THIAMINE BIOSYNTHESIS GENE EXPRESSION ANALYSIS IN *Elaeis guineensis* DURING INTERACTIONS WITH *Hendersonia toruloidea*

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ABSTRACT

Thiamine plays an indispensable role as a cofactor in many metabolic reactions in all living organisms. The active form, thiamine pyrophosphate (TPP) functions as a cofactor in important metabolic reactions including glycolysis, pentose phosphate pathway and the tricarboxylic acid cycle. Besides that, thiamine is also associated with the induction of systemic acquired resistance (SAR) in plants. *Hendersonia toruloidea* is an endophytic fungus originally isolated from oil palm roots which have been shown to have biocontrol activity. Seven months old oil palm seedlings were inoculated with *H. toruloidea* and the response on the expression of thiamine biosynthesis genes were investigated. Microscopy analysis was performed to visualise the colonisation of the fungus. Ribonucleic acid (RNA) was extracted from oil palm leaves at Day 1, 7, 15 and 30 post-treatment. Quantitative Real-time PCR (qRT-PCR) was performed to measure the level of expression of four key thiamine biosynthesis genes, namely *THI4*, *THIC*, *TH1* and *TPK*. The results showed up to 12-fold increase in the expression of all gene transcripts at Day 1 and 7 post-treatment. This work provides first evidence of enhancement of thiamine biosynthesis by endophytic colonisation in oil palm seedlings.

Keywords: oil palm, endophytic fungus, thiamine biosynthesis, gene expression.

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INTRODUCTION

Thiamine (vitamin B1), in its active form of thiamine diphosphate is essential cofactor for key enzymes of central metabolism in cellular organisms. It is synthesised *de novo* only in plants, bacteria and fungi. The thiamine biosynthesis pathway in plants as shown in *Figure 1* consists of two separate branches, which is the synthesis of thiazole and pyrimidine moieties. The condensation

of pyrimidine and thiazole ring moieties will form thiamine monophosphate, which is then converted to the active form thiamine pyrophosphate (TPP). TPP is a cofactor of pyruvate dehydrogenase which participates in the oxidative decarboxylation of pyruvate to acetyl-CoA, synthesis of amino acid such as valine, leucine and isoleucine, 2-oxoglutarate dehydrogenase which is involved in Krebs cycle (Goyer, 2010; Goyer and Haynes, 2011; Tunc-Ozdemir *et al.*, 2009). TPP is also a cofactor for transketolase in the oxidative pentose phosphate pathway, which plays a role in the production of reactive oxidative species (ROS). Downstream reactions of TPP-dependent of Krebs cycle is the oxidative phosphorylation pathway where electron is generated to synthesise ATP, which is important in energy production in all living organisms. Fundamentally, thiamine forms an indispensable role in proper functioning in plants.

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Apart from its role as an enzymatic cofactor, previous studies had shown that thiamine has non-cofactor roles. Thiamine metabolism is involved in plant adaptation towards biotic and abiotic stress in plant and also microorganism (Rapala-Kozik *et al.*, 2012; Sylvander *et al.*, 2013). Specifically, exogenous application of thiamine was reported to confer resistance against biotic stress and abiotic stress (Ahn *et al.*, 2007; Tunc-Ozdemir *et al.*, 2009). Application of thiamine in plants have been shown to activate plant defence responses in *Arabidopsis* (Ahn *et al.*, 2007). Furthermore, thiamine treatment in soyabean also enhances resistance to charcoal rot disease (Monaim, 2011), rice against sheath blight disease (Bahuguna *et al.*, 2012), and grapevine against a disease caused by *Plasmopora viticola* (Boubakri *et al.*, 2012). In *Arabidopsis thaliana*, thiamine treatment activated pathogenesis-related protein (PR-1) and phenylalanine lyase (PAL) (Ahn *et al.*, 2007). Similarly, thiamine treatment in grapevine reduced downy mildew development compared to untreated control in a dose-dependent manner by inducing hydrogen peroxide generation, callose disposition and host resistance (HR) cell death (Boubakri *et al.*, 2012). Therefore, thiamine is involved in priming, which is important to mitigate any crop-damaging diseases and stresses. Recent studies in oil palm have also shown that the infection of *G. boninense* in oil palm upregulated the expression of the first two enzymes in thiamine biosynthesis pathway (Balai Yusof *et al.*, 2015). Various abiotic stresses have also showed to increase the expression of thiamine biosynthesis genes in oil palm (Abidin *et al.*, 2016). Wong *et al.* (2016) also reported that there was an increase of up to two-fold in thiamine biosynthesis gene expression when subjected to salinity stress induced by polyethylene glycol (PEG) in oil palm.

Oil palm is the most profitable oil-bearing crops in the world. However, productivity of oil palm is threatened by basal stem rot disease caused by *Ganoderma boninense* that resulted in major economic losses and yield gap (Barcelos *et al.*, 2015). Therefore, studies on the proper management of the disease have been accelerating. Endophytes, defined as fungi that are present in most plant tissues without causing any visible symptoms were utilised as a biological control agent as a preventive measure against the disease (Wilson, 1995). Several endophytic species have been utilised as a biological control agent namely *Actinomycetes*, *Pseudomonads*, *Trichoderma* and *Hendersonia* (Sundram *et al.*, 2011). It was reported that the application of the endophytes has growth promoting effects besides disease suppression against *G. boninense*. The endophytic fungus *Hendersonia toruloidea*, originally isolated from oil palm trunk and root tissues is used as a biocontrol agent. *In vitro* and nursery trial studies of

application of *H. toruloidea* suppressed the infection of pathogenic fungus *G. boninense* on oil palm (Idris *et al.*, 2012; Nurrashyeda *et al.*, 2011).

Although endophytes generally do not cause visible symptoms, they certainly affect the plant phenotype (Heil, 2011). Recent works demonstrated that fungal endophytes trigger the production of secondary metabolites such as alkaloids, steroids, terpenoids, quinones and phenylpropanoids which act as anti-microbial compounds (Mousa and Raizada, 2013). The production of secondary metabolites therefore increases plant's metabolic fitness and immunity. Since thiamine is associated in its role in plants immunity, the aim of this study is to investigate the response of oil palm seedlings, specifically on the expression of thiamine biosynthesis genes upon colonisation of the endophytic fungus *H. toruloidea*. The morphology and the colonisation pattern of the endophytic *H. toruloidea* was visualised on scanning electron microscopy and transmission electron microscopy.

Source: Adapted from Guan *et al.* (2014).

Figure 1. The thiamine biosynthesis pathway in plants.

MATERIALS AND METHODS

Fungal Strains, Growth Conditions and Granular Bioformulation Preparation

The fungal strain was the endophytic *H. toruloidea* that was previously isolated from healthy oil palm in disease affected areas of MPOB Teluk Intan, Perak, Malaysia (Nur Rashyeda and Idris, 2013). Pure axenic cultures were sub-cultured on potato dextrose agar and incubated at 28°C for 10 days. The conidial spores were scraped and poured into potato dextrose broth containing 9% jaggery. The fungal cultures were incubated at 28°C for four days at 150 rpm in a shaking incubator. The fungal cultures were encapsulated in an alginate formulation containing kaolin, empty fruit bunch and pectin (Idris *et al.*, 2010; 2012; Nur Rashyeda *et al.*, 2011).

Application of *H. toruloidea* to Oil Palm Seedlings

Seven months old seedlings of *Dura x Pisifera* (D x P) variety of oil palm were grown under nursery conditions at MPOB Nursery, Section 15, Bandar Baru Bangi, Selangor, Malaysia. The oil palm was inoculated with *H. toruloidea* by applying 50 g of the bioformulation (10^7 CFU g⁻¹) and drenched with tap water. Control plants were not treated. Sampling of spear leaves and roots was carried out at 0, 1, 7, 15, and 30 days post-treatment with three replicates for each time point. The tissue samples were frozen in liquid nitrogen and stored at -80°C until further analysis.

Reisolation of Endophytic Fungus by Direct Plating Technique

To confirm the presence of *H. toruloidea* in the seedlings, the endophytic fungus was reisolated from the surface-sterilised roots and placed on potato dextrose agar according to the procedure described by Idris *et al.* (2010). The 10 cm root segments were cut and dipped into 10% Clorox for 2 min. The root segments were then dipped into 70% alcohol for 1 min, followed by washing in sterile distilled water twice for 1 min. The root segments were scraped to reveal inner layer, cut into 5 mm pieces, and plated on potato dextrose agar. The agar was incubated at 28°C for seven to 10 days. The physical and morphological structures were examined and confirmed.

Scanning Electron Microscopy Analysis

To further illustrate the morphology of endophytic *H. toruloidea*, root segments were processed for scanning electron microscopy. Briefly, root samples were soaked in 4% glutaraldehyde

solution overnight. The root samples were put in dehydration series of (25%, 50%, 75%, 90%, 100%). Samples were then dried using critical point drying. Each specimen was mounted on aluminium stubs, coated with gold in a sputter, and observed in scanning electron microscopy.

Transmission Electron Microscopy Analysis

Oil palm root sections were cut into 1 mm³ pieces. The root sections were put into separate vials and fixed in fixative 4% glutaraldehyde for two days. Next, the root sections were washed with 0.1 M sodium cacodylate buffer for three changes of 30 min each. The sections were then post-fixed in 1% osmium tetroxide for 2 hr at 4°C and washed again with 0.1 sodium cacodylate buffer for three changes of 30 min each. A dehydration series of acetone 35%, 50%, 75%, 95% was performed for 45 min each series. Final dehydration series of 100% acetone was performed for three changes of 1 hr each. Further steps of processing include infiltration of the specimen with acetone and resin mixture as follows:

Acetone	Resin	Duration (hr)
1	1	12
1	3	12
	100%	12
	100%	4

The specimen was then embedded into beam capsules and filled with resin mixture. It was polymerised in oven at 60°C for 48 hr. A thick sectioning was performed by cutting the polymerised specimen using an ultramicrotome to cut 1 µm thick sections. The thick sections were stained with toluidine blue, dried on a hot plate. The stain was washed under running tap water. The area of interest was examined under light microscope. For the ultrathin sectioning, the ultrathin sections were cut and selected for silver sections. The sections were selected with a grid and dried using filter paper. For the staining procedure, the sections were stained with uranyl acetate for 15 min and washed with double distilled water. Next, the sections were stained with lead stain for 10 min and washed with double distilled water. Lastly, the sections were viewed using transmission electron microscopy.

RNA Isolation and Quantitative Real-time Polymerase Chain Reaction (qRT-PCR) Analysis

Total ribonucleic acid (RNA) was isolated from oil palm spear leaves according to CTAB method with modifications (Zeng and Yang, 2002). Genomic DNA was removed using DNase I (Promega, USA). One µg of purified RNA samples were reverse transcribed using GoScript cDNA

synthesis kit (Promega, USA). The specific primers for qRT-PCR were designed by Primer Premier 6.0 (Primer Biosoft, Palo Alto, CA, USA). In a 10 μ l PCR reaction mixture, 4.0 μ l of cDNA template, 5 μ l of 2 X SYBR SensiFast Hi-Rox (Bioline), 0.4 μ l of 10 mM of forward and reverse primers for each gene. The qRT-PCR was performed using Bio-Rad CFX Connect 96. The cycling conditions used was 2 min at 95°C, followed by 40 cycles of 10 s at 95°C, and 30 s at 60°C. Three biological replicates with three technical replicates each were assayed for each sample.

Analysis of Gene Expression

Transcript levels of each gene were normalised to reference genes tubulin, and glyceraldehyde-3-phosphate using the method by (Vandesompele *et al.*, 2002). The $2^{-\Delta\Delta CT}$ method was used to analyse the relative changes in gene expression of *THI4*, *THIC*, *TPK* and *TH1* (Livak and Schmittgen, 2001).

DNA Sequencing

Purified PCR products were sequenced using 1st BASE DNA sequencing service (1st BASE,

Singapore). The FASTA sequences were then analysed using Basic Local Alignment Search Tool (BLAST) (<https://blast.ncbi.nlm.nih.gov>) to find DNA sequences in NCBI database.

RESULTS AND DISCUSSION

Colonisation of Endophytic *H. toruloidea* in Oil Palm Seedlings

The colonisation ability of the fungus was evaluated by culture dependent method of direct plating technique to re-isolate the fungus into a potato dextrose agar. After 10 days of incubation on potato dextrose agar plate, the endophytic fungus was successfully recovered, revealing the characteristics of greyish-black mycelia on the root sections. The root sections that was sent for scanning electron microscopy analysis also revealed hyphae, conidial spores and ascospores which are sac-contained spores which confirmed the identity of the fungus (Figure 2). Further evaluation of the colonisation ability of *H. toruloidea* involved transmission electron microscopy analyses to

Figure 2. The morphological structures of *H. toruloidea* viewed via scanning electron microscopy analysis. (a) and (b) Are the hyphae structures. (c) Ascospores structures. (d) Conidial spores.

visualise the colonisation and localisation of the fungus inside the root. *Figure 3* reveals transverse section of oil palm root at Day 1 and Day 30 post-colonisation. *H. toruloidea* was found to be residing mostly in the intracellular cells of the cortical tissues.

The Effect of Colonisation of *H. toruloidea* on Expression of Thiamine Biosynthesis Genes in Oil Palm

In this study, the key thiamine biosynthesis genes in oil palm namely *THIC*, *THI4*, *TH1* and *TPK* were identified and sequenced. All primers were designed between 100-200 bp for efficient amplification and quantification (*Table 1*). The expression of thiamine biosynthesis genes in oil palm seedlings during colonisation of endophytic *H. toruloidea* was performed using qRT-PCR at different time points of 24 hr, 7-, 15- and 30-day post-treatment. After 24 hr of application of *H. toruloidea*, the oil palm seedlings showed a significantly increased expression of *THI4*, *TPK*, *THIC*, *TH1* (12.9-fold, 3.65-fold, 1.65-fold, 3.047-fold respectively).

Meanwhile at Day 7, *THI4*, *TPK*, *THIC*, *TH1* were shown to be downregulated (2.85-fold, 0.92-fold, 0.44 and 1.07-fold respectively). After two weeks and four weeks of endophytic treatment, the level expressions of *THI4*, *TPK*, *THIC* and *TH1* decreased lower than control seedlings, which was normalised to 1.

The hypothesis of the study is that the thiamine biosynthesis genes will be upregulated upon endophytic colonisation. Successful colonisation of *H. toruloidea* was visualised by transmission electron microscopy analysis which revealed that the fungus resided in intracellular tissues as shown in *Figure 3*. Overall, *THI4*, *TPK*, *THIC*, and *TH1* were upregulated after 24 hr of inoculation as shown in *Figure 4*. Previous studies have shown that thiamine and its intermediates are involved in systemic acquired resistance in various plant species and acts as a signalling molecule (Rapala-Kozik *et al.*, 2008; Tunc-Ozdemir *et al.*, 2009). The observation of upregulation of thiamine biosynthesis genes as a result of colonisation of endophytic fungus is relatively novel.

Figure 3. Transverse sections of oil palm root colonised by H. toruloidea viewed via transmission electron microscopy analysis. Red arrows showing hyphae and spores colonising intracellular cells at Day 30 of colonisation.

TABLE 1. LIST OF PRIMERS USED IN THIS STUDY

Primer name	Forward (5' to 3')	Reverse (5' to 3')	Amplicon size (bp)
Tubulin F3 R3	ACACGGCATAGATCCAACCG	TGG TTC CAG GCT CCA AATC	147
GADPH F1 R1	GTCCACCTGCTCAAGTACG	CGGACACGACCTTGATGACC	110
THI4 F3 R3	ATGATACCCACGCCGACAC	TGCTCCACGATGGCGACTTG	104
THIC F3 R3	AATGAAGGTCCAGGGCAT	GCTGAGGTGATGTGATCA	188
TH1 F5 R5	CCGACATGCCAGCACGAGTT	ACACCACCACAGCCAATGTAGT	130
TPK F5 R5	GGGAGCACTTGGTGAAGGTTT	TGTGGATCTCATGGCGATGTGT	140

Figure 4. The effect of colonisation of *H. toruloidea* on the expression of *THI4*, *THIC*, *TH1*, and *TPK*. C0: control Day 0, T1: *H.t* inoculated Day 1, T7: *H.t*-inoculated Day 7, T15: *H.t*-inoculated Day 15, T30: *H.t*-inoculated Day 30. Error bars indicate SE between three biological replicates. Asterisks indicate significant difference when compared to control using Student T-test, ($p < 0.05$).

It was observed that the level of expression of *THI4*, which is the first enzyme in the thiamine biosynthesis pathway of the thiazole branch was remarkably high upon 24 hr of endophytic colonisation by *H. toruloidea*, which was about 12-fold. Because of the relatively long duration of observation, the effects of endophytic colonisation, the downregulation of thiamine biosynthesis genes after seven days of inoculation was due to adaptation to the establishment of the *H. toruloidea* in the oil palm seedlings. The level of expression of *TH1*, *TPK* and *THIC* was not prominent compared to *THI4*. This suggest that *THI4*, which is the thiazole synthase is involved in stress responses. In fungal pathogen *Verticillium dahlia*, VdTHI4, which was found to be localised in the mitochondria is required for stress tolerance (Hoppenau *et al.*, 2014).

It was implied that the upregulation of thiamine biosynthesis genes specifically through the

increase of expression of *THI4* was caused by the increased demand of TPP which acts as coenzymes in numerous physiological processes notably glycolysis, pentose phosphate pathway, synthesis of nucleic acid and synthesis of NADH. The increased requirement of the TPP-dependent enzymes was probably due to perturbations in the metabolism to deal with stress and adaptive responses due to *H. toruloidea* inoculation in the oil palm seedlings (Rapala-Kozik *et al.*, 2008).

The application of endophyte is an excellent strategy to activate systemic acquired resistance in plants. Numerous studies have highlighted that potential role of fungal endophytes in plant protection through the upregulation of secondary metabolites namely phytohormones, phenolic compounds, and defence enzymes (Gao *et al.*, 2010; Kumara *et al.*, 2014). Endophyte-mediated systemic acquired resistance could serve as disease control strategies to develop plants with enhanced disease

resistance. The relationship between thiamine and systemic acquired resistance was established by Ahn *et al.* (2007) in which thiamine treatment elicited transient expression of pathogenesis-related (PR) genes and hypersensitive responses in rice, *Arabidopsis* and cucumber. Similarly, thiamine treatment in rice induced priming, which resulted in higher hydrogen peroxide content, total phenolics accumulation and phenylalanine-lyase activity (Bahuguna *et al.*, 2012). Meanwhile, the pathogenic fungus *Sclerotinia* inoculated on *Arabidopsis* upregulated thiamine biosynthesis and correlated with increased accumulation of thiamine, thiamine monophosphate, and thiamine pyrophosphate (Zhou *et al.*, 2013). This implies that there is an increase in *de novo* synthesis of endogenous thiamine upon *Sclerotinia* infection. Thiamine-treated plant confers plant resistance to the pathogenic fungus by producing reactive oxidative species (ROS) to promote defence-signalling. Therefore, in relation to this study, it can be safely implicated that thiamine biosynthesis, specifically the upregulation of *THI4* was due to early adaptive defence responses in the oil palm seedlings upon active colonisation of *H. toruloidea*, as confirmed by scanning electron microscopy and re-isolation studies.

Ideally, it is a minimal requirement that both *THIC* and *THI4*, the upstream enzymes on both branches of pyrimidine and thiazole have to be upregulated to increase the total thiamine pool (Dong *et al.*, 2016; Pourcel *et al.*, 2013). It was shown that *THI4* was significantly upregulated to 12.9-fold after 24 hr of endophytic colonisation. Meanwhile, the increased level of expression of *THIC* upon colonisation of endophytic fungus is not as prominent which is about 1.65-fold. This can be explained as *THIC* is a highly complex energy-expensive enzyme. The synthesis of *THIC* requires a big energy investment as structural studies have shown that *THIC* is a highly complex enzyme, as it is made of an iron-sulphur cluster, S-adenosyl methionine and ferredoxin-thioredoxin redox system (Colinas and Fitzpatrick, 2015). The compound is also involved in oxygenic photosynthesis and is regulated according to the circadian rhythm (Colinas and Fitzpatrick, 2015).

Apart from that, *THIC* which is responsible for synthesis of HMP-PP (hydroxymethylpyrimidine-pyrophosphate), is required for synthesis of TMP by condensation between HMP-PP and HET-P. The unbalanced magnitude of expression of *THIC* and *THI4* could be explained by involvement of other sources of HMP-PP. In yeast (*Saccharomyces cerevisiae*) thiamine biosynthesis pathway, HMP-PP is obtained from pyridoxal 5-phosphate (PLP) biosynthesis pathway (Li *et al.*, 2010). This implies that *THIC* is not significantly upregulated if HMP-PP pool is already sufficient.

It is interesting to note that after seven days and 15 days post-inoculation, the expression of thiamine biosynthesis genes is lower in inoculated seedlings than control seedlings. This suggests tight regulatory processes, where the genes are switched off when thiamine pool is already sufficient. It also implicates that the endophytic fungus *H. toruloidea* also synthesises thiamine, therefore, thiamine biosynthesis machinery in oil palm are repressed. Although plants generally biosynthesise thiamine, it may be more advantageous to obtain from various external sources through biotic interactions (Helliwell *et al.*, 2014). Paerl *et al.* (2015) reported that during co-culturing of auxotrophic picoeukaryotic algae *Ostreococcus lucimarinus* and bacterium *Pseudoalteromonas* sp, the algae was able to salvage thiamine from the bacterium.

CONCLUSION

The major findings presented here showed that successful colonisation by *H. toruloidea* in oil palm seedlings resulted in upregulation of thiamine biosynthesis genes. The attenuation in thiamine biosynthesis signals adaptation, which may be important to maintain optimum growth and function in plants. Further molecular, biochemical and physiological studies are needed to understand the roles and functions of thiamine in oil palm stress protection.

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