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PRODUCTION OF SPECIALIZED TRANSFORMATION VECTORS FOR THE PRODUCTION OF BIODEGRADABLE PLASTICS IN TRANSGENIC *ARABIDOPSIS* AND OIL PALM

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PRODUCTION OF SPECIALIZED TRANSFORMATION VECTORS FOR THE PRODUCTION OF BIODEGRADABLE PLASTICS IN TRANSGENIC *ARABIDOPSIS* AND OIL PALM

By

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Chairman: **Ho Chai Ling, PhD**

Faculty: **Biotechnology and Biomolecular Sciences**

Polyhydroxyalkanoates (PHAs), such as polyhydroxybutyrate (PHB) and polyhydroxybutyrate-co-hydroxyvalerate (PHBV) are bacterial polyesters, which can be used to produce biodegradable products. Since the mass production of PHAs in bacteria via fermentation is expensive, the production of PHAs in plants may be an attractive alternative. The production of PHB in plants required genetic engineering of *phbA*, *phbB* and *phbC* genes of *Ralstonia eutropha*, whereas, the *bktB*, *phbB*, *phbC* genes of *R. eutropha* and *tdcB gene* of *Escherichia coli* were required for PHBV production. In this study, each of these gene was fused with the transit peptide (*Tp*) of oil palm acyl-carrier-protein (*ACP*), and driven by the oil palm leaf-specific promoter (*LSP1*), for targeting into the plastids of leaf cells. In total, four transformation vectors, pLSP15 (PHB) and pLSP20 (PHBV), pLSP13 (PHB) and pLSP23 (PHBV) were constructed for the transformation of *Arabidopsis* and oil palm, respectively. Each vector contained the phosphinothricin acetyltransferase gene (*Bar*) driven by *CaMV35S* promoter in pLSP15 and pLSP20, and *ubiquitin* promoter in pLSP13 and pLSP23, as plant selectable marker. Matrix attachment

region of tobacco (RB7MAR) was also included, to stabilize the transgene expression and to minimize gene silencing due to positional effects. Restriction enzymes, polymerase chain reaction (PCR) and DNA sequencing were used to verify all the constructed vectors. *Arabidopsis* transformation produced T1 transgenic *Arabidopsis* plants with normal phenotypes at a transformation efficiency of 0.2%~1.0%. PCR and Southern analyses were used to confirm the insertion of the transgenes. Nile blue A staining of these T1 plants demonstrated the accumulation of PHB granules in the leaf. The initial screening of Basta-resistant oil palm embryogenic calli transformed with pLSP13 using PCR demonstrated the presence of *Bar* and PHB genes in transformed oil palm.

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

PENGHASILAN VEKTOR-VEKTOR TRANSFORMASI KHAS UNTUK PENGHASILAN PLASTIK BIOMUDAHURAI DI DALAM *ARABIDOPSIS* DAN SAWIT TRANSGENIK

Oleh

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Polihidroxialkanotes (PHAs) seperti polihidroxibutirat (PHB) dan polihidroxi-ko-hidroxivalerat (PHBV) merupakan poliester bakteria yang boleh digunakan untuk menghasilkan produk-produk biomudahurai. Oleh kerana penghasilan PHAs dalam kuantiti yang besar menggunakan bakteria melalui kaedah fermentasi adalah mahal, penghasilan PHAs di dalam tumbuhan mungkin alternatif yang menarik. Penghasilan PHB di dalam tumbuhan memerlukan kejuruteraan genetik keatas gen *phbA, phbB* dan *phbC* daripada *Ralstonia eutropha,* manakala gen *bktB, phbB,* dan *phbC* daripada *R. eutropha* dan *tdcB* daripada *Escherichia coli* diperlukan untuk menghasilkan PHBV. Melalui kajian ini, setiap gen disambung dengan peptid transit (*TP*) daripada proten-pembawa-asil sawit (*ACP*) dan dikawal oleh promoter khusus kepada daun (*LSP1*) untuk ditujukan ke dalam plastid sel daun. Sejumlah 4 vektor transformasi iaitu pLSP15 (PHB) dan pLSP20 (PHBV), pLSP13 (PHB) dan pLSP23 (PHBV) telah dibina, masing-masing ditransformasi ke dalam *Arabidopsis* dan sawit. Setiap vektor mengandungi gen pospinotirisin asetiltransferas (*Bar*) sebagai penanda pemilihan tumbuhan yang dikawal oleh promoter *CaMV35* di dalam pLSP15 dan

pLSP20 serta promoter *ubikuitin* di dalam pLSP13 dan pLSP23. Kawasan pelekatan matrik daripada tembakau (RB7MAR) turut dimasukkan untuk mengstabilkan pengungkap transgen dan meminimumkan penyeyapan gen hasil daripada kesan-kesan posisi. Enzim penyekatan, tindakbalas berantai polimeras (PCR) dan penjujukan DNA telah digunakan untuk mengesahkan vektor-vektor yang dibina. Transformasi *Arabidopsis* menghasilkan pokok *Arabidopsis* transgenik T1 yang normal dengan 0.2%~1.0% efisiensi transformasi. PCR dan pemblotan Southern telah digunakan bagi mengesahkan penyelitan gen. Perwarnaan biru A nile terhadap pokok-pokok T1 tersebut telah menunjukkan pengumpulan gumpalan-gumpalan PHB di dalam daun. Penyaringan awal menggunakan PCR keatas kalus embriogenik sawit yang ditransformasi menggunakan pLSP13 telah menunjukkan kehadiran gen *Bar* dan PHB dalam sawit yang ditransformasikan.

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I certify that an Examination Committee has met on 17th October, 2006 to conduct the final examination of Abdul Masani Mat Yunus on his Master of Science thesis entitled " Production of Specialized Transformation Vectors for the Production of Biodegradable Plastics in Transgenic *Arabidopsis* and Oil Palm " 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:

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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 Science. The members of the Supervisory Committee are as follows:

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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.

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ABDUL MASANI MAT YUNUS

Date: 23 SEPTEMBER 2006

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LIST OF ABBREVIATIONS

ACP	acyl carrier protein
btkB	gene coding for 3-ketothiolase
Bar	gene coding for phosphinothricin acetyltransferase
$CaCl_2$	calsium chloride
CaMV35S	cauliflower mosaic virus 35S
CoA	coenzyme A
CTAB	cetyltrimethylammonium bromide
2,4-D	2,4-dichlorophenoxyacetic acid
DIG	digoxigenin
DMSO	dimetyl sulfoxide
dNTP	deoxynicotinamide triphosphate
dwt	dry weight
EDTA	ethylenediaminetetra acetic acid
fwt	fresh weight
3HB	3-hydroxybutyrate
HCL	hydrochloric acid
3HV	3-hydroxyvalerate
IBA	indole-3-butyric acid
ilvA	gene coding for threonine deaminase
KCL	potassium chloride
KOH	potassium hydroxide
LB	left border
LSP1	leaf-specific promoter
MCL	medium-chain-length

$MgC1_2$	magnesium chloride
$MgSO_4$	magnesium sulfate
MPOB	Malaysian Palm Oil Board
MS	murashige and skoog
NAA	α-naphthaleneacetic acid
NaCl	sodium chloride
NaOH	sodium hydroxide
Nos	gene coding for nopaline syntase
PCR	polymerase chain reaction
PDC	puruvate dehydrogenase complex
PHA	polyhydroxyalkanoate
phbA	gene coding for 3-ketothiolase
phbB	gene coding for acetoacetyl-CoA reductase
phbC	gene coding for PHA synthase
PHB	polyhydroxybutyrate
PHBV	polyhydroxybutyrate-co-hydroxyvalerate
PRP	pathogen-related protein
RB	right border
RB7MAR	RB7 matrix attachment region
$RbCl_2$	rubidium chloride
SCL	short-chain-length
SDS	sodium dedocyl sulfate
TAG	triacylglycerols
TCA	tricarboxylic acid
tdcB	gene coding for threonine dehydratase

T-DNA	transferable-DNA
Tp	transit peptide

CHAPTER 1

INTRODUCTION

Palm oil is the second largest source of edible oil after soy oil in the world, which contributes 23.18 million tonnes (19.8%) of the total world's production of oils and fats (Pushparajah, 2001). Since 1990, Malaysia is the largest producer of palm oil, contributing about 11.8 million tonnes or 50.9% of the total production, while Indonesia produced about 7.5 million tonnes or 32.3%. Malaysia is also the world's largest exporter of palm oil, accounting for about 61.1% or 10.62 million tonnes of the total exports of 17.37 million tonnes in 2001. Palm oil has a wide range of applications, about 80% are used for food industries such as cooking oil, shortenings, margarines, ice creams and cookies while the rest are used as feedstock for a number of non-food applications such a diesel fuel substitute, drilling mud, soaps and epoxidised palm oil products, polyols, polyurethanes and polyacrylates (Salmiah, 2000). In addition to the cost benefit and its multiuse, palm oil was also proven to be nutritious. Studies have indicated that palm oil lowers serum cholesterol levels to the same degree as sunflower oil which is rich in unsaturated fatty acid (Manorama and Rukmini, 1992). Palm oil does not increase the plasma cholesterol or low-density lipoprotein (LDL), where increased level could be harmful but on the other hand, it increases the high-density lipoprotein (HDL), which protects against heart disease (Sundram *et al*., 1992). Furthermore, palm oil has anti-tumor effects especially with the presence of high levels of vitamin E, tocopherols and tocotrienols (Nesretnam *et al*., 1992).

The challenge that the oil palm industry will face in the 21st century is the ability to maintain profitability in the face of labor shortage and limited land resources. World production of palm oil was projected to double from the year 2000 to year 2020 with a total production exceeding 40 million tones and it is expected that nearly 26% of the world's oil and fat demand will be obtained from palm oil (Oil World, 2020: www.mpob.gov.my). Due to this projected demand, it is important to increase the yield of oil palm as well as to improve the palm oil quality at a better rate than that has been achieved by conventional breeding. Accordingly, in early 1990s, the genetic engineering programme has been initiated at Malaysian Palm Oil Board (MPOB), then Palm Oil Research Institute of Malaysia (PORIM), to fulfill this demand (Cheah *et al*., 1995). The primary strategy of this programme is to produce transgenic oil palm with high oleic oil content (Cheah *et al*., 1995). Besides increasing oleic acid, modification of oil quality and production of novel high value products have also been targeted. These targets include increasing stearic acid, synthesizing palmitoleic acid, synthesizing ricinoleic acid and producing biodegradable plastics (Parveez *et al*., 1999). Currently, biodegradable plastics such as polyhydroxyalkanoates (PHAs) are being produced under the trade name Biopol™ by Monsanto in a two-stage glucose / propionate fed batch fermentation process using *Ralstonia eutropha* (Kessler *et al*., 2001). The economics of the manufacturing process is still a major barrier to the widespread use of PHAs. An alternative strategy for lowering production costs which has been proposed is to develop transgenic plants that produce PHAs. This strategy could lead to considerably cheaper production of PHAs because production from plants does not require expensive fermentation equipments and substrates (Poirier *et al*., 1992).

In the last 10 years, MPOB has made significant progress on the production of PHAs [polyhydroxybutyrate (PHB) and polyhydroxybutyrate-co-hydroxyvalerate (PHBV)] in transgenic oil palm, such as development of a transformation system using biolistic-mediated method (Parveez, 1998), isolation of tissue-specific promoters such as mesocarp and leaf specific promoters (Siti Nor Akmar *et al*., 2001), construction of PHB and PHBV transformation vectors driven by constitutives and mesocarp-specific promoters (Masani *et al*., 2001; Masani and Parveez, 2003), transformation and production of transgenic oil palm (Parveez, 2003).

In this study, transformation of oil palm and *Arabidopsis* with the PHB and PHBV genes with the goal of accumulating bioplastics in the leaves was initiated. The objectives of this study are:

1. To construct PHB and PHBV transformation vectors driven by oil palm leaf-specific promoter (*LSP1*).
2. To transform PHB and PHBV transformation vectors into *Arabidopsis* and oil palm.
3. To confirm integration of PHB and PHBV genes in transgenic *Arabidopsis* and oil palm by molecular analysis.

CHAPTER 2

LITERATURE REVIEW

2.1 Polyhydroxyalkanoates (PHAs)

Polyesters like polyhydroxyalkanoates (PHAs) are a large group of polymers of 3-(R)-hydroxy fatty acids linked by an ester bond between the hydroxyl group and the carboxy group of an adjacent monomer (Sudesh *et al*., 2000). PHAs are divided into two groups, i.e. short-chain-length PHAs (SCL-PHAs) that is comprised of PHB and the copolymer PHBV; and medium-chain-length PHAs (MCL-PHAs) that consists of 3-(R)-hydroxyhexanoate / 3-(R)-hydroxytetradecanoate monomers (Figure 1). PHAs are osmotically inert compounds and they are optically active, biocompatible, biodegradable and hydrophobic. The properties of PHAs vary with their compositions (Table 1). PHAs are mainly composed of R-(-)-3-hydroxyalkanoic acid monomers. Each type of PHA generally consists of 1000-10000 monomers, but most are synthesized by SCL monomers. There are many different types of PHAs that are characterized by chain length, type of functional group and degree of unsaturated bonds. A higher degree of unsaturation increases the rubber qualities of a polymer, and different functional groups change the physical and chemical properties of a polymer (Madison and Huisman, 1999).

2.1.1 Polyhydroxybutyrate (PHB)

Most of the knowledge on bacterial production of PHB is generated from *Ralstonia eutropha* (formerly known as *Alcaligenes eutrophus*) because it naturally produces PHB and can produce up to 85% of its dry weight (dwt) when grown in media containing excess glucose.