OPTIMISATION OF *gus* A GENE TRANSFER INTO COCOA (*THEOBROMA CACAO*) VIA *AGROBACTERIUM TUMEFACIENS* – MEDIATED TRANSFORMATION

ANISAH BINTI SAVANTIL

SCHOOL OF SCIENCE AND TECHNOLOGY
UNIVERSITY MALAYSIA SABAH
2011

OPTIMISATION OF *gus* A GENE TRANSFER INTO COCOA (*THEOBROMA CACAO*) VIA *AGROBACTERIUM TUMEFACIENS* – MEDIATED TRANSFORMATION

ANISAH BINTI SAVANTIL

THESIS SUBMITTED IN FULFILLMENT FOR THE DEGREE OF MASTER OF SCIENCE

SCHOOL OF SCIENCE AND TECHNOLOGY
UNIVERSITY MALAYSIA SABAH
2011

OPTIMISATION OF *gus* A GENE TRANSFER INTO COCOA (*THEOBROMA CACAO*) VIA *AGROBACTERIUM TUMEFACIENS* – MEDIATED TRANSFORMATION

ANISAH BINTI SAVANTIL

THESIS SUBMITTED IN FULFILLMENT FOR THE DEGREE OF MASTER OF SCIENCE

SCHOOL OF SCIENCE AND TECHNOLOGY
UNIVERSITY MALAYSIA SABAH
2011

PUMS 99:1

UNIVERSITI MALAYSIA SABAH

BORANG PENGESAHAN TESIS

JUDUL : _____
_____
_____

IJAZAH : _____
_____

SAYA : _____ **SESI PENGAJIAN** : _____
(HURUF BESAR)

Mengaku membenarkan tesis *(LPSM/Sarjana/Doktor Falsafah) ini disimpan di Perpustakaan Universiti Malaysia Sabah dengan syarat-syarat kegunaan seperti berikut:-

1. Tesis adalah hak milik Universiti Malaysia Sabah.
2. Perpustakaan Universiti Malaysia Sabah dibenarkan membuat salinan untuk tujuan pengajian sahaja.
3. Perpustakaan dibenarkan membuat salinan tesis ini sebagai bahan pertukaran antara institusi pengajian tinggi.
4. Sila tandakan (/)

☐ SULIT (Mengandungi maklumat yang berdarjah keselamatan atau kepentingan Malaysia seperti yang termaktub di AKTA RAHSIA RASMI 1972)

☐ TERHAD (Mengandungi maklumat TERHAD yang telah ditentukan oleh organisasi/badan di mana penyelidikan dijalankan)

☐ TIDAK TERHAD

Disahkan oleh:

_____ _____
(TANDATANGAN PENULIS) (TANDATANGAN PUSTAKAWAN)

Alamat Tetap: _____
_____
_____
_____

_____
(NAMA PENYELIA)

TARIKH: _____ TARIKH: _____

Catatan:
*Potong yang tidak berkenaan.
*Jika tesis ini SULIT dan TERHAD, sila lampirkan surat daripada pihak berkuasa/organisasi berkenaan dengan menyatakan sekali sebab dan tempoh tesis ini perlu dikelaskan sebagai SULIT dan TERHAD.
*Tesis dimaksudkan sebagai tesis bagi Ijazah Doktor Falsafah dan Sarjana Secara Penyelidikan atau disertai bagi pengajian secara kerja kursus dan Laporan Projek Sarjana Muda (LPSM).

DECLARATION

I hereby declare that the material in this thesis is my own except for quotations, excerpts, equations, summaries and references, which have been dully acknowledged.

02 November 2011

_____

Anisah Binti Savantil
PS2002-001-551

CERTIFICATION

NAME	:	**ANISAH BINTI SAVANTIL**
MATRIC NO	:	**PS2002-001-551**
TITLE	:	**OPTIMISATION OF *gus*A TRANSFER INTO COCOA VIA *AGROBACTERIUM TUMEFACIENS*-MEDIATED TRANSFORMATION**
DEGREE	:	**MASTER OF SCIENCE (BIOTECHNOLOGY)**
VIVA DATE	:	**4 DECEMBER 2010**

DECLARED BY

1. SUPERVISOR
Prof. Madia Dr. Zaleha A. Aziz Signature

_____

2. CO-SUPERVISOR
Dr. Douglas Bruce Furtek Signature

_____

ACKNOWLEDGEMENT

First and foremost, I would like to express my grateful and thanksgiving to The Mighty GOD, for His mercy, blessing, and unconditional love.

I would like to express my gratitude to Dr. Douglas B. Furtek (Supervisor) and Dr. Zaleha Abdul Aziz (Co – supervisor) whose expertise, understanding, and patience, added considerably to my postgraduate experience. I appreciate their vast knowledge and skill in many areas especially in this field of study, and their assistance in writing this thesis.

A very special thanks goes out to Malaysian Cocoa Board (MCB) for financial support and infrastructure and MCB staff especially Dr. Rosmin Kasran, the Director of Center for Cocoa Biotechnology Research, and also all the tissue culture group especially Mr. Azhar Mohammed, Mdm. Mavis Peter Jaus and Mdm. JainabMadali and molecular group especially Mdm. Lea Johnsiul and Ms. RoslinaMohd Shah for their assistance and support. Not also forgetting to appreciate my former lab mate Helda Souki and my friend Helena Biun for their help and encouragement.

I also would like to thank my family for their spiritual and financial supports they provided throughout my entire life and this course. Thanks also goes out specially to my beloved sister Dairin @ oyoi for her help in some editing on the references part of this thesis and her assistance to look after my two precious princess. Her presence around my family always creates pleasure time for us especially for my children. I also would like to thank my two years and ten months old daughter, Cgyne Angelicca @ ggen and ten months old daughter, Cyrsthy Aenthem @ ddem. Their coming brought indefinite laughter and happiness to my family. I am profoundly grateful to my dear husband, Patrick Assun @ Johnny, for his endless love and support during the period of my Master degree program.

Lastly but not least, thank you very much. I doubt that I will ever be able to convey my appreciation fully, but I owe all of you my eternal gratitude. May GOD bless and be with you always.
Thank you.

Anisah Savantil
02 November 2011

ABSTRACT

OPTIMISATION OF *gus*A GENE TRANSFER INTO COCOA (*THEOBROMA CACAO*) VIA *AGROBACTERIUM TUMEFACIENS*-MEDIATED TRANSFORMATION

This study describes the optimization of parameters (pH, temperature, periods of co-cultivation and age of explant) for *gus*A gene transfer into two cocoa clones (PBC 123 and BR 25) via *Agrobacterium tumefaciens*-mediated transformation by using staminode cocoa bud flower collected one day before anthesis as explants. Super avirulent *A. tumefaciens* strain AGL 1 harbouring the binary vector pGPTV-Kan/GUS was used. The binary vector contains a CaMV 35S-driven β-glucuronidase (GUS) gene and a complete neomycin phosphotransferase II (*npt*II) gene for conferring plant resistance to the antibiotic paromomycin. Callus induction medium (IM) for clone PBC 123 consisted of Driver and Kuniyuki Walnut (DKW) minerals, glucose, vitamins, 2 mg l^{-1} 2, 4-dichlorophenoxyacetic acid (2,4-D) and 0.005 mg l^{-1} Thidiazuron (TDZ) pH5.3. Callus IM for clone BR 25 was identical to IM for clone PBC 123 except the TDZ was replaced with 100 mg l^{-1} 2-iP. Callus was allowed to proliferate on semi-solid callus inducing medium for 14 days, 21 days, and 28 days prior co-cultivation with *A. tumefaciens* on semi-solid co-cultivation media for one, two, and three days at various temperatures (19^{0}C, 21^{0}C, 23^{0}C, and 25^{0}C), and pHs (4.8, 5.3, and 5.8). Each set of parameter was design in triplicate plate. Half amount of treated calli in each tested parameter was examined for GUS expression, while another half was selected on selective medium containing 100 µg ml^{-1} paromomycin. GUS expression analyses were done after 18 weeks selection. Based on the GUS expression analyses, up to 86% of calli had at least one blue sector after 18 weeks on selection media containing 100 µg ml^{-1} paromomycin. The best transformation frequency (86.1%) for clone BR 25 was obtained when explant was 21 days old and transformation condition was with pH 5.8, 25^{0}C, and three days co-cultivation period. For clone PBC 123, the best transformation frequency (83.3%) was obtained when explant was 14 days old and transformation condition was with pH 4.8, 19^{0}C, and three days co-cultivation period. A PCR-walking method (Cottage *et al*. 2001) with modification was used to verify the integration of the T-DNA into genome of the putatively transformed cocoa calli. Based on the sequencing results, this method worked with purified plasmid pGPTV-KAN/GUS but the sensitivity too low for putatively transformed cocoa calli.

ABSTRAK

Kajian ini membincangkan beberapa parameter termasuk suhu, pH, tempoh ko-kultivasi dan umur kalus untuk pemindahan gen gusA ke dalam dua klon koko (PBC 123 dan BR 25) melalui kaedah Agrobacterium tumefaciens dengan menggunakan staminode bunga koko sebagai eksplant. Strain A. tumefaciens yang digunakan adalah dari jenis 'super' avirulen AGL 1 yang membawa vektor binari pGPTV-Kan/GUS. Vektor pGPTV-Kan/GUS ini mengandungi gene β-glucuronidase (GUS) yang dikawal oleh promoter CaMV 35S dan gen lengkap neomycin phosphotransferase II (nptII) yang membawa kerintangan tumbuhan terhadap antibiotik paromomycin. Media mengaruh kalus untuk klon PBC 123 terdiri daripada mineral DKW, glukosa, vitamin-vitamin, 2 mg l^{-1} 2,4-D dan 0.005 mg l^{1} TDZ, pH5.3. Media mengaruh kalus untuk klon BR 25 adalah sama dengan media mengaruh kalus klon PBC 123 kecuali kandungan TDZ yang digantikan dengan 100 mg l^{1} 2-iP. Kalus dibiarkan membesar di atas media mengaruh separa pejal selama 14 hari, 21 hari dan 28 hari sebelum ko-kultivasi dengan A. tumefaciens di atas media ko-kultivasi separa pejal selama satu, dua dan tiga hari pada suhu 19^{0}C, 21^{0}C, 23^{0}C dan 25^{0}C serta pada pH 4.8, 5.3, and 5.8. Setiap set parameter direka dalam tiga replikat piring. Separuh daripada kalus yang telah diko-kultivasi dengan A. tumefaciens dari setiap set parameter diambil untuk tujuan ujian penyataan aktiviti GUS. Manakala yang separuh lagi dikultur di atas media pemilihan mengandungi 100 µg ml^{1} paromomycin. Analisis penyataan GUS dilakukan selepas 18 minggu di atas media pemilihan. Berdasarkan kepada analisis penyataan GUS, sehingga 86% daripada kalus yang diuji mempunyai sekurang-kurangnya satu kawasan bertanda biru selepas 18 minggu di atas media pemilihan. Frekuensi transformasi terbaik (86.1%) untuk klon BR 25 diperolehi daripada kalus yang berumur 21 hari, 3 hari ko-kultivasi pada pH 5.8 dengan suhu 25^{0}C. Bagi klon PBC 123, frekuensi transformasi terbaik (83.3%) diperolehi daripada kalus berumur 14 hari, 3 hari ko-kultivasi pada pH 4.8 dengan suhu 19^{0}C. Kaedah 'PCR-walking' (Cottage et al. 2001) dengan pengubahsuaian digunakan untuk tujuan verifikasi integrasi T-DNA ke dalam genom kalus koko yang secara putatif telah ditransormasi. Berdasarkan kepada keputusan penjujukan, kaedah ini berfungsi bagi plasmid tulin pGPTV-KAN/GUS tetapi kepekaannya adalah terlalu rendah untuk kalus koko yang putatif.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATION

AS	: Acetosyringone
ATP	: Adenosine Triphosphate
A. tumefaciens	: *Agrobacterium tumefaciens*
BAP	: 6-benzylaminopurine
BLAST	: Basic Local Alignment Search Tool
bp	: base pair
BR25	: Balung River 25
$CaCl_2.2H_2O$	: Calcium chloride
$(CaNO_3)_2.4H_2O$	: Calcium nitrate 4-hydrate
CAT	: Chloramphenicol acetyltransferase
Chi	: Chitinase
cm	: Centimeter
CPB	: Cocoa Pod Borer
DNA	: Deoxyribonucleic
DKW	: Driver and Kuniyuki Walnut (1984)
DMF	: Dimethylformamide
DMSO	: Dimethylsulfoxide
E. coli	: *Escherichia coli*
EDTA	: Ethylenediaminetetra acetic acid
EtBr	: Ethidium bromide
g	: gram
GFP	: Gene fluorescent protein
GUS	: β-glucuronidase reporter gene

HPT	: Hygromycin phospgotransferase
hpt	: Hygromycin phospgotransferase
IAA	: 3-indoleacetic acid
IBA	: 3-indolebutyric acid
KAC	: Potassium acetate
Kb	: Kilobase
KCI	: Potassium chloride
KH_2PO_4	: Potassium dihydrogen phosphate
K_2HPO_4	: Dipotassium phosphate
KNO_3	: Potassium nitrate
L	: Liter
LB broth	: Luria Bertani broth
LB	: Left border
LUC	: Luceferase gene
MAS	: Molecular marker assisted selection
MARs	: Matrix attachment regions
MCB	: Malaysian Cocoa Board
MES	: Morpholineethanesulfonic acid
mg	: Miligram
Mg	: Magnesium
$MgSO_4.H_2O$	: Magnesium sulfate
mm	: Milimeter
MS	: Murashige and Skoog (1962)
Mw	: Molecular weight
NAA	: 1-napthylacetic acid

NaCl	: Sodium chloride
$NaH_2PO_4.H_2O$	: Sodium phosphate, sodium dihydrogen phosphate
NaOH	: Sodium hydroxide
NPTII	: Neomycin phosphotransferase II
PBC123	: Prang Besar Clone 123
pGPTV-Kan/GUS	: Plasmid glucuronidase plant transformation vector Kan/GUS
PCR	: Polymerase chain reaction
PPT	: Phosphinothricin
PVP	: Polyvinil pyrolidone
RB	: Right border
SDS	: Sodium dodecyl sulfate
SE	: Somatic embryogenesis
rpm	: Resolution perminute
T-DNA	: Transfer – deoxyribonucleic acid
TDZ	: Thidiazuron
Ti-plasmid	: Tumor-inducing plasmid
UV	: Ultra violet
V	: Volt
Vir	: Virulence
Vol	: Volume
VSD	: Vascular Streak Dieback
X-Gluc	: 5-bromo-4-chlo-3-Indoyl-β-D-glucuronide
2, 4-D	: 2, 4-dichlorophenoxyacetic acid
2iP	: N^6-(2-isopentyl)adenine
%	: Percentage

^0C	: Degree Celcsius
µM	: Micro molar

LIST OF APPENDIX

CHAPTER 1

INTRODUCTION

Theobroma cacao L. (cocoa tree) is a tropical perennial tree, native to rainforest of the Amazon basin, which has been cultivated since pre-Columbian times (Hurst *et al.*, 2002). Cocoa is one of the important commodity crops cultivated in Malaysia. Currently, cocoa is third important crop in Malaysia after oil palm and rubber (Azhar and Lee, 2004). In terms of cocoa beans grinding, Malaysia is the largest grinder in Asia and emerged as fifth major grinder around the globe (Malaysian Cocoa Monitor, 2010). The processed cocoa beans have been used largely in food, pharmaceutical, cosmetic, and world chocolate industries. In ecological benefits, cocoa plantation serves as a component of complex agroecosystem that provide both economic and ecological benefits to the farmers and the producing countries (Wood and Lass, 1985).

Despite its importance, cocoa production is seriously affected by a number of pests and diseases. The Cocoa Pod Borer (CPB) (*Conopomorpha cramerella* Snellen; Lepidoptera, Gracillariidae) is the most serious insect pest of cocoa whereas the Vascular Streak Dieback (VSD) (*Oncobasidium Theobroma*) and Black Pod are two major elements that affecting the cocoa production in Malaysia (Ahmad Kamil *et al.*, 2005). Annual loss due to CPB in cocoa bean production in Malaysia is estimated not less than 15 percent of potential yields (Malaysian Cocoa Board, personal communication). At the current production of about 30,000 tonnes per year, this translates into a production loss of 5,000 tonnes per year which is an economic loss of RM10,000/tonne from the total annual income RM50,000,000 (Malaysian Cocoa Board, personal communication). If left uncontrolled, damage caused by CPB can result in 100% crop loss. Current control measures for pests and diseases for cocoa depend mostly on insecticides, which are expensive and dangerous (Azhar, 1987). Therefore, new environmentally safe and efficient methods for controlling the pests and diseases are greatly needed.

Planting cocoa trees that resistant to pest and disease is the best way to overcome the problem of yield loss due to pests and diseases. There are commercially cultivated cocoa clones various degrees of resistance to CPB or VSD or black pod has been reported (Ahmad Kamil *et al.*, 2005). However, none has been found to be fully resistant and the resistance levels are very much depending on the cocoa clone. It could be highly resistant, partially resistant or susceptible and it may not possess high yield attributes as well. An alternative method to address this problem is creating cocoa tree that carries all the favoured traits (highly resistant and yet high yield) which can be obtained through genetic transformation and tissue culture. Through genetic transformation for example, a B. t. gene or protease inhibitor gene can be inserted into the cocoa genome to create cocoa tree that resistant to CPB (Sharma *et al.*, 2000). However, successful transformation can only be obtained with an efficient in vitro plant regeneration and transformation protocols.

A reliable protocol for *Agrobacterium tumefaciens*-mediated transformation and generation of transgenic cocoa plants has been established (Maximova *et al.*, 2003, 2006). Despite this achievement, cocoa transformation is still remains inefficient. In Malaysia, the most successful work on cocoa transformation is on cocoa somatic embryos using *Agrobacterium* system which was done by the Malaysian Cocoa Board (MCB) scientists. Partially transformed cocoa somatic embryos were produced, unfortunately these somatic embryos failed to germinate into plantlets.

The previous transformations works have set a platform for optimisation of gene transfer system into cocoa genome via *Agrobacterium tumefaciens*. There are many factors affecting transformation efficiency in plant, these include: *Agrobacterium* strain, age of explants, explant type, addition of acetosyrngone, co-cultivation period, co-cultivation pH and co-cultivation temperature (Bernal *et al.*, 2009; Razzaq *et al.*, 2011; Suhakat Ali *et al.*, 2007). Therefore, the objective of this study was to optimise transformation parameters for cocoa clone PBC123 and BR25 via *Agrobacterium tumefaciens*. The scope for this study includes; evaluating the effect of explant age (14-days, 21-d, and 28-d), co-cultivation period (1-day, 2-d,

and 3-d), co-cultivation temperature (19^0C, 21^0C, 23^0C, and 25^0C), and co-cultivation pH (4.8, 5.3, and 5.8) Staminode-derived calli were transformed using *Agrobacterium tumefaciens* strain AGL1 harbouring the vector pGPTV-Kan/GUS. This work also evaluates the integration of the T-DNA into the cocoa genome, using a modified PCR-walking method (Cottage *et al.,* 2001).