UNIVERSITI PUTRA MALAYSIA

IDENTIFICATION AND CHARACTERISATION OF THIAMINE PYROPHOSPHATE RIBOSWITCH IN OIL PALM (*Elaeis guineensis* Jacq.)

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By

ATIQAH BINTI SUBKI

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Faculty : Biotechnology and Biomolecular Sciences

The oil palm (*Elaeis guineensis*) is an important crop due to its diverse uses. In Malaysia, the productivity of this crop is hampered with various stresses ranging from biotic to abiotic stresses. Recent studies suggest the importance of signalling molecules in plants in coping against stresses, which includes thiamine (vitamin B_1). Thiamine (vitamin B_1) is an essential microelement that is synthesised *de novo* by plants and microorganisms. The active form of thiamine, which is known as thiamine pyrophosphate (TPP), plays a prominent role in metabolic activities particularly as an enzymatic cofactor. Recently, thiamine biosynthesis pathways in oil palm have been characterised but the search of novel regulatory element known as riboswitch is yet to be done. Previous studies showed that thiamine biosynthesis pathway is regulated by RNA element known as riboswitch. Riboswitch binds a small molecule, resulting in a change in production of the proteins encoded by the mRNA. TPP binds specifically to TPP riboswitch to regulate thiamine biosynthesis through a variety of mechanisms and they have been found in archaea, bacteria and eukaryotes. This study was carried out to hunt for TPP riboswitch in oil palm's thiamine biosynthesis gene. Riboswitch detection software like RiboSW, RibEx, Riboswitch Scanner and Denison Riboswitch Detector were utilised in order to locate putative TPP riboswitch in oil palm *ThiC* gene sequence that encodes for the first enzyme in the pyrimidine branch of the pathway. The analysis revealed a 192 bp putative TPP riboswitch located at the 3' untranslated region (UTR) of the mRNA. Further comparative gene analysis showed that the 92-nucleotide aptamer region, where the metabolite binds is conserved inter-species. The secondary structure analysis was also carried out using Mfold Web server and it showed a stem-loop structure manifested with stems (P1-P5) with minimum free energy of -12.26 kcal/mol. Besides that, the interaction of riboswitch and its ligand was determined using isothermal titration calorimetry (ITC) and it yielded an exothermic reaction with 1:1 stoichiometry interaction with binding affinities of 0.178 nM, at 30 °C. To further evaluate the ability of riboswitch to control the pathway, exogenous thiamine was applied to four months old of oil palm seedlings and sampling of spear leaves tissue was carried out at day 0, 1, 2 and 3 post-treatment for expression analysis of *ThiC* gene via quantitative polymerase chain reaction (qPCR). Results showed an approximately 5-fold decrease in *ThiC* gene expression upon application of exogenous thiamine. Quantification of thiamine and its derivatives was carried out via HPLC and the results showed that it is correlated to the down regulation of *ThiC* gene expression. The application of exogenous thiamine to oil palm affected *ThiC* gene expression, which supported the prediction of the presence of TPP riboswitch in the gene. Overall, this study provides the first evidence on the presence, binding and the functionality of TPP riboswitch in oil palm. This study is hoped to pave a way for better understanding on the regulation of thiamine biosynthesis pathway in oil palm, which could later be exploited for various purposes especially in manipulation of thiamine biosynthesis pathways in combating stresses in oil palm.

Abstrak tesis yang dikemukakan kepada Senat of Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains

PENGECAMAN DAN PENCIRIAN SUIS-RIBO TIAMINA PIROFOSFAT DALAM KELAPA SAWIT (*Elaeis guineensis* Jacq.)

Oleh

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Kelapa sawit (*Elaeis guineensis*) merupakan tanaman penting kerana kegunaannya yang pelbagai. Di Malaysia, produktiviti tanaman ini telah terancam disebabkan oleh tekanan dari segi biotik dan abiotik. Kajian terbaru telah mencadangkan kepentingan molekul isyarat dalam menangani tekanan dalam tumbuhan termasuk tiamina (vitamin B_1). Tiamina (vitamin B_1) adalah unsur mikro penting yang di sintesis secara '*de novo*' oleh tumbuhan dan mikroorganisma. Bentuk aktif tiamina, yang dikenali sebagai tiamina pirofosfat (TPP), memainkan peranan penting dalam aktiviti metabolik terutamanya sebagai kofaktor enzim. Yang terbaru, gen yang terlibat dalam laluan biosintesis tiamina pada kelapa sawit telah dicirikan namun unsur pengawalan unik yang dinamakan suis-ribo masih belum ditemui. Kajian terdahulu telah membuktikan bahawa biosintesis tiamina dikawalatur oleh unsur asid ribonukleik (RNA) yang dikenali sebagai suis-ribo. Suis-ribo mengikat molekul kecil, menyebabkan perubahan pada penghasilan protein yang di kod oleh asid ribonukleik utusan (mRNA). TPP mengikat secara spesifik pada suis-ribo TPP bagi mengawalatur biosintesis tiamina melalui pelbagai mekanisma dan ianya telah dijumpai di dalam arkea, bakteria dan eukariota. Kajian ini telah dijalankan untuk mencari suis-ribo TPP dalam gen biosintesis tiamina kelapa sawit. Perisian pengesanan suis-ribo seperti 'RiboSW', 'RibEx', 'Riboswitch Scanner' dan 'Denison Riboswitch Detector' telah diguna pakai untuk mengesan lokasi suis-ribo TPP dalam gen *ThiC* kelapa sawit yang mengekod enzim pertama pada cabang pirimidina di dalam laluan biosintesis tiamina. Analisis ini mendapati suis-ribo TPP sepanjang 192 pasangan bes terletak pada hujung 3' kawasan mRNA yang tidak diterjemah. Analisis perbandingan genomik lanjutan menunjukkan bahawa kawasan aptamer bernukleotid 92, di mana kawasan metabolit diikat dipulihara antara spesies. Analisis struktur sekunder pula telah dijalankan menggunakan aplikasi web 'Mfold' yang telah menunjukkan struktur gelung stem (P1-P5) dengan tenaga minima percuma -12.26 kcal/mol. Selain daripada itu, interaksi antara suis-ribo dan ligan telah ditentukan menggunakan titratan isoterma kalorimetri (ITC) dan ianya telah menunjukkan tindakbalas eksoterma dengan interaksi stoikiometri 1:1 bersama afiniti pengikat 0.178 nM pada suhu 30 °C. Untuk mengesahkan dengan lebih lanjut fungsi

suis-ribo sebagai elemen kawalatur laluian biosintesis, pengaplikasian tiamina secara eksogenik telah dijalankan pada anak pokok sawit berusia empat bulan dan persempelan daun telah dijalankan pada hari 0, 1, 2 dan 3 selepas rawatan untuk digunakan dalam analisis ekspresi gen *ThiC* menggunakan reaksi rantai polimeras kuantitatif (qPCR). Keputusan menunjukkan penurunan sebanyak kira-kira 5 lipatan pengungkapan gen *ThiC* kesan daripada rawatan tiamina eksogenik. Pengkuantitian tiamina dan terbitan tiamina telah dijalankan melalui HPLC dan keputusan menunjukkan yang ianya berhubung kait dengan penurunan pengekspresan gen *ThiC*. Rawatan tiamina eksogenik kepada anak sawit telah memberi kesan kepada pengekspresan gen *ThiC*, iaitu menyokong sangkaan tentang kewujudan suis-ribo TPP di dalam gen tersebut. Secara keseluruhan, kajian ini telah membuktikan kewujudan, pengikatan dan fungsi suis-ribo TPP di dalam kelapa sawit. Kajian ini di harapkan dapat membina laluan kepada kefahaman yang lebih baik tentang pengawalaturan laluan biosintesis tiamina di dalam kelapa sawit di mana pada masa akan datang boleh dieksploitasi untuk pelbagai keperluan terutamanya dalam memanipulasi laluan biosintesis thiamina dalam menangkis tekanan dalam kelapa sawit.

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This one is for my parents.

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LIST OF ABBREVIATIONS

α	Alpha
bp	Base pair
β	Beta
BLAST	Basic local alignment search tool
cDNA	Complementary deoxyribonucleic acid
Δ	Delta
dH_2O	Distilled water
DMF	Dimethyl formamide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium bromide
g	Gram
HCl	Hydrochloric acid
HEPES	N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid
HMP-P	Hydroxymethylpyrimidine pyrophosphate
H_3PO_4	Phosphoric acid
ITC	Isothermal titration calorimetry
kb	Kilobase
$K_3Fe(CN)_6$	Potassium ferricyanide
L	Litre
M	Molar
mM	Millimolar
µm	Micrometre
Min	Minute
$MgCl_2$	Magnesium chloride
mRNA	Messenger ribonucleic acid
NaOH	Sodium hydroxide
NCBI	National Centre for Biotechnology Information
PCR	Polymerase chain reaction
ppm	Parts per million
qPCR	Quantitative polymerase chain reaction
RCBD	Randomised complete block design
RNA	Ribonucleic acid
rpm	Revolution per minute
SDS	Sodium dodecyl sulphate
TMP	Thiamine monophosphate
Tris	Tris[Hydroxymethyl)aminomethane
Tris-HCl	Tris[Hydroxymethyl)aminomethane hydrochloride
TPP	Thiamine pyrophosphate
UTR	Untranslated region
UV	Ultraviolet
V	Volt
°C	Degree Celsius
%	Percentage
A_{260nm}	Absorbance at wavelength 260 nanometre
A_{280nm}	Absorbance at wavelength 280 nanometre
µg	Microgram

μL	Microlitre
mg	Milligram

CHAPTER 1

INTRODUCTION

Thiamine (Vitamin B_1) is a necessary microelement merited by its prominent role as cofactor in some central metabolic activities such as in glycolysis and pentose phosphate pathways (Goyer, 2010). In recent years, thiamine has been designated to be related to plant protection studies. The active form of thiamine known as thiamine pyrophosphate (TPP) can directly controls the *de novo* biosynthesis of thiamine through feedback regulation mechanism (Mangel *et al.*, 2017). Other than its role as a cofactor, scientists are interested to divulge on the newly found role of this metabolite in plants as a signaling molecule during unfavorable conditions (Bocobza and Aharoni, 2014).

In Malaysia, palm oil industry is one of the important key economic drivers that secures substantial income for the national economy (Ludin *et al.*, 2014). Nevertheless, environmental stresses have influenced the growth and its production. This issue has to be taken seriously because it has a major impact on the productivity of plants especially in oil palm. To further understand the metabolism that occur during the event, a rigorous study on the stressor effect towards plant regulation and how they cope the stress at genetic level is increasing in number.

Some comprehensive studies on the effects of biotic and abiotic stresses on the regulation of thiamine in oil palm have previously been done. For example, boosting thiamine content could increase plants' resistance towards stresses. Furthermore, application of endophythic fungus upregulated the gene expression of genes involved in thiamine biosynthesis pathway thus increase the total thiamine content in oil palm (Balia Yusof *et al.*, 2015; Rapala-Kozik *et al.*, 2012; Kamarudin *et al.*, 2017). On the other hand, systemic acquired resistance (SAR) in several plants were shown to be induced upon thiamine application, suggesting role of thiamine as stress-responsive molecule (Goyer, 2010).

The regulation of thiamine biosynthesis pathways is uncommon from other type of vitamins. Previous studies by Guan *et al.* (2014) revealed that the energy cost of thiamine synthesis is higher as compared to other vitamin co-factor, suggesting the presence of novel regulatory element called riboswitch. Riboswitch is an RNA molecule that allows direct binding of specific ligand to it, resulting in a change in protein production. The most studied class of riboswitch is TPP riboswitch (Aghdam *et al.*, 2016). The regulation of thiamine biosynthesis via riboswitch has been widely identified in prokaryotes, plants, and certain fungi (Barrick and Breaker, 2007).

TPP riboswitch mechanism is found to be significant in the maintenance of adequate thiamine levels in plants (Bocobza and Aharoni, 2014). In response to the environmental changes, this mechanism will cause the cells to sense the intracellular concentration of TPP metabolites and cause the conformational changes to occur which will then lead to several mechanisms of regulation to take place (Proshkin *et al.*, 2014). Generally, riboswitch function in the modulation of gene expression by executing transcription and splicing activity (Roth *et al.*, 2009). Currently, riboswitches present themselves as viable candidates for a sophisticated mechanism of regulatory control in RNA-based life (Lynch *et al.,* 2007).

Evidently, the elucidation of thiamine biosynthesis and identification of TPP riboswitch have been widely conducted in other organisms but not in oil palm. Recently, the biosynthesis of thiamine in oil palm has been characterised, but to further understand the mechanism that occurs during the event, a rigorous study on how thiamine biosynthesis in oil palm is regulated should be conducted since there are very limited amount of studies involving thiamine in oil palm is available, let alone the studies on TPP riboswitch. Although the total genome of oil palm was currently revealed, the utilisation of this information on localisation of novel regulatory element like riboswitch is yet to be done.

The core objectives in hunting for riboswitch elements is to further understand if riboswitch actually exist as an alternative system in regulating gene expression. The search of new novel RNA regulatory element like riboswitch is crucial to fully divulge how thiamine is made as it has been seen as a important signalling molecule in modulating stresses in oil palm.

Therefore, the objectives of this study are;

1. To identify and locate the position of TPP riboswitch in the genes involved in thiamine biosynthesis pathway of oil palm (*Elaeis guineensis*) through *in-silico* analyses.
2. To verify the interaction of the predicted TPP riboswitch with its ligand using isothermal titration calorimetry (ITC).
3. To determine the effect of exogenous thiamine application on *ThiC* gene expression via quantitative polymerase chain reaction.

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(Signature of Chairman of Supervisory Committee)
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