang et al., 2020). THAUMATIN is a gene that has the ability to reduce fungal cell wall activity and is also associated with endo-b-1,3-glucanase (Liu *et al*., 2010). While CHALCONE is an enzyme that is responsible for the biosynthesis of flavonoids in the resistance process by plants (Zuk *et al*., 2016). SENESCENCE is a gene that induces a hypersensitive response and plays a role in PCD (programmed cell death) when plants are infected with pathogens (del Duca *et al.*, 2014). The Arabidopsis gene SENESCENCE-ASSOCIATED GENE 13 (SAG13) has been widely employed as a marker of plant senescence, though SAG13's function is uncertain. Senescence and hypersensitive reaction, a form of programmed cell death brought on by pathogens, are two examples of mechanisms that result in SAG13 induction in plants (Nikhilesh *et al*., 2020; Zhang *et al*., 2013). The difference between the results in this study and those proposed by Zuhar *et al*., (2021), thought to be due to differences in year of planting of oil palm in which Zuhar *et al*., (2021) used seeds (Nakazato *et al*., 2000).

2. Gene ontology analysis defining each gene role in Ganoderma response

The relationships between those seven indicators are highlighted by gene ontology of process enrichment. The top ten terms found are related to the metabolism of flavonoids (FDR 2.88E-16), reactions to hormones (FDR 5.45E-16), reactions to organic substances (FDR 7.41E-16), reactions to chemicals

(FDR 1.15E-13), reactions to stimuli (FDR 5.50E-13), reactions to jasmonates (FDR 8.84E-13), reactions to injury (FDR 2.29E-12), and the negative regulation of ethylene-a (FDR 1.33E-11) (Figure 4). The accuracy of terminology taken from a literature database is represented by the FDR score.

Figure 4. Gene ontology related to the molecular function related to the seven markers
Gambar 4. Ontologi gen terkait proses biologis dari tujuh marker gen

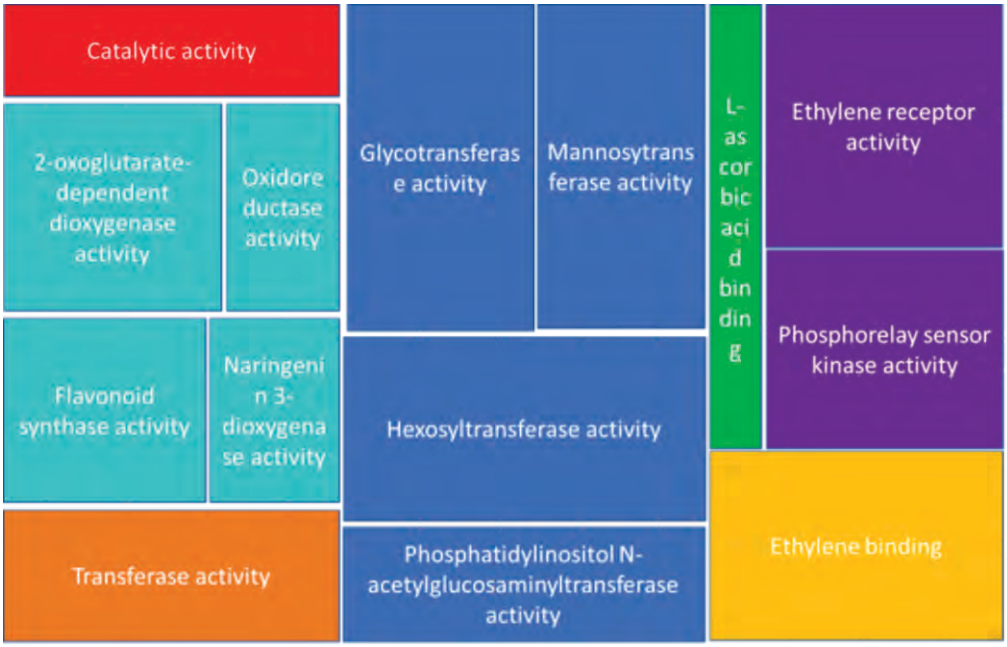

Figure 5. Gene ontology related to the enrichment function related to the seven markers
Gambar 5. Ontologi gen terkait fungsi molekular dari tujuh marker gen

Furthermore, the seven markers from gene ontology's function enrichment are strongly linked to the transferase activity for the hexosyl and glycosyl groups, with FDR scores of 1.057E-07 and 3.66E-07, respectively. In addition, the nomenclature refers to mannosyltransferase activity, phosphorelay sensor kinase activity, and ethylene receptor activity and binding (FDR 1.46E-06) (Figure 5). The majority of terminology describes the way each marker acts during the infection phase.

The GO analysis data emphasize that enrichment process exhibit that every terminology playing roles as response to infection. The terms such as response to wounding, to chemical, to organic substance and hormone revealing that when the Ganoderma infection occurs, the oil palm tree show self-defense systems. In response to a number of environmental variables, plants create a variety of primary and secondary metabolites.

Increased primary metabolism alters the signaling pathways that trigger plant defense reactions. Plants activate a number of genes linked to basic metabolic pathways, including those involved in the synthesis or breakdown of lipids, carbohydrates, and amino acids, upon exposure to pathogens or elicitors. Genetic analyses have further supported the role of these metabolic pathways in plant defensive responses (Rojas *et al*., 2014). While secondary metabolic pathways in plants result in a variety of substances known as plant secondary metabolites (PSMs). PSMs contain a significant number of structurally varied chemicals that were either produced as intermediates in the biosynthetic pathways of these primary metabolites or as primary metabolites themselves (Piasecka et al., 2015). PSMs are typically categorized into five significant molecular families based on their biosynthesis pathways i.e. phenolics, terpenes, steroids, alkaloids, and flavonoids (Kessler & Kalske, 2018).

CONCLUSION

The study found that the location of the infection and the age of the oil palm affected the marker variation of early infection Ganoderma. The only marker that consistently appears in the early stages of the Ganoderma infection from three separate locations is CHALCONE. Despite this, gene ontology data support the concept of seven gene markers created for the early detection of Ganoderma infection, which play critical roles in the infection-induced plant defense response. This work was successful in confirming early infection in three places in oil palm, elucidating the variables influencing the efficacy and sensitivity of molecular detection, and elucidating the purpose and significance of particular genes for detection.

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