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TRANSCRIPTOMIC AND PROTEOMIC STUDIES TO INVESTIGATE THE BASAL STEM ROT DISEASE IN OIL PALM SEEDLINGS

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ABSTRACT

When oil palm is exposed to, and infected by Ganoderma boninense*, the initial defence system is launched in the roots to reduce the damage caused by the disease. The present work described the transcript and protein profiles in roots of 18 months old oil palm seedlings that were exposed to the fungal pathogen for 12 months, following artificial inoculation at six months old. Three different phenotypes were observed; control (uninoculated), asymptomatic, and symptomatic (inoculated). It was found that the transcripts from the ubiquitin-mediated proteolysis pathway were common in control and asymptomatic seedlings; while proteins involved in cellular processes, and protein and sugar metabolisms were higher in abundance in asymptomatic seedlings. The transcripts involved in carbon fixation, glycolysis/gluconeogenesis, and pyruvate metabolism together with proteins responsible for stress response were identified in symptomatic seedlings. By integrating these omics data, it was observed that symptomatic seedlings were moving towards generating and storing energy for a possible defence strategy, and at the same time emitting stress signals and responses. This was in contrast with asymptomatic seedlings where regular functions such as cellular processes and carbohydrate metabolisms were found to be active.*

Keywords: asymptomatic, disease tolerance, oil palm, omics technologies, symptomatic.

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INTRODUCTION

The basal stem rot (BSR) disease, caused by *Ganoderma boninense*, is prevalent in Malaysia's oil palm plantations, especially in the coastal region (Mercière *et al*., 2017). The transmission and control of the disease are still ambiguous, thus making an accurate diagnosis and carrying out suitable treatments difficult (Hushiarian *et al*., 2013). The accepted mode of invasion by *Ganoderma* into the oil palm is through its massive network of roots. The fungal pathogen will attack the root at a point of entry, and slowly reaches the base of the oil palm (Pilotti, 2005; Rees *et al*., 2009).

There have been many unsuccessful attempts to investigate the thorough infection process at the point of entry at the roots and approaching the bole, up towards the effects of the disease by way of foliar symptoms. Most earlier studies had compared diseased and non-diseased (control *vs.* treated) young seedlings, following a short exposure time using a specific technology *e.g.,* transcriptome or genome sequencing, where the data are subsequently analysed using a combination of bioinformatic tools (Ho *et al*., 2019; Qistina Othman *et al*., 2018). Protein studies related to BSR were also conducted on leaf and root tissues (Daim *et al*., 2015a; 2015b).

Other than targeting candidates that are frequently studied in plant root-fungal interaction, plant immunisation is an alternative process for studying the defence system induced by biotic or

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abiotic stresses. In this case, asymptomatic seedlings, those that do not exhibit foliar symptoms following artificial inoculation with *Ganoderma*, could be considered as 'immunised' against the disease. Other scientists classify an asymptomatic host as 'resistant' to a specific disease, while a symptomatic host as 'susceptible'. Nevertheless, asymptomatic or resistant plants could still host the pathogen as the latter grows as a biotroph (Crone *et al*., 2013).

Omics technologies have long been used in many plant research areas, especially in investigating crop diseases, and understanding how these diseases affect crop health and yield. Previous studies were often conducted using a single method to probe into abiotic or biotic stresses (Bahari *et al*., 2018; Dai *et al*., 2019). The findings revolved around either gene expression/regulatory analysis, protein identification, interaction network, or metabolite profiling in the target tissues. A subset of these findings was then routinely validated through various methods. The major findings of omics experiments are the presence and expressions of these specific candidate genes (genomics), proteins (proteomics), or metabolites (metabolomics) in the target tissues.

The research using a multi-omics approach by combining several omics technologies to investigate disease ecology has allowed detailed representations of plant-disease interactions and plant-disease and environment interactions (Crandall *et al*., 2020; Neik *et al*., 2020). The plant stress response and defence system can be better understood by integrating data from multi-omics experiments. Specific functions that are expressed in a combined network are far more informative as compared to functions that are obtained using individual omics approaches.

The major problem in the estimation of root disease progression is connected to the fact that data are only collected when the oil palm is destructed. Observations on the changes in the site-specific tissues will then be determined. However, once the plant is destructed, it is practically impossible to track any further changes. Therefore, the present work used omics technologies to simultaneously examine the global transcript and protein levels in the roots of asymptomatic and symptomatic seedlings after prolonged exposure to *Ganoderma*.

MATERIALS AND METHODS

Materials

Elaeis guineensis Jacq. germinated seeds from a single cross were obtained from Sime Darby Plantation Research Sdn. Bhd. A subset of these samples was also used for expression analyses in a separate study (Tan *et al*., 2016). The parental cross came from Progeny Trial 59 in Dusun Durian Estate, Banting, Selangor, Malaysia planted in May 1979. The palm showed no signs and symptoms of *G. boninense* infection and was still producing bunches at the time of sampling. A total of 790 seedlings were screened in this trial, where 700 of them were subjected to *G. boninense* inoculation, while the remaining 90 seedlings served as control.

Preparation of Fungal Inoculum

Ganoderma boninense strain PER71 was obtained from the Malaysian Palm Oil Board (MPOB), and maintained at the Crop Protection Unit, Sime Darby Plantation Research Sdn. Bhd. The rubber wood block (RWB) inoculation process using *G. boninense* cultures, Disease Severity Index (DSI) and Disease Severity (Internal) (DI) analyses were conducted by Crop Protection Unit (Breton *et al*., 2006; Sariah and Zakaria, 2000). A customised RWB with a dimension of 6 × 6 × 6 cm was used as a source of inoculum for *G. boninense*. The RWB was sterilised at 80°C overnight, and then autoclaved at 121°C for 1 hr. A plastic bag containing 120 mL of malt extract agar and one RWB was sealed, autoclaved at 121°C for 15 min, and left to solidify overnight at room temperature. A seven-day-old *G. boninense* culture, maintained on a potato dextrose agar plate, was divided into half, where one part was inoculated onto the sterilised RWB. Inoculated RWBs were sealed in individual plastic bags and incubated in the dark at 25°C-28°C for 150 days, for mycelia to fully colonise all six surfaces of the RWB.

Artificial Inoculation

Oil palm germinated seeds (*Dura* × *Dura*) were sowed in 10" × 12" polybags containing topsoil and sand, at a ratio of 2:1. They were maintained in a shaded nursery for six months, in accordance with the Oil Palm Nursery Technique Good Agricultural Practices (Sime Darby Plantation). Two stringent selection processes were done when the seedlings were three and six months old, where those that were abnormal were culled before the infection process using inoculated RWB. Only those that were normal and healthy were subjected to nursery trials. During transplanting, *Ganoderma*-inoculated RWB was placed in the middle of 15" × 18" polybags that were half-filled with soil. The six months old seedlings were then placed in close contact above the RWB, before more soil was used to fill up the polybags. Control seedlings were prepared in a similar manner using sterilised, uninoculated RWB instead of inoculated ones. Each polybag was positioned above a concrete slab to obstruct the geotropic growth of roots into the soil. All seedlings were maintained in a nursery under the artificial shade with a 50% light cut-off. They were monitored until 18 months old (12 months post-inoculation).

TABLE 1. DISEASE PROGRESSION BASED ON THE DISEASE SEVERITY INDEX (DSI)

Class	Symptom
0	Healthy (green, healthy leaves)
1	Presence of white mycelium, with or without chlorotic/necrotic leaves
2	Presence of basidioma initials at the stem base with 1-3 chlorotic/necrotic leaves
3	Formation of basidioma at the stem base with more than 3 chlorotic/necrotic leaves
4	Presence of fruiting body with 50% necrotic leaves; plant is dried/dead

The progression of the disease was also assessed based on DSI (*Table 1*).

Sampling

The boles and roots of three seedlings from three phenotypes were sampled and washed under running water to remove excess soil. These phenotypes were control (uninoculated seedlings), asymptomatic (inoculated seedlings without foliar symptoms), and symptomatic (inoculated seedlings with foliar symptoms). The boles were cut into half, to identify the extent of severity of rotten tissues in inoculated oil palm seedlings (*Table 2*). Roots were dipped in liquid nitrogen and frozen at -80°C. All samples were pulverised using a mortar and pestle, into fine powder under constant liquid nitrogen, and frozen at -80°C until further analysis. The roots of three biological replicates from each phenotype were used for both transcriptomic and proteomic studies.

TABLE 2. THE SEVERITY OF BOLE TISSUES IN INOCULATED OIL PALM SEEDLINGS BASED ON INTERNAL DISEASE SEVERITY

Class	Symptom
0	Healthy (no internal rot)
1	Less than 20% of rotten tissues
2	20%-50% rotten tissues
3	51%-90% rotten tissues
4	More than 90% of rotten tissues

'Omics' Technologies

RNA extraction. The CTAB method was used to extract total RNA from the roots of oil palm seedlings with several modifications (Asemota and Shah, 2004; Tan *et al*., 2016). Briefly, 4 mL of extraction buffer (2% CTAB; 0.1 M Tris-HCl, pH 8; 2 M NaCl; 25 mM EDTA; 2% β-mercaptoethanol; 2% PVPP) was added to 2 g of frozen ground material, and mixed well using a vortex. An equal volume of phenol (pH 4.3): chloroform: isoamyl alcohol was added, and the mixture was centrifuged at 10 000 rpm for 15 min at 4°C. The supernatant was transferred into a new tube and precipitated twice in equal volumes of 2 M LiCl. The mixture was centrifuged at 10 000 rpm for 20 min at 4°C, and the pellet was resuspended in RNase-free water. A total of 0.1 vol of 3 M sodium acetate and 2.5 vol of absolute ethanol was added to the solution, and incubated at -80°C for 3 hr. The tube was centrifuged at 12 000 rpm at 4°C for 20 min. The resulting pellet was then washed in 70% ethanol, and thereafter, DNaseI (Epicentre) was applied before the RNA was precipitated in ethanol. Quantification was achieved using a Nanodrop ND-1000 spectrophotometer, where the 260/280 and 260/230 ratios between 1.8-2.0 were accepted. The RNA was run on 1% agarose gels to check the integrity of 18S and 28S rRNA.

Sequencing library preparation. The cDNA sequencing libraries were prepared using ScriptSeq™ v2 RNA-Seq Library Preparation Kit (Epicentre, Illumina), according to the manufacturer's instructions. The RNA was treated with terminator 5'-phosphate dependant exonuclease (Epicentre, Illumina) to deplete the rRNA.

Bioinformatics analysis. *De novo* assembly of raw counts was performed using Trinity software (Broad Institute, Hebrew University of Jerusalem), and unigenes were generated by CAP3 (Huang and Madan, 1999). The NOIseq analysis was used to determine the differential expression between the samples (Tarazona *et al*., 2011). The RPKM (normalised counts) for each sample was first calculated, and the M value, which is the $\log_2$ fold change, and D value, which is the absolute difference of the comparison, were later determined. With this, the noise distribution was generated for the background, M-D, using the available replicates. The probability value was calculated by comparing the M-D of the gene *vs.* the noise distribution and was set at >0.75.

iTRAQ analysis. The TCA-acetone-phenol method was used to extract the total protein (Daim *et al*., 2015b). The pellets were air-dried prior to labelling using iTRAQ reagents. The samples were then precipitated using acetone, reduced and alkylated, and finally digested using trypsin. A total of 100 µg of protein was then labelled using the iTRAQ reagents (4-plex). All procedures were conducted according

to the manufacturer's instructions (iTRAQ, AB Sciex). Three separate iTRAQ experiments were conducted to analyse all nine biological samples; one sample from each group (control, asymptomatic, and symptomatic) was labelled with a dye, while the fourth dye was labelled with the same control sample.

A pooled sample was prepared by combining all labelled samples. The peptides were desalted using a reversed-phase column (Strata-X 33 μm polymeric, Phenomenex). They were then dissolved in 10 mM KH_2PO_4 (pH 3) containing 10% acetonitrile. The peptides were separated using a strong cation exchange liquid chromatography column (PolySulfoethyl column, 4.6 × 100 mm, 5 μm, 300 A) on an Agilent 1100 HPLC system.

The peptide elution was done with a linear gradient of 0-400 mM KCl. Eight fractions were collected and desalted on Strata-X columns. The fractions were analysed using the 1260 Infinity HPLC system (Agilent) coupled to a 1260 Chipcube Nanospray interface (Agilent) on a QTOF 6540 mass spectrometer (Agilent). The peptides were separated in a ProtID-Chip-150 C_{18} column with a linear gradient of acetonitrile/0.1% formic acid (v/v). The spectral data were analysed against the NCBI Viridiplantae database, and protein identifications were done with ProteinPilot™ 4.5 Software (AB Sciex). The database was downloaded in July 2013 and contained 2 747 034 sequences.

Proteins with a relative abundance of >5 and with a *p*-value <0.05 were selected as statistically significant to ensure up- and downregulation. The protein lists were further analysed by the UniProt database. A global FDR was set at <0.1%.

RESULTS AND DISCUSSION

Phenotypic Evaluation

The trial was conducted on 18 months old seedlings to assess the disease severity of one single cross of interest. These seedlings were selected after a final round of culling right before they were exposed to *Ganoderma*-inoculated RWB at the six months old seedling. Results showed that this cross had an estimated disease severity of 5.32% and 10.12% severity of the bole tissues.

Seedlings were chosen for omics studies based on their DSI and disease severity (internal) (*Figure 1*). They were destructed to confirm the presence of lesions in the boles. Inoculated seedlings that were classified as Class 0 for both DSI and disease severity (internal) were considered asymptomatic, while those that were classified as Class 2 and above for DSI, and Class 1 and above for disease severity (internal) were considered symptomatic. Uninoculated seedlings with Class 0 for both DSI and disease severity (internal) served as control. The roots of three biological replicates that represented the three different phenotypes (control, asymptomatic and symptomatic) were used to construct the libraries for both transcriptomic and proteomic studies.

Destructive sampling was conducted when the seedlings were 18 months old, when there was positive root contact with RWB, in control and treated seedlings (data not shown). This situation (positive root contact with RWB) occurred by using polybags that are suitable for six to nine months old seedlings instead of APM polybags that are bigger in size. Roots of 18 months old seedlings were forced to expand and grow in a confined space within the polybag.

The mode of infection of BSR is through the roots. The progression of the disease is slow and varies for each individual, especially for older seedlings. Once the infection is established in the roots, the disease progresses towards the boles, and the seedlings may or may not show signs of foliar symptoms. Signs and symptoms of infection are prominent and occur early in germinated or younger seedlings. For six months old seedlings, their immune system is more developed than the much younger seedlings. They would have acquired the ability to defend themselves from the same source of fungal infection (the same inoculum source and size).

Asymptomatic seedlings do not show lesions in the boles, but infection is established in the roots (roots that were rotten or starting to rot were visible). DSI and DI data were collected from the foliar and bole, respectively. Control (uninoculated RWB) was put in place to ensure that it was treated in the same manner as inoculated seedlings. In this condition, both control and treated seedlings were treated equally, except for the inoculated RWB in treated (asymptomatic and symptomatic) seedlings. All treated seedlings were exposed to the same fungal pathogen at the same time with the same inoculum size.

The plant's defence system will entirely dictate its susceptibility (or tolerance) to the disease. Furthermore, infection is more often atopic and might have stopped progressing at the time the seedlings were sampled in the present work. In the case of asymptomatic seedlings, the infection has progressed to the roots but not the boles. Hence, they were identified as asymptomatic (fronds were healthy and green with a few roots that were rotten or started to rot). Symptomatic seedlings showed foliar symptoms but did not present lesions in the boles.

Unigenes in Control, Asymptomatic and Symptomatic Seedlings

Comparisons were made between asymptomatic *vs.* control, and symptomatic *vs.* control seedlings

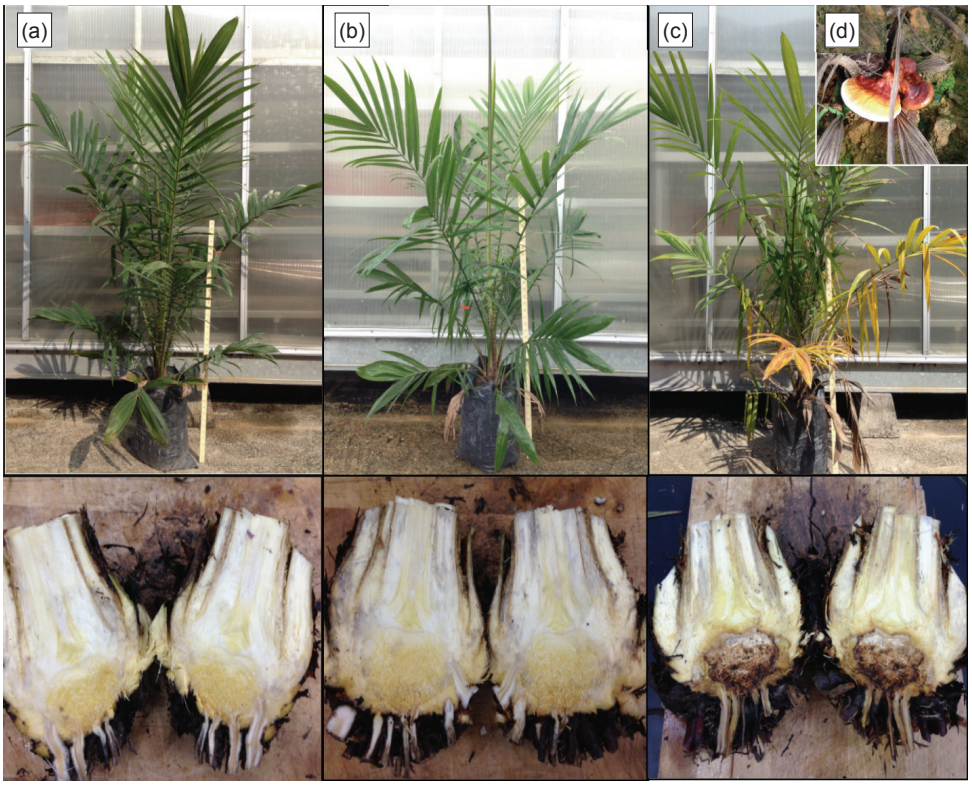

Figure 1. Oil palm seedlings were destructed to observe the severity of rotten tissues in the boles. Seedlings were chosen based on three different categories. (a) control seedling with class 0 DSI, and class 0 DI (internal); (b) asymptomatic seedling with class 0 DSI, and class 0 DI (internal); (c) asymptomatic seedling with class 3 DSI, and class 3 DI (internal), (d) basidiocarp growing at the base of a seedling.

in terms of differential expression of transcripts. The cDNA libraries were constructed from three biological replicates from the three groups (*Table 3*). A total of 464 701 946 paired-end reads with an average read length of 150 nucleotides (nt) were obtained. Annotations were performed by using BLAST against the UniProt database at http://www.uniprot.org/. Unigenes from control, asymptomatic, and symptomatic seedlings were identified and categorised into KEGG pathways via https://www.genome.jp/kegg/pathway.html.

A subset of the expressed genes was validated using both real-time quantitative PCR and NanoString in the same samples (data not shown). A part of the present work was also filed for a patent (WO2017155384A1, 2017).

A Venn diagram analysis was performed to group unigenes that appeared only in control, symptomatic, and asymptomatic seedlings (*Figure 2*). The unigenes were grouped into three different phenotypes based on the KEGG pathways (*Table 4*). Eight pathways from the unigenes appeared in all three phenotypes. Most of the pathways that appeared in either one or two phenotypes were common in cell metabolism, and carboxylic and amino acid metabolisms.

The ubiquitin-mediated proteolysis pathway was detected in the roots of asymptomatic seedlings and control. It made use of receptors that recognised and deciphered the ubiquitin code to regulate most cellular processes, including host-pathogen interactions (Li *et al.*, 2016). Modifications of the genes that are regulated by the ubiquitin system have been shown to manipulate the host defence system. Increased resistance against the root-infecting fungal pathogen *Fusarium oxysporum* has

TABLE 3. SUMMARY OF PRE- AND POST-PROCESSED READS OF RNA-SEQUENCING LIBRARIES

	RNA-Seq libraries		
	Control	**Asymptomatic**	**Symptomatic**
Raw reads	221 907 420	199 546 208	148 818 010
Raw nucleotides	33 286 113 000	29 931 931 200	22 322 701 500
Processed reads	137 203 583	197 032 111	137 203 582
Processed nucleotides	30 148 229 470	26 032 716 787	18 391 549 644
Mapped (%)	52	57	50

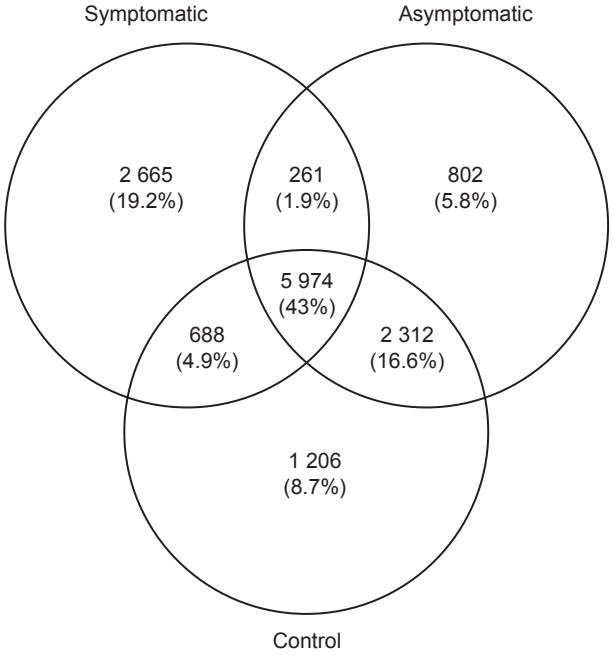

Figure 2. Venn diagram of unigenes from root tissues of oil palm seedlings that displayed three different phenotypes; control, asymptomatic, and symptomatic.

been displayed by the pub22/23/24 triple mutant. This suggested a role in root-mediated defence by PUB22/23/24 against soil-borne pathogens (Chen *et al*., 2013; Zhou and Zeng, 2017).

Transcriptional Expression in Roots of Symptomatic and Asymptomatic Seedlings

Most of the transcripts detected in symptomatic and asymptomatic seedlings were linked to cell walls, transporters, disease resistance, and phytohormone signalling pathways. A total of 20 upregulated and 20 downregulated transcripts were found in symptomatic and asymptomatic seedlings (*Table 5*).

Cell wall-related transcripts were differentially expressed in symptomatic seedlings. Endoglucanase, which is required for secondary cell wall formation in the developing xylem (Glass *et al*., 2015), was found to be downregulated. Glucanases also belong to the pathogenesis-related protein family (PR-2). They were also found to be involved in the infection of rice roots by *Azoarcus* sp. strain BH72. The visible sites of expression of the endoglucanase gene *eglA* were the points where lateral roots and root tips emerged, which are also the primary points of entry into the roots (Reinhold *et al*., 2006).

The ATP-binding cassette (ABC) proteins transport secondary metabolites and plant hormones that regulate the overall development of plants (Hwang *et al*., 2016). The ABCC transporters respond to pathogen-specific biotic stress where particular changes in ABCC transcript levels are found to induce distinct signalling cascades (Wanke and Kolukisaoglu, 2010). The ABCB proteins may serve as basal auxin transporters (Cho and Cho, 2013). The ABCI and ABCF transporters which are found down-regulated in asymptomatic seedlings are not directly involved in the transport process but likely function in ribosome recycling and translational control (Kang *et al*., 2011).

The transcripts of resistance proteins were detected in the roots of inoculated oil palm seedlings. Resistance gene analogues (RGAs) are a large class of potential R-genes (resistance genes) that enable plants to recognise specific races of pathogens and to mount an effective localised defence response (Jones, 1996). Disease resistance and abiotic stress tolerance in *Nicotiana benthamian* were enhanced in the overexpressed pathogen-induced grapevine *VaRGA1* gene (Li *et al*., 2017). In strawberries, the *Phytophthora cactorum* infection in crown rot showed that the gene transcriptional profiles of most RGAs were stronger and quicker in the resistant Bukammen than in the susceptible FDP821 genotype during the early infection stage (Chen *et al*., 2016).

Gene expression is regulated by gibberellins by promoting the degradation of transcriptional regulator proteins (Murase *et al*., 2008). The earliest findings of gibberellic acid (GA) in plant immunity were related to the decreased levels of GA in infected rice caused by Phytoreovirus (Zhu *et al*., 2005). However, exogenous GA was found to lower the resistance to two rice pathogens *Magnaporthe oryzae* and *Xanthomonas oryzae* (Qin *et al*., 2013; Yang *et al*., 2008). The rice GA signalling also appears to promote resistance to necrotrophs, and susceptibility to hemibiotrophs (De Bruyne *et al*., 2014).

Unique Proteins with Different Abundance

The iTRAQ analysis provides a relative quantification and was used for comparing control and diseased roots of oil palm seedlings in the present work. A total of 53 proteins were found to be higher in abundance, while 69 proteins were lower in abundance. The number of proteins that were higher in abundance was found in roots of asymptomatic seedlings, compared to symptomatic seedlings, while proteins that were lower in abundance were found in symptomatic seedlings. The activities of an increased number of proteins could be suppressed in the roots of symptomatic seedlings when the plants are showing signs of disease symptoms. When necrotic tissues in roots of symptomatic seedlings increase with the progression of disease infection, the functions of proteins in healthy tissues will decrease.

These proteins were subsequently grouped based on their functions (*Table 6*). The number of proteins with the following functions is lower in abundance in the roots of symptomatic seedlings;

TABLE 4. CLASSIFICATION OF UNIGENES BASED ON KEGG PATHWAYS

a) Appear in all phenotypes

(KO00520) Amino sugar and nucleotide sugar metabolisms

(KO00190) Oxidative phosphorylation

(KO04075) Plant hormone signal transduction

(KO04626) Plant-pathogen interaction

(KO04141) Protein processing in the endoplasmic reticulum

(KO03010) Ribosome

(KO03013) RNA transport

(KO00230) Purine metabolism

b) Appear in roots of control and asymptomatic seedlings

(KO04120) Ubiquitin-mediated proteolysis

c) Appear in roots of control and symptomatic seedlings

(KO00710) Carbon fixation in photosynthetic organisms

(KO00010) Glycolysis/gluconeogenesis

(KO00620) Pyruvate metabolism

d) Appear in roots of asymptomatic and symptomatic seedlings

Aspartate and glutamate metabolisms

e) Appear in one phenotype only

Control	Asymptomatic	Symptomatic
(KO00330) Arginine and proline metabolisms	(KO00020) Citrate cycle (TCA Cycle)	(KO00250) Alanine
(KO00270) Cysteine and methionine metabolisms	(KO04144) Endocytosis	(KO00720) Carbon fixation pathways in prokaryotes
(KO00051) Fructose and mannose metabolisms	(KO00564) Glycerophospholipid metabolism	(KO00071) Fatty acid metabolism
(KO03015) mRNA surveillance pathway	(KO03015) mRNA surveillance pathway	(KO00680) Methane metabolism
(KO00030) Pentose phosphate pathway	(KO03008) Ribosome biogenesis in eukaryotes	(KO04146) Peroxisome
(KO03050) Proteasome	(KO00072) Synthesis and degradation of ketone bodies	(KO00240) Pyrimidine metabolism
(KO03040) Spliceosome	(KO00900) Terpenoid backbone biosynthesis	(KO03018) RNA degradation
(KO00500) Starch and sucrose metabolisms	(KO00280) Valine	(KO00500) Starch and sucrose metabolisms

carbohydrate metabolism, cellular processes, protein metabolism and sugar metabolism. These main metabolic processes were also identified in soybean root diseases, Chinese cabbage and wheat (Bai *et al*., 2019; Kang *et al*., 2019; Lan *et al*., 2019). Carbohydrate metabolism, cellular processes, energy and protein metabolisms, signalling and stress response were higher in asymptomatic seedlings. The difference in protein abundances and identities in the symptomatic and asymptomatic seedlings indicated that specific protein requirements were needed when these seedlings were under stressed conditions. A proteomic profile of banana roots infected with *F. oxysporum* revealed that differentially regulated proteins involved in different defence pathways are likely associated with different resistant levels of the three banana cultivars (Li *et al*., 2013).

Proteins in Roots of Symptomatic and Asymptomatic Seedlings

Many proteins of lower abundance were detected in the roots of symptomatic seedlings (*Table 7*). Most of these proteins are involved in defence, stress response, and signalling. Unique, 14-3-3 proteins are phosphate-binding proteins

TABLE 5. UPREGULATED AND DOWNREGULATED TRANSCRIPTS FOUND IN ROOTS OF SYMPTOMATIC AND ASYMPTOMATIC SEEDLINGS

Contig	Differential expression (log2)	Uniprot
Downregulated transcripts		
Singleton1	-7.39	ABC transporter C family member 10 OS=*Arabidopsis thaliana*
Singleton2	-6.58	NAC domain-containing protein 68 OS=*Oryza sativa* subsp. *japonica*
Singleton3	-6.07	Adenosine kinase 2 OS=*A. thaliana*
Singleton4	-6.04	BI1-like protein OS=*A. thaliana*
Singleton5	-5.57	DDB1- and CUL4-associated factor homolog 1 OS=*A. thaliana*
Singleton6	-5.49	Pentatricopeptide repeat-containing protein At3g63370 OS=*A. thaliana*
Singleton7	-5.48	COP9 signalosome complex subunit 6a OS=*A. thaliana*
Singleton8	-5.30	Endoglucanase 25 OS=*A. thaliana*
Singleton9	-5.10	Putative lipoxygenase 5 OS=*O. sativa* subsp. *japonica*
Singleton10	-5.04	Cleavage and polyadenylation specificity factor CPSF30 OS=*A. thaliana*
Singleton11	-4.52	Plastidic glucose transporter 4 OS=*A. thaliana*
Singleton12	-4.37	Ammonium transporter 3 member 3 OS=*O. sativa* subsp. *japonica*
Singleton13	-4.28	Chalcone synthase 3 OS=*Ruta graveolens*
Singleton14	-4.25	40S ribosomal protein S4-3 OS=*A. thaliana*
Singleton15	-4.19	Probable serine/threonine-protein kinase CCRP1 OS=*Zea mays*
Singleton16	-4.19	Zinc finger A20 and AN1 domain-containing stress-associated protein 8 OS=*O. sativa* subsp. *japonica*
Singleton17	-4.17	ABC transporter B family member 11 OS=*A. thaliana*
Singleton18	-4.09	Putative late blight resistance protein homolog R1B-17 OS=*Solanum demissum*
Singleton19	-4.02	Proteasome subunit alpha type-6 OS=*Nicotiana tabacum*
Singleton20	-4.00	Phosphoenolpyruvate carboxylase kinase 1 OS=*A. thaliana*
Upregulated transcripts		
Singleton21	9.18	Alpha, alpha-trehalose-phosphate synthase _UDP-forming_1 OS=*A. thaliana*
Singleton22	9.08	Gibberellin receptor GID1 OS=*O. sativa* subsp. *japonica*
Singleton23	9.07	Xyloglucan endotransglucosylase/hydrolase protein 22 OS=*A. thaliana*
Singleton24	9.07	Protein GAST1 OS=*S. lycopersicum*
Singleton25	8.95	Sphingosine-1-phosphate lyase OS=*O. sativa* subsp. *japonica*
Singleton26	8.76	Pentatricopeptide repeat-containing protein At3g02650, mitochondrial OS=*A. thaliana*
Singleton27	8.75	Probable peptide/nitrate transporter At1g33440 OS=*A. thaliana*
Singleton28	8.68	Transcription factor AS1 OS=*A. thaliana*
Singleton29	8.64	Probable receptor-like protein kinase At5g18500 OS=*A. thaliana*
Singleton30	8.60	Long chain acyl-CoA synthetase 4 OS=*A. thaliana*
Singleton31	8.40	Shaggy-related protein kinase beta OS=*A. thaliana*
Singleton32	8.40	Phosphoenolpyruvate carboxylase 1 OS=*A. thaliana*
Singleton33	8.35	Peptide transporter PTR1 OS=*A. thaliana*
Singleton34	8.31	Dehydrin COR410 OS=*Triticum aestivum*
Singleton35	8.21	DNA-directed RNA polymerase D subunit 1 OS=*A. thaliana*
Singleton36	8.18	Malate dehydrogenase, chloroplastic OS=*A. thaliana*
Singleton37	8.18	Regulatory protein NPR1 OS=*A. thaliana*
Singleton38	8.15	Putative disease resistance protein RGA1 OS=*S. bulbocastanum*
Singleton39	8.10	12-oxophytodienoate reductase 3 OS=*A. thaliana*
Singleton40	8.09	Uncharacterised protein At4g10930 OS=*A. thaliana*

TABLE 6. ABUNDANCE OF UNIQUE PROTEINS PERFORMING SPECIFIC FUNCTIONS

Function	Symptomatic		Asymptomatic	
Protein abundance	**Higher**	**Lower**	**Higher**	**Lower**
Carbohydrate metabolism	-	4	2	1
Cell wall-related defence	1	2	-	1
Cellular processes	-	12	9	5
Energy	1	1	5	-
Lipid metabolism	-	-	1	-
PR protein	1	1	1	2
Protein metabolism	2	7	6	1
Signalling	1	1	2	1
Stress response	1	11	2	-
Sugar metabolism	-	6	6	-
Transporter	-	2	2	2

that regulate multiple signalling pathways. These proteins control hormone signalling, cell division, and disease resistance. The 14-3-3 isoform protein family are key targets of bacterial effectors for *Xv3* disease resistance in tomatoes (Dubrow *et al*., 2018). The acireductone dioxygenase-like protein contributes to the production of ethylene, a product of the methionine salvage pathway (Friedman *et al*., 2011). It is important in maintaining the S-adenosylmethionine levels for ethylene production (White and Flashman, 2016). Ethylene is important in the hormonal regulation of plant development, as a virulence factor of fungal and bacterial pathogens, and as a signalling compound in disease resistance (Loon *et al*., 2006).

Beta-1,3-glucanases are abundant in plants; they work either alone or with chitinases and other antifungal proteins to protect plants against fungal pathogens (Balasubramanian *et al*., 2012). Beta-1,3-glucanases inhibit the growth of fungal pathogens when they catalyse the hydrolytic cleavage of beta-1,3-glucans, a major structural component present in the fungal cell wall (Su *et al*., 2016).

Cyclophilins have multiple functions and are highly versatile. Many growth and development processes in plants, particularly hormone signalling and stress responses, are regulated by cyclophilins (Singh *et al*., 2020). In soybean, the *CYP1* transcript level was reduced in response to *Phytophthora sojae* infection (Mainali *et al*., 2017). Since isoflavonoids are involved in plant stress resistance against biotic and abiotic factors, the interaction of *GmCYP1* with the isoflavonoid regulators *GmMYB176* and 14-3-3 protein, suggests its role in the defence system of soybean.

Enolase is essential for the growth and development of plants. Altered ENO2 functions in *Arabidopsis* plants have reduced cell size and defective cell differentiation with restricted lignification (Eremina *et al*., 2015).

The 40S ribosomal protein S18-like protein is highly abundant in asymptomatic seedlings (*Table 8*). Ribosomal proteins are important in maintaining the stability of the ribosomal complex and mediating protein synthesis (Moin *et al*., 2016). Rice infected with *X. oryzae* and *Rhizoctonia solani* have induced the up-regulation of several ribosomal proteins. The transcript levels of *RPS4* are also higher during biotic stresses (Saha *et al*., 2017).

The cellular membrane potential in plants is established by plasma membrane (PM) H^+-ATPases (Elmore and Coaker, 2011). They interact with *RIN4*, a negative regulator of disease resistance, to manage stomatal apertures during *Pseudomonas syringae* invasion of leaf tissues in *Arabidopsis* (Liu *et al*., 2009). Furthermore, PM H^+-ATPase activities have been manipulated by evolving pathogens during infection, and are targeted by pathogens to increase plant susceptibility (Elmore and Coaker, 2011).

Receptor kinases are important in plant immunity, growth and development. They deploy many receptor kinases as pattern recognition receptors that detect microbe- and host-derived molecular patterns as the first layer of inducible defence (Tang *et al*., 2017). The serine/threonine protein phosphatase 2A controls pathogenesis responses in various plant species. It contributes to the regulation of receptor signalling, organellar signalling, gene expression, metabolic pathways, and cell death, all of which essentially impact plant immunity (Durian *et al*., 2016).

CONCLUSION

Research using a combination of omics technologies is useful for providing a universal view of understanding the molecular systems that underlie various functions in oil palm seedlings infected with BSR. The present work explored both the transcripts

TABLE 7. PROTEINS WITH DIFFERENT ABUNDANCE PRESENT IN THE ROOTS OF SYMPTOMATIC OIL PALM SEEDLINGS

Accession	UniProt	Name	Fold change	Unused ProtScore	Total ProtScore	% Coverage	Peptide (95%)
a) Proteins with higher abundance							
gi|310689155	E3V1H6	6-phosphogluconate dehydrogenase [*Pinus sylvestris*]	87.90	2.00	4.42	17.4	7
gi|514814998	K4A649	PREDICTED: 5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase-like [*Setaria italica*]	87.90	3.01	8.83	18.7	7
gi|192910882	B3TLW8	Beta-1,3-glucanase [*Elaeis guineensis*]	6.14	12.64	12.64	38.1	28
gi|7271955	Q9M511	Cytosolic pyruvate kinase [*Lilium longiflorum*]	6.08	3.16	3.17	8.4	2
gi|192910890	B3TLX2	Early flowering protein 1 [*E. guineensis*]	2.11	16.06	16.06	55.1	17
gi|192911934	B3TLZ4	Temperature-induced lipocalin [*E. guineensis*]	1.29	2.03	2.03	15.4	1
gi|502136998	A5BUU4	PREDICTED: 40S ribosomal protein SA-like [*Cicer arietinum*]	1.19	8.00	8.00	20.9	7
gi|148927382	A6N8F8	Cysteine proteinase [*E. guineensis*]	1.18	4.76	4.76	19.2	7
gi|75259589	Q6I544	RecName: Full=Germin-like protein 5-1; Flags: Precursor	1.14	5.87	5.87	18.6	8
gi|22535406	Q6F2Y7	Heat shock protein 101 [*O. sativa*]	1.06	2.34	2.34	4.6	1
b) Proteins with lower abundance							
gi|164652940	B6RF01	14-3-3d protein [*Gossypium hirsutum*]	90.09	2.00	2.00	6.5	1
gi|192913044	B3TM49	Aci-reductone dioxygenase-like protein [*E. guineensis*]	90.09	2.44	2.44	1.3	1
gi|448872690	M1H922	Alpha-1,4-glucan-protein synthase [*E. guineensis*]	90.09	4.00	4.00	27.8	2
gi|192910882	B3TLW8	Beta-1,3-glucanase [*E. guineensis*]	90.09	2.16	2.16	6.4	2
gi|192910744	B3TLP9	Cyclophilin [*E. guineensis*]	90.09	2.00	2.00	4.0	1
gi|255539693	B9R9N6	Enolase isoform X2, putative [*Ricinus communis*]	89.29	1.57	1.59	10.7	2
gi|255539693	B9R9N6	Enolase isoform X2, putative [*R. communis*]	77.52	3.48	9.68	26.5	7
gi|511537412	R9VV88	FVE protein [*Litchi chinensis*]	42.02	2.22	4.30	23.8	2
gi|508785294	A0A061GRR9	General regulatory factor 7, NU [*Theobroma cacao*]	40.16	2.00	5.13	17.1	3
gi|192910866	B3TLW0	Glutathione S-transferase [*E. guineensis*]	36.36	4.05	4.05	5.2	2
gi|82400215	Q2XQF4	Glyceraldehyde 3-phosphate dehydrogenase [*E. guineensis*]	33.11	15.00	15.00	62.4	12
gi|302805526	D8SMA1	Hypothetical protein SELMODRAFT_156635 [*Selaginella moellendorffii*]	25.84	2.42	2.42	10.8	1
gi|192910730	B3TLP2	Light-inducible protein ATLS1 [*E. guineensis*]	22.08	2.85	7.17	9.3	5
gi|351724907	Q71EW8	Methionine synthase [*Glycine max*]	18.18	4.01	10.91	59.3	9
gi|192910736	B3TLP5	Mitochondrial F0 ATP synthase D chain [*E. guineensis*]	16.00	3.62	3.62	19.8	1
gi|897618	C0HF32	Mitochondrial F-1-ATPase subunit 2 [*Zea mays*]	15.70	5.89	5.89	21.9	3
gi|192910872	B3TLW3	Pathogenesis-related protein [*E. guineensis*]	12.24	11.21	11.21	30.3	9
gi|211906450	D2D2Z8	Phosphoglycerate kinase [*Gossypium hirsutum*]	9.73	4.16	4.16	12.5	2
gi|508712850	A0A061EK37	Phosphoglycerate mutase, 2,3-bisphosphoglycerate-independent isoform 2 [*Theobroma cacao*]	9.63	2.00	2.00	7.2	1
gi|359482944	F6HYG1	PREDICTED: 97 kDa heat shock protein-like [*Vitis vinifera*]	8.47	12.60	12.60	39.1	8
gi|470102123	Q8S9S4	PREDICTED: IAA-amino acid hydrolase ILR1-like 4-like [*Fragaria vesca*]	8.02	4.21	4.21	8.6	3
gi|514779322	K3XJN7	PREDICTED: malate dehydrogenase, mitochondrial-like [*Setaria italica*]	7.45	5.33	5.33	30.7	3
gi|514814448	K4A6V8	PREDICTED: phosphoglucomutase, cytoplasmic 2-like isoform X2 [*S. italica*]	6.92	8.93	8.93	35.3	8
gi|225452974	Q42290	PREDICTED: probable mitochondrial-processing peptidase subunit beta [*V. vinifera*]	5.34	15.75	15.75	36.2	12
gi|514798087	K3YJL3	PREDICTED: proteasome subunit beta type-1-like [*S. italica*]	5.01	4.05	23.44	49.2	28

Note: Unused (ProtScore)- A measure of the protein confidence for a detected protein, calculated from the peptide confidence for peptides from spectra that have not already been completely "used" by higher scoring winning proteins. For 95% confidence, the required Unused ProtScore is 1.3; Total (ProtScore)- A measure of the total amount of evidence for a detected protein; % Coverage- The percentage of matching amino acids from identified peptides divided by the total number of amino acids in the sequence.

TABLE 8. PROTEINS WITH DIFFERENT ABUNDANCE PRESENT IN THE ROOTS OF ASYMPTOMATIC OIL PALM SEEDLINGS

Accession	UniProt	Name	Fold change	Unused ProtScore	Total ProtScore	% Coverage	Peptide (95%)
a) Proteins with higher abundance							
gi|502093390	P34788	PREDICTED: 40S ribosomal protein S18-like [*Arabidopsis thaliana*]	99.1	1.48	1.48	17.8	1
gi|159465541	P55195	Phosphoribosylaminoimidazole carboxylase, eukaryotic-type [*Chlamydomonas reinhardtii*]	87.9	1.35	1.35	3.2	1
gi|46430485	Q75N96	Plasma membrane H+-ATPase [*Daucus carota*]	87.9	2	5.24	7	3
gi|514778521	K3XF69	PREDICTED: probable inactive receptor kinase At2g26730-like [*Setaria italica*]	87.1	2	2	2.5	1
gi|514728147	Q38950	PREDICTED: serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A beta isoform-like isoform X2 [*S. italica*]	86.3	2.7	2.7	9.1	1
gi|310689155	E3V1H6	6-phosphogluconate dehydrogenase [*Pinus sylvestris*]	77.3	2	4.42	17.4	7
gi|192911934	B3TLZ4	Temperature-induced lipocalin [*Elaeis guineensis*]	6.9	2.03	2.03	15.4	1
gi|374256013	H6TNN9	Putative methionine synthase protein, partial [*E. guineensis*]	5.4	7.68	10.61	28.9	7
gi|192910908	B3TLY1	Fructose-bisphosphate aldolase [*E. guineensis*]	5.1	18.23	18.23	51.4	20
gi|48527431	Q5J1K3	Elongation factor 1-alpha 1 [*E. guineensis*]	5	12.62	12.62	27.3	14
gi|25396545	A9TW09	Alpha tubulin [*Physcomitrium patens*]	4.4	19.07	19.07	36.3	14
gi|47026989	Q677B2	Nucleoside diphosphate kinase [*Hyacinthus orientalis*]	4	6	6	19.5	4
gi|192910730	B3TLP2	Light-inducible protein ATLS1 [*E. guineensis*]	3.9	4.09	4.09	42.6	2
gi|192910882	B3TLW8	Beta-1,3-glucanase [*E. guineensis*]	3.8	16.88	16.88	36	28
gi|4126976	Q9ZWI8	Vacuolar H+-pyrophosphatase [*Chara corallina*]	3.5	1.47	1.47	4.3	1
gi|50251203	Q6EUQ9	Putative vacuolar proton-ATPase [*Oryza sativa*]	3.5	15.45	15.45	26.7	14
gi|192910674	B3TLL4	Triose phosphate isomerase cytosolic isoform [*E. guineensis*]	2.8	4.49	4.54	26	5
gi|81686725	Q6ZI55	NAD-dependent isocitrate dehydrogenase c;2 [*O. sativa*]	2.5	4.03	4.03	16.9	2
gi|357167236	P08926	PREDICTED: ruBisCO large subunit-binding protein subunit alpha, chloroplastic-like [*Brachypodium distachyon*]	2.3	9.85	9.85	19.9	8
gi|192910890	B3TLX2	Early flowering protein 1 [*E. guineensis*]	2	13.68	13.68	56.3	15
gi|502082135	P54774	PREDICTED: cell division cycle protein 48 homolog [*Cicer arietinum*]	1.7	6.77	6.77	13.1	4
gi|225435632	P28723	PREDICTED: formate--tetrahydrofolate ligase-like [*Vitis vinifera*]	1.4	2.79	2.79	4.9	1
gi|77553679	Q2QXR7	Pyruvate kinase family protein expressed [*O. sativa*]	1.4	4	4	12.5	2
gi|470107271	Q6K9N6	PREDICTED: succinyl-CoA ligase [*ADP*-forming] subunit beta, mitochondrial-like [*Fragaria vesca* subsp. *vesca*]	1.2	4.13	4.13	22	4
gi|353441078	G8FGH1	Putative enolase [*E. guineensis*]	1.2	4.01	10.91	59.3	9
gi|192910928	B2CZK7	Plasma membrane intrinsic protein [*E. guineensis*]	1.2	5.07	5.07	22.3	5
gi|508722839	A0A061G0U1	Sugar isomerase (SIS) family protein isoform 2 [*Theobroma cacao*]	1.1	5.12	5.12	9.5	3
gi|192910860	B3TLV7	Adenine phosphoribosyltransferase 1 [*E. guineensis*]	1.1	2.59	2.59	18.8	1
gi|353441104	G8FGJ2	Putative cytosolic malate dehydrogenase [*E. guineensis*]	1.1	1.51	6.32	32	4
gi|225452974	Q42290	PREDICTED: probable mitochondrial-processing peptidase subunit beta [*V. vinifera*]	1.1	4.53	4.53	9.9	2
gi|192912964	B3TM09	Protein disulphide isomerase 2 precursor [*E. guineensis*]	1.1	10.67	10.67	25.7	6
gi|897618	C0HF32	ATP synthase subunit beta [*Zea mays*]	1	38.01	38.01	56.2	42
b) Proteins with lower abundance							
gi|8698725	Q42605	Bifunctional UDP-glucose 4-epimerase and UDP-xylose 4-epimerase 1 [*A. thaliana*]	89.3	1.79	1.79	12	2
gi|192910924	B3TLY9	Annexin P35 [*E. guineensis*]	5.1	4.76	4.76	33.7	6
gi|508774440	A0A061FWS9	P-type H(+)-exporting transporter [*T. cacao*]	4.8	4.88	4.88	6.1	2
gi|192910890	B3TLX2	Early flowering protein 1 [*E. guineensis*]	4.7	15.76	15.76	65.8	23
gi|192912982	B3TM18	Pathogenesis-related protein 10c [*E. guineensis*]	4.5	9.36	11.54	52.2	7
gi|192912964	B3TM09	Protein disulphide isomerase 2 precursor [*E. guineensis*]	4.3	25.16	25.16	41.3	13
gi|502136998	A5BUU4	PREDICTED: 40S ribosomal protein SA-like [*Cicer arietinum*]	4.2	8	8	20.9	7
gi|474160132	M7ZC83	Adenosylhomocysteinase [*Triticum urartu*]	4.1	4.66	4.66	6.9	3
gi|448872668	M1GS77	5′-methylthioadenosine/s-adenosylhomocysteine nucleosidase 1-like protein [*E. guineensis*]	3.4	4.33	4.33	13.1	3
gi|192911934	B3TLZ4	Temperature-induced lipocalin [*E. guineensis*]	3.3	2.59	2.59	42.6	1
gi|508774440	A0A061FWS9	H(+)-transporting ATPase plant/fungi plasma membrane type [*T. cacao*]	2.5	7.38	7.38	4.9	4
gi|192910866	B3TLW0	Glutathione S-transferase [*E. guineensis*]	1.9	5.33	5.33	30.7	3
gi|148872938	A6MZ92	ATP citrate lyase alpha subunit [*Glycyrrhiza uralensis*]	1	6.16	6.16	19.2	4

Note: Unused (ProtScore)- A measure of the protein confidence for a detected protein, calculated from the peptide confidence for peptides from spectra that have not already been completely "used" by higher scoring winning proteins. For 95% confidence, the required Unused ProtScore is 1.3; Total (ProtScore)- A measure of the total amount of evidence for a detected protein; % Coverage- The percentage of matching amino acids from identified peptides divided by the total number of amino acids in the sequence.

and proteins in artificially inoculated seedlings after a 12 months prolonged exposure to *Ganoderma*. The biological functions of asymptomatic seedlings resembled those of healthy, uninoculated ones where cellular processes for regular growth and development were observed. Stress-responsive proteins and transcripts related to carbon fixation, glycolysis and pyruvate metabolism were detected in symptomatic seedlings. The profiles of asymptomatic and symptomatic seedlings can be used to understand the behaviour of host plants towards the mechanisms of disease infection after prolonged exposure. These findings add value to previous publications where seedlings were usually destructed after a three to four months exposure to the disease, and comparisons were made only for control and treated seedlings. Further investigations into the mechanisms of disease tolerance in oil palm could be proposed, where asymptomatic seedlings are thoroughly scrutinised. Integration of transcriptomic and proteomic approaches will also provide a better understanding of host-pathogen interactions, leading to new insights which would not be easy to attain using either the protein or mRNA differential analysis alone.

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