

Full Length Research Paper

Osmotic stress upregulates the transcription of thiamine (vitamin B1) biosynthesis genes (THIC and THI4) in oil palm (*Elaeis guineensis*)

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Thiamine or vitamin B₁ comprises a pyrimidine moiety and a thiazole moiety. Thiamine pyrophosphate (TPP), the active form of thiamine, acts as a cofactor for various major enzymes, for example, transketolase (TK), α -ketoglutarate dehydrogenase (KGDH) and pyruvate dehydrogenase (PDH). It is suggested that TPP plays another important role, which is protecting plants against abiotic and biotic stresses such as osmotic stress. In this study, the gene transcripts of first two enzymes in thiamine biosynthesis pathway, THIC and THI1/THI4 were identified and amplified from oil palm tissues. Primers were designed based on sequence comparison of the genes from *Arabidopsis thaliana*, *Zea mays*, *Oryza sativa* and *Alnus glutinosa*. Oil palm's responses in terms of the expression profiles of these two thiamine biosynthesis genes to an osmotic stress inducer, polyethylene glycol (PEG) were examined. The level of gene transcripts was analyzed using reverse transcriptase polymerase chain reaction (RT-PCR) and both THIC and THI1/THI4 gene transcripts were successfully amplified. The levels of transcription were measured and the results showed that the expression of THIC gene transcripts showed an increase of up to 200% in 1% PEG treated plant as compared to non-treated plant while the expression of THI1/THI4 gene transcripts showed an increase of up to 100% in treated plant as compared to non-treated plant. However, increase in concentration of PEG showed decrease in amount of transcription level for both gene transcripts. The results support the suggestion that thiamine may play an important function in plant defense against stresses as these findings may lead to an overexpression of thiamine in general.

Key words: Thiamine, vitamin B₁, osmotic stress, oil palm, gene expression.

INTRODUCTION

Thiamine is important for all living organism as it serves vital functions in carbohydrate metabolism,

nicotinamide adenine dinucleotide phosphate (NADPH) and adenosine triphosphate (ATP) synthesis and also

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nucleic acids formation (Nosaka, 2006). Thiamine pyrophosphate (TPP), the active form of thiamine acts as a cofactor for various key enzymes for example pyruvate dehydrogenase, transketolase and α -ketoglutarate dehydrogenase (Frank et al., 2007). Thiamine and its derivative, thiamine pyrophosphate (TPP) play important roles in human nutrition and central metabolism (Tunc-Ozdemir et al., 2009; Guan et al., 2014). In previous studies, thiamine and TPP are reported as crucial stress-response molecules in plant adaptations to counteract different abiotic stress conditions (Tunc-Ozdemir et al., 2009; Rapala-Kozik et al., 2012). Thiamine enhances resistance to oxidative stress via salicylic acid (SA) signaling pathway (Ahn et al., 2007).

Naturally, osmotic stress occurs through drought, salinity or even cold stress which lowers the water potential in plant cell (Hoffmann, 2002). Studies have shown that deficit in water status in plants has led to the rapid changes in gene expression (Yamaguchi-Shinozaki and Shinozaki, 2006; Osakabe et al., 2011). Besides that, osmotic stress in plants has been shown to inhibit the plant growth via efflux of potassium ion (K^+) which is essential for osmoregulation and plant growth (Maggio et al., 2006; Zonia and Munnik, 2007). K^+ aid in guard cells' functions in opening and closure of stomata for osmoregulation (Schroeder and Hagiwara, 1989; Pei et al., 1997; Ache et al., 2000; Hosy et al., 2003; Kim et al., 2010) and regulates the sap flow from roots to shoots in plants with nutrients for plant growth (Lebaudy et al., 2007). Upregulation of phytohormone synthesis, such as abscisic acid, under osmotic stress has also been reported (Osakabe et al., 2013). Apart from that, osmotic stress has also showed evidence in formation of oxygen radical species via the upregulation of antioxidant enzymes and accumulation of compounds which neutralize those radical species (Upadhyaya and Panda 2004; Upadhyaya et al., 2008, 2011; Gill and Tuteza 2010). Severe exposure to osmotic stress to plants will lead to wilting and necrosis (Upadhyaya et al., 2013).

Thiamine biosynthesis

Animals and humans must consume thiamine through their diets because they cannot synthesize it, while plants and microorganism can biosynthesize it *de novo* (Moulin et al., 2013). The pyrimidine and thiazole moieties are synthesized in distinct branches of the pathway and are then condensed to form thiamine monophosphate (TMP). TMP is then phosphorylated by a specific kinase to form TPP. Plants synthesize TPP from elementary precursors via *de novo* biosynthetic pathways that are analogous to both bacteria and yeast (Goyer, 2010). The initial phases of TPP biosynthesis include two parallel pathways. One is like the mechanism found in bacteria in which the pyrimidine branch of thiamine (4-amino-2-methyl-5-hydroxymethylpyrimidinemonophosphate, HMP-

P) is synthesized from 5-aminoimidazole ribonucleotide (AIR) and is catalysed by an enzyme, which is encoded by THIC gene and has been identified in *Arabidopsis thaliana* (Raschke et al., 2007). The other pathways are similar to the mechanism found in yeast (Chatterjee et al., 2008) wherein the thiazole branch of thiamine (4-methyl-5-(2-hydroxyethyl)-thiazole phosphate, HET-P) is synthesized from glycine, nicotinamide adenine dinucleotide (NAD^+) and a sulphur donor protein. The THI1 gene, which has been identified in *Zea mays* and *Arabidopsis thaliana* (Machado et al., 1996) and its orthologue, the THI4, which is found in bacteria encodes for the main enzyme that synthesizes HET-P. Studies on THI4 gene in yeast proved that it has two functions, in thiamine biosynthesis and also in DNA damage tolerance when subjected to abiotic stress (Machado et al., 1997). Research by Rapala-Kozik et al. (2008) revealed the regulation of thiamine metabolism in *Z. mays* seedlings under different abiotic stresses. In the study, *Z. mays* seedlings were exposed to drought, salinity and oxidative stress. The total thiamine content in the maize seedlings increased under different stress conditions. Experiments by Tunc-Ozdemir et al. (2009) proved that under oxidative stress, high salinity, sugar deprivation and hypoxia, the regulation of THI1 gene transcript was increased. Thiamine and TPP serve as essential stress-response molecules during different abiotic stress conditions to combat the condition. Accumulation of thiamine and TPP was observed in *Arabidopsis* when exposed to abiotic stresses like high light intensity, low temperature, osmotic, salinity and oxidative treatment. Enhanced tolerance towards oxidative stress was observed when plants were supplemented with exogenous thiamine. Studies by Ahn et al. (2005) and Zhang et al. (1998) also showed that thiamine affects the defence-related genes or systemic acquired response (SAR)-related genes expression in plants for example tobacco, *Arabidopsis* and wheat. The SAR-related genes were expressed enormously in thiamine treated plants. Recently, a study by Balia Yusof et al. (2015) proved that there is an upregulation in the expression of THIC and THI1/THI4 genes transcripts in *Ganoderma boninense* infected oil palm.

Since the previous studies have shown that thiamine can improve the immune system of plants, it is believed that sustained accumulation of thiamine can make plants immune to severe diseases. Oil palm is an economically valuable crop for its oil and has become one of the leading oil crops in the world. Malaysia, the world's second largest producer and exporter of palm oil and its by-products, produced nearly 18 million tons in 2011. However, as sessile organism, oil palm faces a whole lot of stresses. Both biotic and abiotic stresses have negative influences on oil palm survival, palm oil production and crop yield. Common environmental stresses in Malaysia include water deficit, high

temperature and salinity. Among the abiotic factors, water deficit is the most common stress that restricts oil palm growth, survival, distribution and productivity.

In this study, the changes in the transcription level of thiamine biosynthesis genes (THIC and THI1/THI4) in oil palm when subjected to polyethylene glycol (PEG)-induced osmotic stress was analyzed. Since thiamine may play an important function in plant protection against stress, it is hypothesized that there will be an increase in the transcription level of THIC and THI1/THI4 gene transcripts once subjected to osmotic stress.

MATERIALS AND METHODS

Plant materials and stress treatment

A total of 12 six-month old commercial Dura × Pisifera oil palm seedlings were obtained from Sime Darby, Banting. The palms were arranged in a shaded area using randomized complete block design (RCBD) and subjected to the general nursery practices. A set of 3 seedlings were used as control (0% PEG) and different concentrations of polyethylene glycol 6000 (PEG) were given as treatments (1% PEG, 3% PEG and 5% PEG) for its respective sets.

Sampling

Sampling was scheduled at 3, 7 and 30 days post-treatment. Spear leaves were taken as tissue samples from 3 seedlings at each sampling point. The spear leaves were cleaned and cut before being frozen in liquid nitrogen and kept at -80°C for further use.

Data mining and primer designing

Data mining includes the gathering of genes sequences of thiamine biosynthesis pathway (THIC and THI1/THI4) from different plant species from the GenBank of NCBI database. The nucleotide sequences were aligned using ClustalW program and the consensus regions were identified and used to design PCR primers.

Total RNA isolation and quantitation

Total RNA was isolated using the modified RNA extraction protocol by Li and Trick (2005). The tissues from spear leaves at 3-, 7- and 30- days post-treatment were used for RNA extraction. The assessment of RNA quality and integrity was done using a NanoPhotometer (Implen, Germany) and the purified RNA was stored at -80°C.

Amplification of THIC and THI1/THI4 genes

RT-PCR was performed using Tetro cDNA Synthesis Kit (Bioline, USA). Complementary DNA (cDNA) was prepared by mixing 5 µg/µl of total RNA, 4 µl of 5× Reverse Transcriptase Buffer, 1 µl of 10 mM dNTP mix, 1 µl of Oligo (dT)₁₈ Primer, 1 µl of Ribosafe RNase Inhibitor, 1 µl of Tetro Reverse Transcriptase and up to 20 µl of DEPC-treated water. The solution was mixed gently via pipetting prior subjection to incubation at 45°C for 30 min. The solution was then incubated at 85°C for 5 min to stop the reverse

transcriptase reaction. The cDNA prepared was then chilled in ice and stored in -20°C until used for analysis with PCR.

PCR reaction was performed by using MyTaq™ Red Mix (Bioline, USA). A 25 µl reaction was prepared by mixing 1 µl of cDNA Template, 0.5 µl of forward primer, 0.5 µl of reverse primer, 12.5 µl of MyTaq Red Mix and up to 25 µl of deionized water. The reaction mixture was mixed and placed inside the thermocycler (Biometra, Germany). The PCR cycling requirements included the initial denaturation step which was set at 95°C for 2 min for 1 cycle, followed by 28 repetitive cycles of denaturation step at 95°C for 45 s, annealing step at 55°C for 45 s and extension step at 72°C for 1 min. The final cycle of extension was set at 72°C for 5 min for 1 cycle and then held at 4°C. The optimized annealing temperature for THIC primer F3 and THI1/THI4 primer F8 are 55°C while for THIC primer F2 is 48°C. The PCR product was then kept in the -20°C freezer for further use.

Analysis of PCR products

PCR products were analyzed using gel electrophoresis to detect amplification of gene fragments. The gel was run alongside actin gene fragment which acts as control. The bands intensities were calculated using ImageJ software (<http://imagej.nih.gov/ij/>).

PCR product purification

The PCR products were purified using FavorPrep™ Gel Purification Kit (Favorgen). 100 µl of PCR products were transferred in to a microcentrifuge tube and mixed with 500 µl of FADF buffer by vortexing for 12 min. The mixture was then transferred into FADF column in a collection tube and centrifuged for 30 s at 13,000 rpm. The flow-through was discarded and 750 µl of wash buffer was added into the FADF column and centrifuged for 30 s and further centrifuged for 3 min at 13,000 rpm. The column was then placed into a new microcentrifuge tube where the purified PCR product was collected by adding 30 µl of elution buffer to the centre of the column and centrifugeD for 2 min at 13,000 rpm after 2 min standing at room temperature. Each purified PCR product was analyzed with NanoDrop-spectrophotometer for DNA purity and concentration prior to sending them to 1st Base Company (<http://base-asia.com/dna-sequencing-services/>) for sequencing.

DNA sequencing

Purified PCR products were sent for sequencing at 1st BASE DNA sequencing service (1st BASE, Singapore). The sequencing results were then analyzed using Basic Local Alignment Search Tool (BLAST) (<https://blast.ncbi.nlm.nih.gov>) to verify the amplified gene fragments.

RESULTS

Amplification of gene transcripts

Primers were designed based on sequence alignments of different plant species as shown in Table 1. Table 2 shows the designed primers used for this study. Level of transcription was shown visually in Figure 2 by which both THIC and THI1/THI4 gene transcripts were successfully amplified in non-treated oil palm tissues using the designed primers. The fragment size for THIC amplified using primer F2 is 410 bp, while the fragment

Table 1. Data from selected plant species that were used for primer designing and their accession number from GenBank of NCBI database.

Gene	Data from GenBank	Accession no.
THIC	<i>Arabidopsis thaliana</i> phosphomethylpyrimidine synthase mRNA, complete cds	NM_001202705.1
	<i>Arabidopsis thaliana</i> phosphomethylpyrimidine synthase mRNA, complete cds	NM_128517.3
	<i>Arabidopsis thaliana</i> phosphomethylpyrimidine synthase mRNA, complete cds	NM_179804.2
	<i>Zea mays</i> clone 378320 thiamine biosynthesis protein thiC mRNA, complete cds	EU972242.1
	<i>Oryza sativa</i> Japonica Group Os03g0679700 (Os03g0679700) mRNA, complete cds	NM_001057432.1
THI1/THI4	<i>Arabidopsis thaliana</i> thiazole biosynthetic complete cds	NM_124858.3
	<i>Arabidopsis thaliana</i> Thi1 protein mRNA, complete cds	U17589.1
	<i>Zea mays</i> thiamine biosynthesis1 (thi1), mRNA	NM_001112226.1
	<i>Oryza sativa</i> Japonica Group mRNA for thiamine biosynthetic enzyme, complete cds, clone: 12YPR001	AB110170.1
	<i>A. glutinosa</i> mRNA for thiazole biosynthetic enzyme	X97434.1

Table 2. Primers designed for the amplification of THIC, THI1/THI4 and Actin gene transcripts.

Description	Primer's name	Sequences
Primers designed for THIC gene	THIC F1	5'- CATTCTCTTACAGCAAAG -3'
	THIC R1	5'- GTGATGTGATCATAACCAGG -3'
	THIC F2	5'- CTTACAGCAAAGAGAATGAC -3'
	THIC R2	5'- GTGATGTGATCATAACCAGG -3'
	THIC F3	5'- GGGATCATAACTGAGGCGGG -3'
	THIC R3	5'- ATCAGCTCCCCACATGGTTG -3'
	THIC F4	5'- CATAACTGAGGCGGGCGATT -3'
	THIC R4	5'- ACATGGTTGCCCAACTTCCT -3'
	THI4 F5	5'- CATGACGGACATGATCAC -3'
	THI4 F6	5'- CTCTCTTACCTCAACCATC -3'
Primers designed for THI1/THI4 gene	THI4 R6	5'- GCCAATACTCTTCAGCCTC -3'
	THI4 F7	5'- CCAGCTTTTGGCTCGTCCTA -3'
	THI4 R7	5'- GTCAACCGCACAATAGCGTC -3'
	THI4 F8	5'- GACGCTATTGTGCGGTTGAC -3'
	THI4 R8	5'- TCCGTCAATAGCATTCGGCA -3'

size for amplified using THIC primer F3 is 156 bp. The fragment size for THI1/THI4 amplified using primer F8 is 180 bp.

Sequencing analysis

Purified PCR products were sent for sequencing and the sequencing results were verified using BLAST. The verifications are based on the alignment identity and E-value score values where it determines the quality and similarity between the query sequence (sequencing result) and sequences available in the Genbank. The lower the E-value score, the higher the relevancy of the sequences are. However, the higher the identity score,

the higher the similarity of the sequences are. Sequencing result of actin gene fragment showed 98% identity with *E. guineensis* actin (ACT1) mRNA, complete cds (Accession number: AY550991.1) with an E value score of $5e^{-57}$. The fragment size amplified by the designed primer was 135 base pairs. Apart from that, sequencing result of THIC gene fragment amplified using primer F2 showed 99% identity with *E. guineensis* phosphomethylpyrimidine synthase, chloroplastic (LOC105046270), transcript variant X10, mRNA (Accession number: XM_010924818.1) with an E value score of 0.0. The fragment size amplified by the primer was 371 bp. Sequencing result of THIC gene amplified using primer F3 showed 92% identity with *E. guineensis* phosphomethylpyrimidine synthase, chloroplastic

(LOC105046270), transcript variant X10, mRNA (Accession number: XM_010924818.1) with an E value score of $1e^{-72}$. The fragment size amplified by the primer was 201 bp. All the mentioned sequencing results showed a high value of identity and acceptable E-value score which significantly identified the amplified fragments. However, sequencing was unsuccessful for THI1/THI4 PCR products which may be due to low PCR product concentration.

Analysis of PCR products

The level of transcription of both THIC and THI1/THI4 genes in treated and non-treated samples were analyzed using ImageJ software. In theory, it is suggested that the higher the band intensities, the more the copy number of the respective gene present in the cDNA mixture which indicates high level of transcription activity. The difference in the level of transcription in the control and PEG-treated tissue samples for both gene transcripts could be visually observed as shown in Figure 3.

Three days after the application of 1% PEG, high transcription level of THIC gene transcript was observed where there was 200% increase as compared to the control (Figure 4). However, on day 7, an increased transcription level up to 65% was observed for THIC gene transcript as compared to the control. The level of transcription of THI1/THI4 gene transcript 3 days after application of 1% PEG showed similar results as THIC gene transcript, where it showed 100% increase as compared to the control, followed by an increment of just 57% on day 7 post-treatment and only up to 28% increase on day 30 post-treatment. The THIC and THI1/THI4 genes transcriptions level were also altered in the presence of 3% PEG with a day profile similar to that of 1% PEG stress condition but the increase were only up to 1.7-fold. The gene expression pattern in the presence of 5% PEG was somehow lower as compared to the other concentrations of PEG. In short, under osmotic stress, the highest gene transcription level for both THIC and THI1/THI4 gene transcripts were observed on day 7 post-treatment (1.2-fold).

DISCUSSION

The level of gene transcription of two thiamine biosynthesis genes namely THIC and THI1/THI4 in oil palm was compared between normal condition and osmotic stress. It is believed that stress-triggered damage affects the important pathways and therefore apparently need to upregulate the thiamine biosynthetic process which is necessary (Rapala-Kozik et al., 2008, Tunc-Ozdemir et al., 2009). This study evaluated the genes encoding for

the biosynthesis of the pyrimidine and thiazole moieties of thiamine (THIC and THI1/THI4). In determining the upregulation or downregulation of the genes of interest, an osmotic stress inducer, PEG was used where oil palm seedlings were given various concentrations and tissue samples were collected at 3 time points.

The results obtained showed that there was an increase in THIC and THI1/THI4 gene transcription in the earlier stage specifically at day 3 after exposure to stress. This indicated that thiamine was needed to be synthesized in order to combat the damaging effect of osmotic stress. This is not surprising as activation of thiamine biosynthesis in plants is well established in response towards abiotic stresses (Ribeiro et al., 2005; Rapala-Kozik et al., 2012).

However, the decrease of the transcription level of THIC and THI1/THI4 gene transcripts along the period of stress treatment could be due to the fact that the palms are starting to adapt to the stress conditions. There is yet no proof that thiamine being directly involved in combating stresses, is known to have major roles in carbohydrate catabolism, NADPH and ATP synthesis and in the formation of nucleic acids (Rapala-Kozik et al., 2012) which are indirectly involved in the production of compounds involved in defence mechanism. Results from this study showed that the level of transcription for both gene were highly increased as compared to control up to day 7 post-treatment where it indicates thiamine was urgently needed. The decrease of the level of transcription of thiamine biosynthesis genes (same level with control) after day 7 probably showed the less need for thiamine due to the synthesis of stress-combating molecules which leads to the reduction of stress affects. This may show that hormonal signaling mechanism is responsible rather than water availability that affect transcriptional regulation under osmotic stress (Munns et al., 2000).

Apart from that, level of THIC gene transcript was observed to be higher as compared to THI1/THI4 gene transcript level. As previously mentioned, THIC and THI1/THI4 are the genes that encode the first enzymes of pyrimidine and thiazole moieties respectively of the thiamine biosynthesis pathway as shown in Figure 1. These enzymes play a crucial role in thiamine biosynthesis, yet they seem to have a non-cofactor function in DNA damage tolerance induced by abiotic and biotic stresses in plants (Goyer, 2010). Studies on THI4 gene in yeast have proved that it has a dual role, in thiamine biosynthesis and also in DNA damage tolerance when subjected to abiotic stress (Machado et al., 1997).

This may be the reason why somehow the upregulation of the THI1/THI4 gene transcript level was not as high as THIC gene. As a whole, the results showed that indeed, the transcription level for both genes transcript were upregulated in PEG-treated oil palm seedlings which proved this study hypothesis.

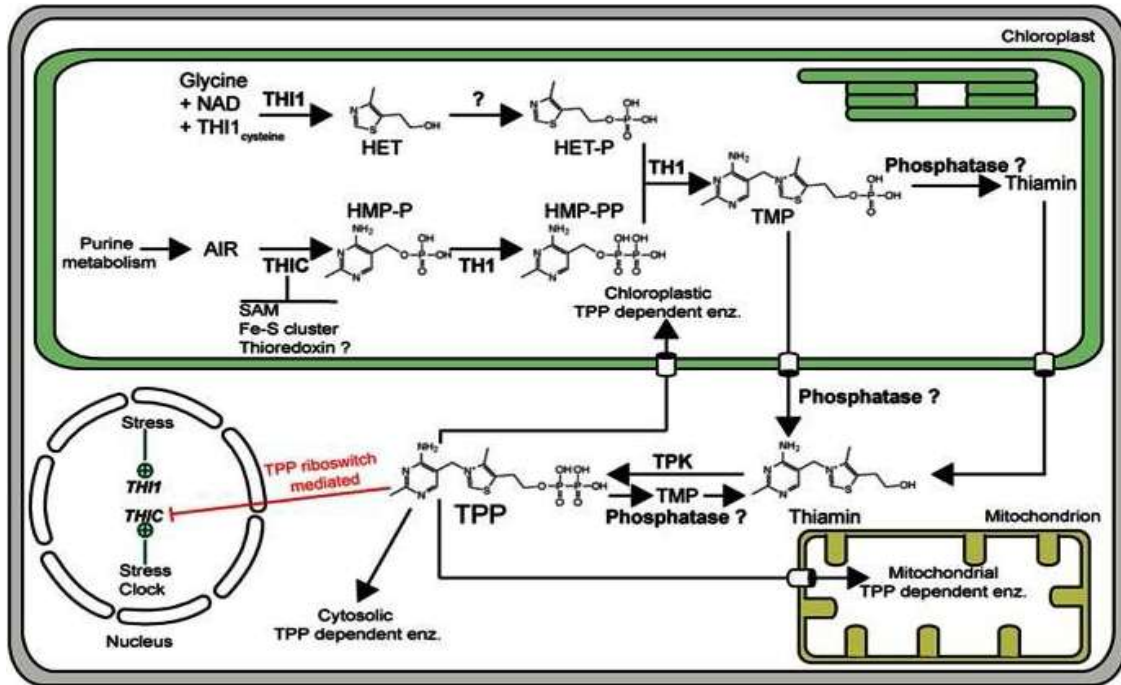


Figure 1. Thiamine biosynthesis pathway of *A. thaliana*. TMP is produced by the condensation of HMP-PP and HET-P. To form the active cofactor, TPP, TMP is first dephosphorylated by a phosphatase and then subsequently pyrophosphorylated by TPK (Pourcel et al., 2013).

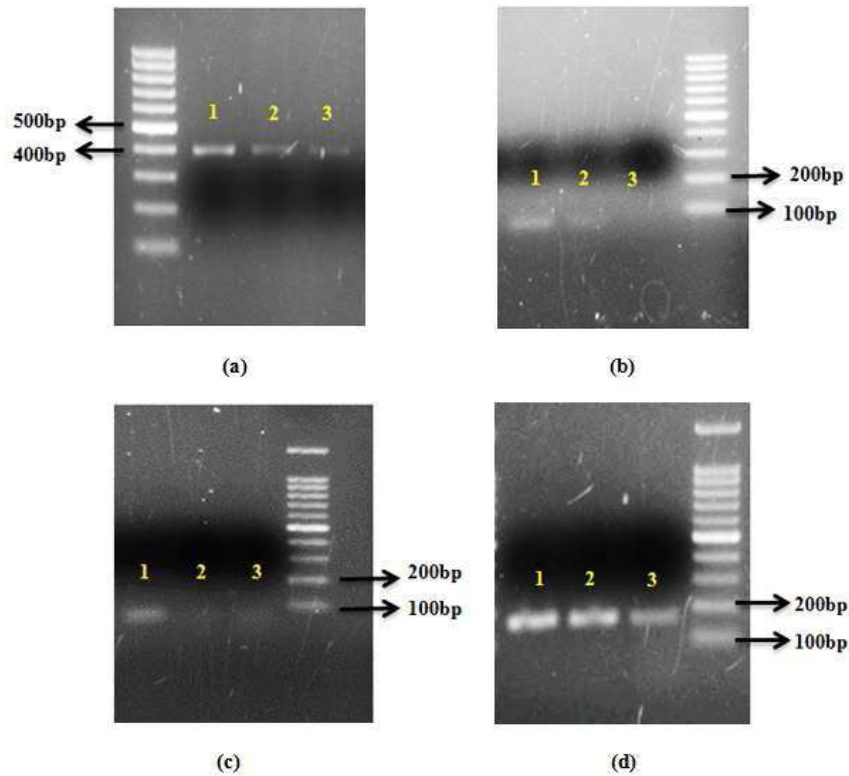


Figure 2. Amplification of (a) THIC primer F2; (b) THIC primer F3; (c) TH1/TH14 primer F8; (d) Actin from non-treated oil palm. Lane 1: spear leaf 3-days; lane 2: spear leaf 7-days; lane 3: spear leaf 30-days post-treatment.

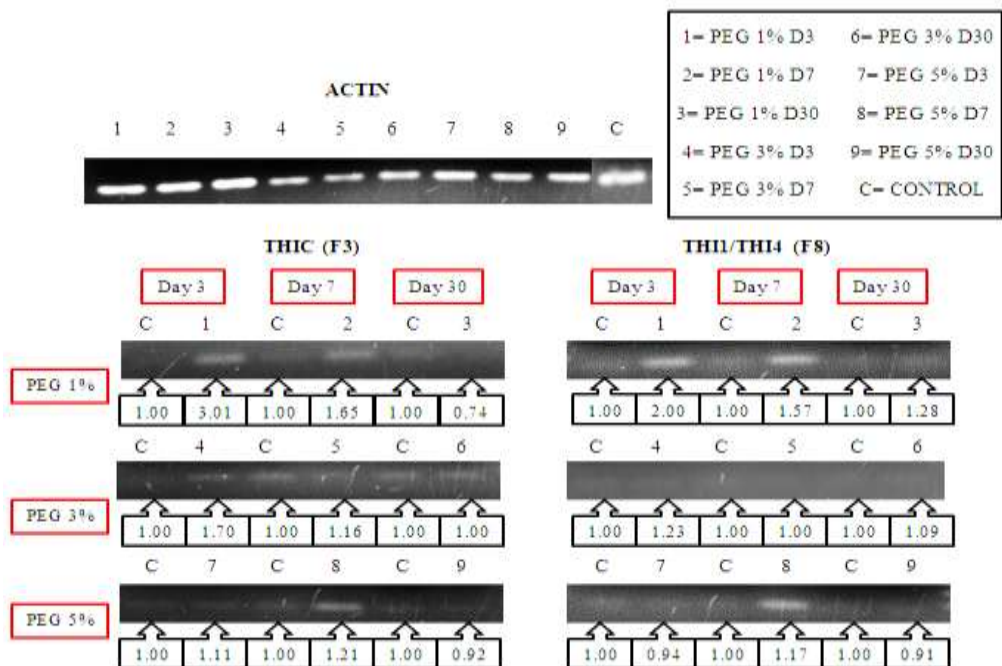


Figure 3. The level of gene expression for THIC and THI1/THI4 in treated and non-treated oil palm spear leaf tissue samples analyzed using ImageJ software. Lane C represents non-treated oil palm spear leaf tissue sample while lane 1-9 represent treated oil palm spear leaf tissue samples.

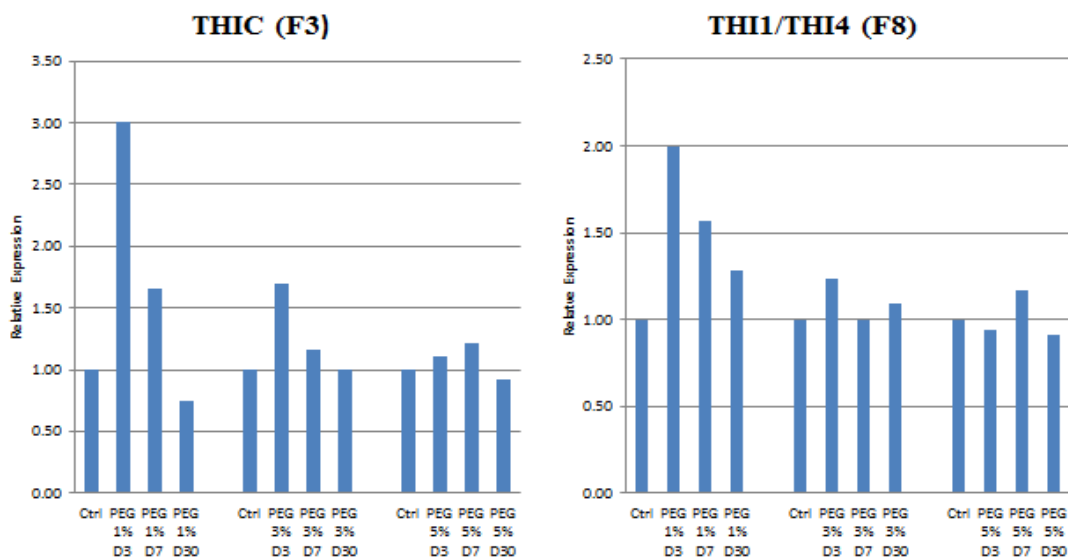


Figure 4. Day-dependent response of oil palm seedlings to osmotic stress revealed through THIC and THI1/THI4 gene expression changes.

Conclusion

Overall, in response to stress, oil palm showed an increase in the level of gene transcripts of the first two

enzymes in the thiamine biosynthesis pathway. The results proved that thiamine biosynthesis genes, namely THIC and THI1/THI4, were upregulated under osmotic stress. An increase of transcription is therefore needed

to maintain the proper thiamine cellular levels as it is crucial to develop new adaptation strategies and to overcome the impacts of stress. These suggest that thiamine biosynthesis in plants is tightly modulated during stress sensing and adaptation.

However, this is a preliminary study which only involves thiamine biosynthesis study at transcriptional level. For future work, total thiamine content determination via HPLC analysis should be carried out to confirm the increase in thiamine accumulation. Apart from that, real-time PCR should also be carried out to confirm the upregulation of the expression of thiamine biosynthesis genes. Besides that, studies on the other genes encoding for other enzymes in the thiamine biosynthesis pathway in oil palm will be useful in elucidating the overall regulation of thiamine biosynthesis in oil palm. It is believed that overexpression of thiamine or the specific enzymes in thiamine biosynthesis pathway will contribute to a more stress-tolerant oil palm variety.

Conflict of Interests

The authors have not declared any conflict of interests.

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