UNIVERSITI PUTRA MALAYSIA

EXPRESSION OF PHENYLALANINE AMMONIA LYASE EgPAL2 FROM *Elaeis guineensis* Jacq. AND ITS MUTANT EgPAL2ASG IN *Escherichia coli*

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By

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July 2020

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Phenylalanine ammonia lyase (PAL) catalyses the conversion of L-phenylalanine to *trans*-cinnamic acid, the first step in the phenylproponoid pathway. The phenylproponoid pathway plays an important role in the production of diverse plant secondary metabolites important for defense and development. PAL is encoded by a small multiple gene family. The specific function of each PAL isoform is not easily predicted based on its expression pattern. In oil palm (*Elaeis guineensis Jacq)*, PAL is encoded by five genes namely *EgPAL1-EgPAL5.* The EgPAL2 have no Ala-Ser-Gly (ASG) catalytic triad, highly conserved amino acids motif involved in the catalytic activity of PAL. The catalytic ability, the true substrate and the role of ASG triad in EgPAL2 were unknown. The objectives of this study were to determine the functionality of EgPAL2 at the protein level and to confirm that the ASG triad loss in EgPAL2 is the prime cause that converts EgPAL2 to a pseudogene. The ASG catalytic triad was inserted in EgPAL2 through a site-directed mutagenesis generating mutant EgPAL2 (EgPAL2ASG) to determine the role of ASG triad in EgPAL2 catalytic activity. The DNA coding sequence of *EgPAL2* and its mutant *EgPAL2* were subcloned into pET51b and expressed in *E. coli* BL21 (DE3). The protein expression was induced by isopropyl β-D-1-thiogalactopyranoside (IPTG). The recombinant protein was purified by using His-tag Ni-NTA purification column. The activities of EgPAL2 and EgPAL2ASG were determined by using L-phenylalanine, L-tyrosine and L-histidine as their substrates. Results showed that EgPAL2 did not have any activities on all tested substrates while EgPAL2ASG showed activities on L-phenylalanine and L-histidine but not L-tyrosine which suggested that the ASG triad was important for PAL catalytic activity. To date, there is no PAL activity on L-histidine was

reported in plants which indicated that EgPAL2 was an ancient PAL which could have evolved from histidine ammonia lyase (HAL) from bacteria.

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

PENGHASILAN FINILALANINA AMMONIA LYASE EgPAL2 DARIPADA *Elaeis guineensis* Jacq. DAN MUTANNYA EgPAL2ASG DALAM *Escherichia coli*

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Finilalanina ammonia lyase (PAL) merupakan pemangkin yang menukarkan L-finilalanina kepada asid *trans*-cinnamik, langkah pertama dalam tapak jalan finilproponoid. Tapak jalan finilproponoid memainkan peranan penting dalam penghasilan pelbagai metabolit sekunder tumbuhan yang penting untuk pertahanan dan pertumbuhan. PAL telah dikodkan oleh satu famili kecil pelbagai gen. Fungsi spesifik setiap PAL isoenzim tidak mudah untuk diramalkan berdasarkan corak ekspresinya. Dalam pokok kelapa sawit, (*Elaeis guineensis Jacq)*, PAL dikodkan oleh lima gen iaitu *EgPAL1-EgPAL5*. EgPAL2 tidak mempunyai Ala-Ser-Gly (ASG) triad, motif asid amino yang dikekalkan terlibat dalam aktiviti pemangkin PAL. Keupayaan pemangkinan, substrat dan peranan triad ASG dalam EgPAL2 tidak diketahui. Objektif kajian ini adalah untuk menentukan kefungsian EgPAL2 di peringkat protein dan untuk mengesahkan kehilangan triad ASG adalah penyebab utama yang menukarkan EgPAL2 kepada pseudogen. Triad ASG telah dimasukan ke dalam EgPAL2 secara 'site-directed mutagenesis' menghasilkan mutan EgPAL2 (EgPAL2ASG) untuk menentukan peranan triad ASG dalam aktiviti pemangkin EgPAL2. Jujukan DNA yang mengkod *EgPAL2* dan mutannya telah disubklonkan dalam pET52b dan diekspres dalam *E. coli* BL21 (DE3). Penghasilan protein telah didorong oleh isopropil β-D-1-thiogalaktopiranosida (IPTG). Penulenan protein rekombinan telah dilakukan dengan menggunakan kolum penulenan His-tag Ni-NTA. Aktiviti EgPAL2 dan EgPAL2ASG telah ditentukan dengan menggunakan L-finilalanina, L-tyrosina dan L-histidina sebagai substrat. Dapatan menunjukan EgPAL2 tidak mempunyai sebarang aktiviti ke atas semua subsrat yang diuji sementara EgPAL2ASG telah menunjukan aktiviti ke atas L-finilalanina dan L-histidina tetapi tidak ke atas L-tyrosina ini menunjukan triad ASG adalah penting untuk aktiviti pemangkinan

PAL. Setakat ini, tidak ada aktiviti PAL terhadap L-histidina dalam tumbuhan telah direkodkan. Ini menunjukan EgPAL2 adalah PAL lama yang telah berevolusi daripada histidina amonia lyase (HAL) dalam bakteria.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

PAL	Phenylalanine ammonia lyase
DNA	Deoxyribonucleic acid
IPTG	Isopropyl β- d-1-thiogalactopyranoside
BLAST	Basic Local Alignment Search Tool
Bp	Basepair
BSA	Bovine serum albumine
dNTP	Deoxynucleoside triphosphate
EDTA	Ethylenediaminetetraacetic acid
LB	Luria-Bertani
PCR	Polymerase chain reaction
rpm	Revolution per minute
SDS	Sodium dodecyl sulphate
TAE	Tris-acetate-EDTA
TAL	Tyrosine ammonia lyase
HAL	Histidine ammonia lyase
U	Unit
UV	Ultraviolet
V	Volt
w/v	Weight per volume
v/v	Volume per volume

CHAPTER 1

INTRODUCTION

Phenylalanine ammonia lyase (PAL) catalyses the conversion of L-phenylalanine to *trans*-cinnamic acid and ammonia, the first step in the phenylproponoid pathway (Cochrane et al, 2004; De Jong et al, 2015). PAL is the rate-limiting step of the pathway which plays an important role for the production of diverse plant secondary metabolites such as lignin and flavonoids (Dixon and Paiva 1995; MacDonald and D' Cunha 2007). One study that shows the role of this enzyme in lignification was in *Brachypodium distachyon* where a knockdown of a PAL gene (BdPAL) reduced the lignin content in the stem to 43% (Cass et al., 2015). In plants, lignin gives the structural support to the plants and also involves in plant defence against pathogen attack (Ithal et al., 2007; Schuetz et al., 2014). This can be observed in *Lotus japonicas* where a knockdown of its PAL gene (LjPAL1) increased the infection thread and caused the structural changes in the roots (Chen et al., 2017). Apart from lignin, PAL is also directly involved in the production of flavonoids. Flavonoids are important for plant as they facilitate plant response towards various environmental stresses (Cass et al., 2015). For example, PAL activity and the flavonoid content were increased in *P. glandulosa* leaves when treated with Copper and Cadmium (González-Mendoza et al., 2018).

The PAL enzyme was encoded by a small multiple gene family (Cochrane, Davin and Lewis, 2004). Different plants have different numbers of PAL gene family members. For examples, Arabidopsis thaliana has four copies of PAL genes while cucumber has 13 copies (Cochrane, Davin and Lewis, 2004; Dong et al., 2016). Expression analyses of PAL families shows that members of the PAL gene family in different plants are differentially expressed and regulated (Cochrane, Davin and Lewis, 2004). The expression patterns of PALs can be observed in many plants as demonstrated in Pyrus bretschneideri, where two members of the family (PbPAL1 and PbPAL2) were highly expressed in the root and stem while a different family member (PbPAL3) was expressed in different parts of the plant with lower expression levels (H. Wang, Cheng, Zhao, Jin, & Lin, 2019).

In oil palm (Elaeis guineensis Jacq), its PAL gene family has five family members namely EgPAL1- EgPAL5 (Yusuf et al., 2018). Of these, one member is unique (EgPAL2) that it has no Ala-Ser-Gly catalytic triad, an important motif that supports substrate binding. These three highly conserved amino acids were known to be involved in the catalytic activity of PAL (Alunni, Cipiciani,

Fioroni, & Ottavi, 2003). The elimination of serine residue at the ASG triad of PAL from parsley exhibited a significant reduction of PAL activity, an experimental proof that supports the important role of the ASG triad in PAL catalytic activities (Kong, 2015; Schuster & Rétey, 1994). Since EgPAL2 gene was expressed in oil palm at different stages of its growth and development (Yusuf et al., 2018), the disappearance of the ASG from EgPAL2 is something interesting. While the gene remains functional at the gene level, it may not be at the protein level. Transcription and translation decode genes into linear polypeptides of amino acids but folding of the polypeptides into 3D structures determines the functionality of decoded genetic products (functional proteins).

It was hypothesized that EgPAL2 does not have the catalytic activity and EgPAL2 is a pseudogene. To test this hypothesis, a recombinant protein of EgPAL2 was made in E. coli where a purified form of the recombinant protein was evaluated for its catalytic activities. The hypothesis is supported if there are no PAL activities observed when the purified recombinant EgPAL2 was assayed using different known substrates of PAL. In addition, a mutant of EgPAL2 with the ASG triad restored was made to suggest that during evolution, the first step in which a functional protein may lose its functionality is through a degradation of its substrate binding site rather than a degradation that ceases its polypeptide and transcript formation.

In this study, the DNA sequences encoded for EgPAL2 and the mutated EgPAL2 which contain Ala- Ser-Gly catalytic triad were ligated to the expression vector pET51b. The expression contructs were introduced to the E. coli BL21 (DE3) by transformation. The expression was induced by isopropyl β-D- 1-thiogalactopyranoside (IPTG). The extracted His-tagged recombinant PAL enzymes were purified by using Ni-NTA purification columns and PAL enzymatic activities were evaluated using three subsrates namely L-phenylalanine, L-tyrosine and L-histidine.

The specific objectives of this study were:

1. To determine the functionality of EgPAL2 at the protein level.
2. To confirm that the ASG triad loss in EgPAL2 is the prime cause that converts EgPAL2 to a pseudogene.

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