UNIVERSITI PUTRA MALAYSIA

FUNCTIONAL ANALYSIS OF THE OIL PALM METALLOTHIONINELIKE GENE PROMOTER USING TRANSIENT EXPRESSION ASSAY

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By

AMIR IZADFARD

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

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In The Name of Allah, the Most Gracious, the Most Merciful

Specially dedicated to:

My kind Family

Abstract of thesis presented to the senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

FUNCTIONAL ANALYSIS OF THE OIL PALM METALLOTHIONINE-LIKE GENE PROMOTER USING TRANSIENT EXPRESSION ASSAY

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AMIR IZADFARD

April 2009

Chairperson: Associate Professor Datin Siti Nor Akmar Abdullah, PhD

Faculty: Agriculture

The metallothionein-like gene, *MT3-A*, is specifically and abundantly expressed in the mesocarp tissue of the oil palm, *Elaeis guineensis*. In order to characterize the *MT3-A* promoter, a functional analysis containing bioinformatics and deletion analysis were carried out. The *MT3-A* promoter was subjected to bioinformatics analysis using three online data bases including *PLACE*, *TESS* and *Softberry* and with the assistance of the *DNASIS* software to identify and determine the position of the various regulatory motifs. Bioinformatics survey of the *MT3-A* promoter showed the presence of more than 50 reported regulatory motifs and the motifs of interest includes an I-box from Monocots/ Dicots at position -944, G-box from *Phaseolus vulgaris* at position -745 and ERE reverse from *Lycopersicon esculentum* at position -317.

To gain further insight into the mechanism that regulates the transcriptional activity of the metallothionein-like gene, analysis of the 5'-deletion series of the 986 bp oil palm *MT3-A* promoter using transient expression assay was carried out. Six promoter fragments of 823, 620, 470, 332, 181, 115 bp were generated by PCR and cloned into promoter-less pEGFP-1 carrying green fluorescent protein (GFP) by introducing *Hind* III and *Pst* I sites. The vector construct containing the different *MT3-A* promoter fragment was co-bombarded with a vector construct carrying CaMV promoter linked to GUS (pBI221) reporter gene into oil palm mesocarp and leaf tissues. Each deletion construct was bombarded to samples from each tissue type. The ratio of GFP/GUS or green/blue fuci was determined in each construct bombarded and taken as the percentage of the construct giving the highest expression.

Based on the transient assay analysis, it was found that the 620 bp and 823 bp truncated versions of the *MT3-A* promoter gave higher expression in the mesocarp compared with the whole 986 bp promoter. But the 470 bp and 332 bp fragments showed decreasing level of expression compared to the full length. These results suggest that at least there are two negative regulatory elements upstream of the positions -823 as well as between -620 and -823. It was noted that production of the 823 bp promoter fragment involves removing the I-box which has been reported to act as a negative regulatory element in a fruit-specific promoter. The

decrease in expression level suggests that there is a positive regulatory element upstream of the -470 region as well as between -332 and -470 of the *MT3-A* promoter. The expression in the leaves was only detectable in the four smallest fragments upon the removal of mesocarp-specific regulatory element upstream of the position -470.

Alignment of the *MT3-A* promoter with the promoter of a closely related *MT3-B* gene which is expressed in both mesocarp and root suggested that there is a ten base-pair motif (AATTTCCTtC) in both of these promoters in the *MT3-A* promoter region (between -332 and -470) which has lost its mesocarp specificity. This motif is located at about the same position [-360 (*MT3-A*) and -346 (*MT3-B*)] from the transcription start site in both promoters. To have an insight on the role of this novel motif, an eight tandem repeat of this motif were synthesized. The constructs carrying this tandem repeats of the motifs in the forward and reverse orientation fused to minimal *MT3-A* promoter in pEGFP-1 were produced and used to bombard mesocarp and leaf tissues of the oil palm. The results suggest that this tandem repeat motif in both forward and reverse orientation could increase the expression of GFP reporter gene in the mesocarp. It was observed that both the forward and reverse orientation of the motif could decrease the level of GFP reporter gene expression in leaf tissue slices.

Abstrak tesis dikemukakan kepada senat Universiti Putra Malaysia sebagai memenuhi keperluan Ijazah Master Sains

ANALISIS FUNGSI PROMOTER GEN METALLOTHIONINE-LIKE MENGGUNAKAN SISTEM ASAI TRANSIEN

Oleh

AMIR IZADFARD

April 2009

Pengerusi : Profesor Madya Datin Siti Nor Akmar, PhD

Fakulti : Pertanian

Gen bak-metallothionine, *MT3-A*, diekspreskan dengan banyak dan spesifik didalam tisu mesokarp kelapa sawit, *Elaeis guineensis*. Dalam usaha untuk mencirikan promoter *MT3-A*, satu analisis fungsi secara bioinformatik dan penyingkiran bersiri telah dijalankan. Analisis bioinformatik terhadap promoter *MT3-A* telah dilaksanakan dengan menggunakan tiga pangkalan data dalam talian iaitu PLACE, TESS dan Softberry digandingkan dengan perisian DNASIS untuk mengenalpasti dan menentukan posisi motif-motif pengawalaturan. Analisis bioinformatik promoter *MT3-A* menunjukkan kehadiran lebih daripada 50 motif-

motif pengawalaturan yang telah dilaporkan. Motif-motif menarik yang telah ditemui adalah termasuk; I-box daripada Monokot/Dikot pada posisi -944, G-box yang juga ditemui pada *Phaseolus vulgaris* di posisi -745; dan juga motif ERE pembalik pada *Lycopersicon esculentum* di posisi -317.

Demi mendapatkan maklumat yang lebih mendalam tentang mekanisma yang mengawalatur aktiviti traskripsi gen metallothionine-like, analisis penyingkiran jujukan 5' pada promoter 986 bp *MT3-A* dilakukan melalui asai pengekspresan transien. Enam serpihan promoter; 823, 620, 470, 332, 181 dan 115 telah dihasilkan menggunakan PCR dan diklonkan ke dalam plasmid pEGFP-1 tanpa promoter yang membawa protein pendarflour floresen hijau (GFP) dengan memperkenalkan tapak-tapak *Hind* III dan *Pst* I. Konstruk vektor yang mengandungi fragmen promoter *MT3-A* yang berlainan telah dibedilkan bersama konstruk vektor pembawa promoter CaMV yang dicantumkan dengan gen pelapor GUS (pBI221) kedalam tisu-tisu daun dan mesokarp kelapa sawit. Setiap konstruk penyingkiran telah dibedilkan kepada 15 sampel kajian daripada setiap jenis tisu. Tiga eksperimen telah dilakukan secara berasingan. Nisbah GFP/GUS atau hijau/biru fuci telah dikenalpasti dalam setiap sampel yang dibedilkan dan nilai tersebut diambil sebagai peratusan konstruk yang memberikan ekspresi yang tertinggi

Berdasarkan analisis asai transien, didapati serpilan 620 bp dan 823 bp yang telah dipotong daripada promoter *MT3-A* memberikan ekspresi yang tinggi didalam mesokarp berbanding dengan fragmen promoter penuh, 986 bp. Tetapi serpilan 470 bp dan 332 bp menunjukkan pengurangan tahap ekspresi berbanding dengan promoter penuh. Keputusan ini menunjukkan bahawa terdapat tidak kurang daripada dua unsur pengawalaturan negatif di bahagian awal jujukan pada posisi -823 dan juga diantara -620 dan -823. Daripada keputusan tersebut ia juga menunjukkan bahawa penghasilan serpihan promoter 823 bp melibatkan penyingkiran I-box yang bertindak sebagai unsur pengawalaturan negatif didalam promoter spesifik-buah. Pengurangan dalam tahap ekspresi ini menunjukkan terdapatnya unsur pengawalaturan positif pada jujukan awal di bahagian -470 dan juga diantara -332 dan -470 promoter *MT3-A*. Pengekspressan di dalam daun hanya dapat dikenalpasti pada empat serpilan terkecil, ini mungkin disebabkan oleh penyingkiran unsur pengawalaturan spesifik-mesokarp dibahagian awal jujukan sebelum posisi -470.

Daripada analisis penjajaran promoter *MT3-A* bersama promoter gen yang hampir berkait yang diekspreskan dalam mesokarp dan akar iaitu *MT3-B* menunjukkan motif AATTTCCTtC didalam kedua-dua promoter didalam bahagian promoter *MT3-A* diantara -332 dan -470 telah kehilangan kespesifikan mesocarp. Motif ini terletak pada posisi yang agak sama [-360 (*MT3-A*) dan -346 (*MT3-B*)] daripada

tapak permulaan transkripsi pada kedua-dua promoter. Untuk mendapatkan maklumat lanjut tentang fungsi motif novel ini, lapan ulangan tandem motif tersebut telah disintesiskan. Konstruk yang membawa ulangan tandem motif tersebut pada orientasi jujukan ke hadapan dan jujukan berbalik dicantumkan kepada promoter minima *MT3-A* dalam pEGFP-1 dan digunakan untuk dibedilkan kepada tisu-tisu mesokarp dan daun kelapa sawit. Keputusan menunjukkan motif ulangan tandem pada kedua-dua orientasi jujukan ke hadapan dan jujukan berbalik boleh meningkatkan ekspresi pelapor gen GFP dalam mesokarp. Didapati bahawa kedua-dua jujukan ke hadapan dan jujukan berbalik motif tersebut merendahkan paras ekspresi pelapor gen GFP dalam tisu daun.

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I certify that an Examination Committee has met on 1st November 2008 To conduct the final examination of Amir Izadfard on his Master of Science thesis entitled "Functional Analysis of the Regulatory Regions Found in the Oil Palm Metallothionine-like Gene Promoter *via* Transient Assay System" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The committee recommends that the student be awarded the Master Science.

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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 Supervisory Committee were as follows:

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DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

AMIR IZADFARD
Date: 23 April 2009

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LIST OF ABBREVIATIONS

bp	Base pair
CaMV35S	Cauliflower mosaic virus
cDNA	Complementary deoxyribonucleic acid
Cy	cysteine
CaMV	Cauliflower mosaic virus
DPE	Downstream promoter elements
DNase	Deoxynuclease
dNTP	Deoxynucleoside triphosphate
GFP	Green fluorescent protein
GUS	ß –glucuronidase
LUC	Luciferase
MCS	Multiple cloning sites
mRNA	Messenger Ribonucleic acid
MSP1	Mesocarp-specific promoter
MTS	Metallothioneins
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RNase	Ribonuclease
WAA	Week after anthesis

CHAPTER 1

INTRODUCTION

The oil palm species *Elaeis guineensis* is an important oil producing crop. The species *Elaeis guineensis* is one of the largest palm species in the world and has an old trading history. The regular oil palm trade between West Africa and Europe started in the early nineteenth century at the same time as the industrial revolution. The mesocarp and kernel oils were produced by traditional extraction method from fruits collected in semi-wild palm groves (Parveez *et al.,* 1998).

The world's statistic of oil production shows a total of 154 million tonnes of oils and fats were produced in 2007 compared to 149.6 and 140.7 million tonnes in 2006 and 2005, respectively (MPOC, 2007). Palm oil is the leading vegetable oil with the highest production of 38.5 million tonnes. Malaysia accounted for more than 40% of the total world palm oil production. In fact, in the recent decades, Malaysia's oil palm plantation land and crude palm oil production have been increasing; the production of crude palm oil is expected to further increase to 16.3 million tonnes in 2008 due to maturity of more trees (MPOB, 2007). This unique production situation of palm oil in Malaysia needs an extensive support from R&D (Lam *et al.,* 2009).

Genetic engineering is a method of modifying plants for the improvement of characters by introducing foreign genetic material or by enhancing the expression of endogenous genes. Genetic engineering shortens the time required for the production of new varieties and hybrids. Genetic engineering of plants offers new opportunities for agrochemical, food processing, and specialty chemical and pharmaceutical industries to develop new products (Sambanthamurthi *et al.,* 2002).

The specific physiological and biochemical functions of different tissues in plants rely on the specificity of tissue-specific gene expression, which is highly controlled by tissue-specific promoters (Tang *et al.,* 2004). Tissue-specific promoter has a specific DNA sequences generally upstream of the core promoter, which is called *cis*-element which has interaction with special proteins. Since the nucleotide sequence of the *cis*-element is basically the same in every cell, it is highly attractive to determine them in promoter's sequence and characterize their effects in regulation of specific gene expression (Minetoki *et al.,* 1996).

Heterologous or homologues promoter can be transiently introduced into any kind of plant. It is possible to analyze rapidly the effects of promoters on reporter gene expression (Agius *et al.,* 2005). It is very useful to study the effects of the tissue-specific expression of transgene constructs in mature target tissues such as

flowers or fruits (Blumenthal *et al.,* 1999). Many fruit specific promoters have been isolated like *2A11* promoter from tomato (Van Haren and Houck, 1993) or *cucumisin* promoter from Melon (Yamagata *et al.,* 2002) and *GalUR* promoter from strawberry (Agius *et al.,* 2005). Characterization of identified oil palm tissue-specific promoter particularly for mesocarp tissue which is specialized in oil producing, is completely required for future manipulation of this industrially important crop.

Transient gene expression based on biolistic method has been developed for functional analysis of promoters. Transient expression can be easily obtained at very high levels using particle bombardment with most plant species. This approach has been used to examine the effectiveness of various designs of gene constructs prior to stable transformation. Transient expression in cultured cells is a useful tool for studying gene expression networks in plant cells (Hibberd *et al.,* 1998).

This study involves the analysis of the expression pattern with the different lengths of truncated mesocarp-specific promoter in oil palm tissues using transient reporter gene expression system. The biolistic approach was used as a tool for transient transformation of promoter constructs into oil palm tissues.