TRANSIENT ASSAY FOR PROMOTER ACTIVITY OF METALLOTHIONEIN-LIKE GENE FROM OIL PALM IN DRIVING TISSUE-SPECIFIC EXPRESSION OF POLYHYDROXYBUTYRATE AND REPORTER GENES

By

VAHID OMIDVAR

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

November 2006

Specially Dedicated

To my

Father

Mother

Wife

For their Love & Supports

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

TRANSIENT ASSAY FOR PROMOTER ACTIVITY OF METALLOTHIONEIN-LIKE GENE FROM OIL PALM IN DRIVING TISSUE-SPECIFIC EXPRESSION OF POLYHYDROXYBUTYRATE AND REPORTER GENES

By

VAHID OMIDVAR

November 2006

Chairman: Associate Professor Siti Nor Akmar Abdullah, PhD

Faculty: Agriculture

Polyhydroxybutyrate is the most important type of polyhydroxyalkanoates (PHA), which is naturally synthesized by several microorganisms. Production of PHB in plants is based on the consumption of acetyl-CoA as an initial substrate, therefore oil palm being an oil crop serve as potential target due to its high flux of acetyl-CoA during the oil synthesis stage. In oil palm, mesocarp is the specialized tissue for oil synthesis. Targeting the expression of PHB genes in mesocarp tissue requires the mesocarp-specific regulation of these genes by a mesocarp-specific promoter, since accumulation of PHB in other tissues may have deleterious effects on the plant. Pervious efforts have resulted in isolation of promoter sequence of the oil palm mesocarp-specific metallothionein-like gene (designated MSP1) and preparation of the gene construct carrying PHB genes (*pMS29* vector) to drive the specific expression of PHB genes (*phb*A, *phb*B, and *phb*C) in mesocarp tissue.

Analysis of the specificity and strength of MSP1 and evaluating the expression pattern of the PHB genes in oil palm mesocarp tissue in a transient expression system will provide insight information prior to the stable transformation and supports time-saving strategy and more targeted and precise use of the technique.

In this study, expression of PHB genes under the regulation of MSP1 was evaluated in a biolistic-based transient assay in transiently transformed oil palm mesocarp and leaf (control) tissue slices. Transcriptional and translational analysis of PHB genes was carried out by Reverse-transcriptase PCR (RT-PCR) and western blot techniques. RT-PCR analysis revealed that the engineering of PHB biosynthetic pathway genes under the regulation of MSP1 in transformed mesocarp tissue has resulted in successful transcription of *phb*A and *phb*B genes; however the *phb*C gene failed to produce any transcription product. In addition, RT-PCR analysis in non-transformed (non-bombarded) mesocarp tissues showed that there is no endogenous expression of PHB genes, indicating that the expression of PHB genes in transformed mesocarp tissues is the result of the PHB pathway engineering. Furthermore, no expression level of PHB genes was detected in leaf tissue slices after bombardment.

Western blot analysis using polyclonal antibodies specific for *phb*B and *phb*C genes in transformed mesocarp tissues confirmed the successful translation of *phb*B mRNA transcript into protein product, however clearly revealed the failure in translation of *phb*C gene. Western blot analysis of *phb*B and *phb*C gene products in transformed leaf tissues revealed that regulation by MSP1 did not result in translation of these genes. Further analysis in non-transformed tissues showed that

*phb*B and *phb*C gene products do not exist in mesocarp and leaf tissues prior to the transformation. As a conclusion, although engineering of PHB biosynthetic pathway genes in mesocarp tissue did not result in an entirely functional pathway, it resulted in transcription of *phb*A and *phb*B genes and successful translation of *phb*B gene. This demonstrated that bacterial genes irrespective of their source and functions can be transcribed and translated in plant tissues under the regulation of plant tissue-specific promoters.

The tissue-specificity and strength of MSP1 was evaluated *via* transient reporter assays of GUS and GFP reporter genes in different oil palm tissues, including mesocarp, leaf, and root. The constitutive CaMV35S promoter was used as a reference in all analysis. Histochemical GUS assay showed the expression of GUS driven by MSP1 in transformed mesocarp tissue slices, while no expression of GUS gene was detected in transformed leaf and root tissue slices. Using the CaMV35S promoter, GUS expression was observed strongly in all transformed mesocarp, leaf, and root tissue slices. Quantitative analysis of GFP driven by MSP1 in transformed tissues revealed that GFP was expressed dominantly in mesocarp tissue slices (2.7 times more than it was expressed in leaf, and 86 times more than root), however there was some expression level of GFP directed by MSP1 in transformed leaf and root tissues. Comparative analysis of GFP expression, driven by the CaMV35S and MSP1 promoters showed that the CaMV35S promoter has the stronger activity with the average of 1.4 times more than the activity of MSP1 in transformed mesocarp tissue. This result indicated that although MSP1 is a strong promoter and has a great tendency to up-regulate the gene expression in mesocarp tissue, but the promoter does not behave in a hundred percent tissue-specific manner.

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

ANALISIS PROMOTER GEN BAK METALLOTHIONIEN KELAPA SAWIT DALAM MENGAWAL PENGEKSPRESAN SPESIFIK-TISU GEN-GEN POLIHIDROKSIBUTIRAT DAN GEN PELAPOR MELALUI KAEDAH ASAI TRANSIEN

Oleh

VAHID OMIDVAR

Julai 2006

Pengerusi: Profesor Madya Siti Nor Akmar Abdullah, PhD

Fakulti: Pertanian

PHB adalah polihidroksialkanoat (PHA) yang terpenting, ia disintesiskan secara semulajadi oleh beberapa jenis mikroorganisma. Penghasilan PHB di dalam tumbuhan adalah berasaskan penggunaan asetil-CoA sebagai substrat permulaan, maka kelapa sawit yang merupakan tanaman minyak berpotensi sebagai sasaran berikutan kadar asetil-CoA yang tinggi di peringkat sintesis minyak. Di dalam kelapa sawit, mesokarpa adalah tisu khusus yang mengsintesiskan minyak. Penyasaran pengekspresan gen-gen PHB di dalam tisu mesokarpa memerlukan pegawalaturan gen spesifik-mesokarpa oleh promoter spesifik-mesokarpa kerana pengumpulan PHB di dalam tisu lain boleh menjejaskan tumbuhan tersebut. Usaha sebelum ini telah menghasilkan jujukan promoter (MSP1) gen bak metallothionein spesifik-mesokarpa yang telah digunakan untuk membentuk gen mengandungi gen PHB (vektor *pMS29*) untuk mengawal ekspresi khusus gen PHB (*phb*A, *phb*B dan *phb*C) di dalam tisu mesokarpa.

Analisis pengkhususan dan keamatan promoter spesifik-mesokarpa kelapa sawit dan penilaian corak pengekspresan gen PHB di dalam tisu mesokarpa kelapa sawit melalui sistem pengekspresan transien akan memberikan maklumat yang lebih lanjut tentang kestabilan transformasi dan penjimatan masa, seterusnya peggunaan teknik yang lebih jitu.

Dalam kajian ini, pengekspresan gen PHB yang dikawalatur oleh MSP1 dinilai menggunakan kaedah asai transien ke atas hirisan tisu-tisu mesokarpa dan daun (sebagai kawalan) kelapa sawit yang telah ditransformasi secara transien. Analisis transkripsi dan terjemahan gen PHB telah dilaksanakan menggunakan teknik transkriptase berbalik-tindakbalas berantai polimerase (RT-PCR) dan teknik 'Western Blot'. Daripada analisis RT-PCR, didapati bahawa kejuruteraan tapakjalan biosintetik gen-gen PHB dibawah pengawalaturan MSP1 di dalam tisu mesokarpa yang telah ditransformasi, menghasilkan transkripsi gen-gen *phb*A dan *phb*B; namun begitu gen *phb*C gagal menghasilkan sebarang produk transkripsi. Selain itu, analisis RT-PCR di dalam tisu mesokarpa yang tidak ditransformasi (tiada pembedilan) menunjukkan tiada pengekspresan gen-gen PHB yang berlaku, ini menunjukkan bahawa pengekspresan gen PHB endogenus di dalam tisu mesokarpa yang ditranformasi adalah hasil daripada kejuruteraan tapakjalan PHB. Tambahan lagi, tiada pengekspresan gen PHB dikesan dalam hirisan tisu-tisu daun selepas pembedilan.

Analisis tekapan western menggunakan antibodi poliklon spesifik untuk gen *phb*B dan *phb*C ke atas tisu mesokarpa yang ditrasformasi telah mengesahkan kejayaan dalam translasi transkrip mRNA *phb*B kepada produk protein, tetapi ia gagal

menghasilkan produk protein gen *phb*C. Analisis tekapan western produk gen *phb*B dan *phb*C di dalam tisu daun yang ditransformasi mendapati bahawa pengawalaturan gen tersebut oleh MSP1 tidak memberikan hasil proses translasi. Analisis selanjutnya di dalam tisu yang tidak ditransformasi menunjukkan yang produk gen *phb*B dan *phb*C tidak wujud di dalam mesokarpa dan daun sebelum transformasi. Kesimpulannya, walaupun kejuruteraan gen PHB di dalam tisu mesokarpa tidak memberikan tapakjalan yang berfungsi, ia telah memberikan hasil transkripsi gen *phb*A dan *phb*B dan kejayaan translasi gen *phb*B. Ini menunjukkan bahawa gen bakteria, tanpa mengira sumber dan fungsinya, boleh ditranskripsi dan ditranslasi dalam tisu tumbuhan di bawah kawalan promoter khusus tisu tumbuhan.

Spesifisiti-tisu dan kekuatan promoter spesifik-mesokarpa kelapa sawit dinilai menggunakan kaedah asai gen pelapor GUS dan GFP dalam tisu kelapa sawit yang berbeza termasuk mesokarpa, daun dan akar. Promoter konstitutif CaMV35S telah digunakan sebagai rujukan di dalam semua analisis. Analisis histokimia GUS, menunjukkan pengekspresan GUS di bawah kawalan MSP1 pada hirisan tisu-tisu mesokarpa yang telah ditransformasi, manakala tiada pengekspresan gen dikesan pada hirisan tisu akar dan daun. Dengan menggunakan promoter CaMV35S, ekspresi GUS pada kadar yang tinggi telah dilihat di dalam semua hirisan mesokarpa, daun dan akar yang telah ditransformasi. Analisis kuantitatif ke atas GFP yang dikawal oleh MSP1 di dalam tisu yang ditransformasi menunjukkan bahawa GFP telah diekspreskan secara dominan di dalam hirisan tisu mesorkarpa (2.7 kali lebih tinggi daripada ekspresi di dalam daun, dan 86 kali lebih tinggi daripada akar). Bagaimanapun, terdapat sedikit pengekspresan GFP yang dikawal oleh MSP1 di dalam tisu daun dan akar yang telah ditransformasi. Analisis secara

perbandingan ke atas ekspresi GFP, dimangkinkan oleh promoter CaMV35S dan MSP1 menunjukkan promoter CaMV35S mempunyai aktiviti 1.4 kali lebih tinggi berbanding MSP1 di dalam tisu mesokarpa yang telah ditransformasi. Keputusan ini menunjukkan walaupun MSP1 adalah promoter yang kuat dan mempunyai kecenderungan untuk meningkatkan ekspresi gen di dalam tisu mesokarpa, tetapi promoter tersebut tidak bertindakbalas 100% secara spesifik-tisu.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and appreciation to my supervisor, Assoc. Prof. Dr. Siti Nor Akmar Abdullah for her guidance, generous assistance and valuable advice during my study and the preparation as well as completion of this master thesis. Her criticism and suggestion have been most constructive and are highly appreciated. Her patience, trust and enthusiasm have left a deep impression that could not be expressed in words.

Special thanks to Assoc. Prof. Dr. Maheran Abd Aziz and Prof. Dr. Marziah Mahmood for their valuable advice, immense assistance and full support at all stages of the research work. I would like also to thank the Gene Technology Laboratory students for their help and friendship.

The last not the least I would like to express my sincere gratitude and thanks to my parents and my wife for their encouragements and uncountable supports throughout my whole life.

I hope anyone who is not mentioned by name may recognize my gratitude for their kindness, advice and moral support in completion of my master degree at University Putra Malaysia.

I certify that an Examination Committee has met on to conduct the final examination of Vahid Omidvar on his Master of Agriculture Science thesis entitled "Transient Assay for Promoter Activity of Metallothionein-like Gene from Oil Palm in Driving Tissue-specific Expression of Polyhydroxybutyrate and Reporter Genes" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

Chairman, PhD
Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Chairman)

Examiner 1, PhD
Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Internal Examiner)

Examiner 2, PhD
Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Internal Examiner)

External Examiner, PhD
Professor
Faculty of Agriculture
Universiti Kebangsaan Malaysia
(External Examiner)

_____
HASANAH MOHD. GHAZALI, PhD
Professor/Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia
Date:

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

Siti Nor Akmar Abdullah, PhD
Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Chairman)

Maheran Abd Aziz, PhD
Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Member)

Marzieh Mahmood, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

_____
AINI IDERIS, PhD
Professor / Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 16 JANUARY 2007

DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

_____

VAHID OMIDVAR

Date: 19 DECEMBER 2006

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

bp	Base pair
CaMV35S	Cauliflower mosaic virus
cDNA	Complementary deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
dwt	Dry weight
fwt	Fresh weight
KDa	Kilo Dalton
MCS	Multiple cloning sites
MPOB	Malaysian Palm Oil Board
mRNA	Messenger Ribonucleic acid
MSP1	Mesocarp-specific promoter
PCR	Polymerase chain reaction
PHA	Polyhydroxyalkanoates
PHB	Polyhydroxybutyrate
PHBV	polyhydroxybutyrate-*co*-hydroxyvalerate
*phb*A	*β*-ketothiolase
*phb*B	Acetoacetyl-CoA Reductase
*phb*C	PHB synthase
PLA	Polyactides
RNA	Ribonucleic acid
RNase	Ribonuclease
RT-PCR	Reverse-transcriptase PCR