UNIVERSITI PUTRA MALAYSIA

AGROBACTERIUM-MEDIATED TRANSFORMATION OF WILD 'SENDUDUK' (*MELASTOMA MALABATHRICUM*) AND BLUE 'SENDUDUK' (*TIBOUCHINA SEMIDECANDRA*) WITH SENSE AND ANTISENSE DIHYDROFLAVONOL-4-REDUCTASE GENES

WILSON YONG THAU LYM

FBSB 2007 3

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MASTER OF SCIENCE
UNIVERSITI PUTRA MALAYSIA

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By

WILSON YONG THAU LYM

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

June 2007

DEDICATED TO:

FATHER, MOTHER, BROTHERS, SISTER & CHAI HOON

WHO ALWAYS HAVE FAITH IN ME

AND

THEIR SUPPORTS HAVE GUIDED ME TO GONE THROUGH

ALL THE OBSTACLES IN LIFE

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

AGROBACTERIUM-MEDIATED TRANSFORMATION OF WILD 'SENDUDUK' (_MELASTOMA MALABATHRICUM_) AND BLUE 'SENDUDUK' (_TIBOUCHINA SEMIDECANDRA_) WITH SENSE AND ANTISENSE DIHYDROFLAVONOL-4-REDUCTASE GENES

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June 2007

Chairman: **Janna Ong Abdullah, PhD**

Faculty: **Biotechnology and Biomolecular Sciences**

Flower industry has now emerged into a very profitable commercial enterprise. In Malaysia, *Melastomataceae* spp. is identified as a potentially important flowering ornamental and they are grown for commercialization purposes. However, only a few limited flower colours reduced their commercial value. In order to improve the qualities of plants as well as develop new varieties, genetic transformation was suggested. In this study, transformation of potentially flowering ornamental *Melastoma malabathricum* and *Tibouchina semidecandra* with sense and antisense dihydroflavonol-4-reductase (DFR) genes using *Agrobacterium*-mediated method was carried out. Minimal inhibitory concentrations of kanamycin on regeneration of *M. malabathricum* and *T. semidecandra* explants were obtained for the selection of putative transformants. Kanamycin concentrations at 500 and 400 mg/L were suggested to be used in the selection media for *M. malabathricum* shoot and node explants transformation, whereas 400 and 300 mg/L kanamycin were suggested to select for transformed shoots and nodes

of *T. semidecandra*, respectively. Parameters such as bacterial strain, bacterial concentration, pre-culture period, co-cultivation period, immersion time, acetosyringone concentration and wounding type known to influence the transformation efficiency were assessed using green fluorescent protein (GFP) as marker. Results obtained were based on the percentage of GFP expression which was observed three days post-transformation. *Agrobacterium tumefaciens* strains LBA4404 and EHA105 at 1×10^7 cfu mL^{-1} (OD_{600nm} 0.8) showed the highest infectivity on *M. malabathricum* and *T. semidecandra*, respectively. Four days of pre-culture and two days of co-cultivation were optimum for *M. malabathricum* transformation, while three days of pre-culture and co-cultivation were observed for *T. semidecandra*. Results also showed that 60 min of immersion with the addition of 200 µM acetosyringone gave the highest percentage of positive transformants for both *M. malabathricum* and *T. semidecandra*. Mild wounding of explants prior to transformation also significantly enhanced the transformation event but for *M. malabathricum* only. With the optimized transformation protocol established, plasmids pBETD10 and pBETD11, each harbouring DFR gene at different orientations (sense and antisense) and selectable marker *npt*II for kanamycin resistance, were used to transform *M. malabathricum* and *T. semidecandra*. The putative transformants were selected in the presence of kanamycin with their respective optimized concentration. Approximately 4.0% of shoots and 6.7% of nodes for *M. malabathricum* regenerated after transforming with pBETD10, whereas only 3.7% (shoots) and 5.3% (nodes) regenerated with pBETD11 transformation. For the selection of *T. semidecandra*, 5.3% of shoots and 9.3% of nodes regenerated with pBETD10 transformation, while only 4.7% (shoots) and 8.3% (nodes) regenerated after being transformed with pBETD11. Presence and integration of the sense and antisense DFR genes into the genome of *M.*

malabathricum and *T. semidecandra* were confirmed by polymerase chain reaction (PCR), nucleotide sequence alignment and southern blot analysis. Regenerated putative transformants were acclimatized to glasshouse conditions. Approximately 31.0% pBETD10-transformed and 23.1% pBETD11-transformed *M. malabathricum* survived in the glasshouse, whereas 69.4% pBETD10-transformed and 57.4% pBETD11-transformed *T. semidecandra* survived. These putative regenerated transformants were subsequently grown on soil, awaiting flowering. In the present study's time frame, both pBETD10- and pBETD11-transformed *T. semidecandra* had attained maturity and flowering stages. The colour changes caused by transformation were observed at the budding stage where greenish buds were produced by both *T. semidecandra* harbouring the sense and antisense DFR transgenes. Besides, production of four-petal flowers by putative *T. semidecandra* transformants also indicated the morphological difference from wild type plants.

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

TRANSFORMASI BAGI SENDUDUK LIAR (*MELASTOMA MALABATHRICUM*) DAN SENDUDUK BIRU (*TIBOUCHINA SEMIDECANDRA*) MELALUI KAEDAH *AGROBACTERIUM* DENGAN *SENSE* DAN *ANTISENSE* BAGI GEN DIHIDROFLAVONOL-4-REDUKTASI

Oleh

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Jun 2007

Pengerusi: **Janna Ong Abdullah, PhD**

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Industri bunga kini telah berkembang menjadi perusahaan komersial yang membawa keuntungan. Di Malaysia, *Melastomataceae* spp. dikenalpasti mempunyai potensi untuk menjadi bunga hiasan penting dan ia ditanam untuk tujuan kemersialisasi. Akan tetapi, bilangan warna bunga yang terhad telah mengurangkan nilai komersialnya. Untuk tujuan peningkatan kualiti tumbuhan serta penghasilan variasi yang baru, transformasi genetik telah dicadangkan. Dalam penyelidikan ini, transformasi untuk *Melastoma malabathricum* dan *Tibouchina semidecandra* yang berpotensi menjadi bunga hiasan dengan *sense* dan *antisense* bagi gen dihidroflavonol-4-reduktasi (DFR) melalui kaedah *Agrobacterium* telah dilaksanakan. Kepekatan kanamicin terendah sebagai perencat terhadap regenerasi bagi keratan-keratan pokok *M. malabathricum* dan *T. semidecandra* telah diperolehi untuk tujuan pemilihan transforman putatif. Kanamicin dalam kepekatan 500 dan 400 mg/L telah dicadangkan supaya digunakan dalam media pemilihan untuk transformasi keratan-keratan pucuk dan ketiak bagi *M. malabathricum*,

manakala kanamicin dalam 400 dan 300 mg/L masing-masing dicadangkan untuk memilih keratan-keratan pucuk dan ketiak yang ditransformasi bagi *T. semidecandra*. Faktor-faktor seperti strain bakteria, kepekatan bakteria, jangka masa pre-kultur, jangka masa ko-kultivasi, jangka masa rendaman, kepekatan asetosiringon dan taraf pencederaan yang diketahui mempengaruhi keberkesanan transformasi telah ditentukan dengan menggunakan *green fluorescent protein* (GFP) sebagai penanda. Keputusan yang diperolehi berdasarkan kepada peratusan ekspresi GFP pada hari ketiga selepas transformasi. Strain bagi *Agrobacterium tumefaciens* LBA4404 dan EHA105 pada 1×10^{7} cfu ml^{-1} (OD_{600nm} 0.8) masing-masing menunjukkan tahap penjangkitan yang tertinggi untuk *M. malabathricum* dan *T. semidecandra*. Empat hari jangka masa pre-kultur dan dua hari jangka masa ko-kultivasi telah dioptimumkan untuk transformasi *M. malabathricum*, di mana tiga hari jangka masa pre-kultur dan ko-kultivasi telah diperhatikan untuk *T. semidecandra*. Keputusan juga menunjukkan bahawa rendaman selama 60 min dengan penambahan 200 µM asetosiringon memberi peratusan tertinggi kepada transforman positif untuk kedua-dua *M. malabathricum* dan *T. semidecandra*. Pencederaan ringan terhadap keratan pokok sebelum transformasi juga jelas meningkatkan aktiviti transformasi tetapi untuk *M. malabathricum* sahaja. Dengan penghasilan protokol transformasi yang optimum, plasmid-plasmid pBETD10 dan pBETD11, masing-masing membawa gen DFR dalam orientasi yang berlainan (*sense* dan *antisense*) dan penanda pemilihan *npt*II bagi rintangan kanamicin telah digunakan dalam transformasi *M. malabathricum* dan *T. semidecandra*. Transforman-transforman putatif telah dipilih dalam kehadiran kanamicin dengan kepekatan yang ditentukan masing-masing. Sebanyak 4.0% keratan pucuk dan 6.7% keratan ketiak bagi *M. malabathricum* mengalami regenerasi selepas ditransformasi dengan pBETD10,

manakala hanya 3.7% (keratan pucuk) dan 5.3% (keratan ketiak) mengalami regenerasi dengan transformasi pBETD11. Untuk pemilihan bagi *T. semidecandra*, 5.3% keratan pucuk dan 9.3% keratan ketiak telah mengalami regenerasi dengan transformasi pBETD10, di mana hanya 4.7% (keratan pucuk) dan 8.3% (keratan ketiak) mengalami regenerasi selepas ditransformasi dengan pBETD11. Kemunculan dan integrasi untuk *sense* dan *antisense* bagi gen DFR ke dalam genom *M. malabathricum* dan *T. semidecandra* telah ditentukan melalui *polymerase chain reaction* (PCR), susunan jujukan nukleotida dan analisis *southern blot*. Transforman-transforman putatif yang mengalami regenerasi telah disesuaikan untuk pertumbuhan di bawah keadaan rumah kaca. Sebanyak 31.0% pBETD10-transforman dan 23.1% pBETD11-transforman daripada *M. malabathricum* terselamat di dalam rumah kaca, manakala 69.4% pBETD10-transforman dan 57.4% pBETD11-transforman daripada *T. semidecandra* terselamat. Transforman-transforman putatif yang mengalami regenerasi ini kemudian ditumbuh atas tanah untuk pembungaan. Dalam jangka masa pengajian terkini, kedua-dua pBETD10- dan pBETD11-transforman bagi *T. semidecandra* telah mencapai peringkat matang dan berbunga. Perubahan warna akibat transformasi telah diteliti pada peringkat pembentukan kuntum bunga di mana kuntum hijauan telah dihasilkan oleh kedua-dua *T. semidecandra* yang membawa *sense* dan *antisense* bagi transgen DFR. Selain itu, penghasilan bunga yang berkelopak empat daripada transforman-transforman putatif bagi *T. semidecandra* juga menunjukkan perbezaan dari segi morfologi berbanding dengan tumbuhan semulajadi.

ACKNOWLEDGEMENTS

I am grateful to God that giving me the physical and mental strength to carry on my study up to completion. He always there for me and lead me gone through my life.

I would like to express my deepest gratitude to my supervisor, Dr. Janna Ong Abdullah, for the time, encouragement and patient given throughout my study. Her guidance and invaluable advice are truly appreciated and without which, the outcome would have been not possible. I also wish to thank my co-supervisor, Prof. Dr. Maziah Mahmood for her support, advice and helpful suggestion. She also kindly gives me the permission to use all the equipments and facilities in her lab without any constraint.

My sincere appreciation is extended to Dr. Tan Chon Seng (MARDI) and Dr. Wee Chien Yeong (MARDI) for their technical assistances given during my study. Special acknowledgement also given to Dr. Ryutaro AIDA (National Research Institute of Vegetables, Ornamental Plants and Tea, Japan) for his invaluable assistance and helpful suggestion. Special thanks also due to Dr. Masako Mizutani (Suntory Limited, Japan) representing her institute providing the source of plasmids pBETD10 and pBETD11 for my study.

I also wish to thank all my ex- and present labmates for their patience and supports, especially Khairul Anuar Khairoji, Noorman Affendi Marzukhi, Kenneth Way, Ng Chea Yee, Vun Khai Yan, Chew Yee Chern, Lai Kok Song, Azzreena Mohamad Azzeme, Thong Weng Hing, Chan Wai Sun, Yap Wai Sum and Pua Teen Lee. Their willingness

to share their idea, wisdom and experience had guided me to achieve my objectives. I had been gone through so much with them during my study and gained many invaluable experiences from working together with them. Acknowledgement also extended to Shen Fung for her assistance in glasshouse acclimatization of transgenics which make my research more successful.

Last but not least, my deepest appreciation extended to my father, mother, brothers, sister and Chai Hoon for their understanding and support. Their love and care in many ways really kept me going and contributed to the accomplishment of my study.

I certify that an Examination Committee has met on 18 June 2007 to conduct the final examination of Wilson Yong Thau Lym on his Master of Science thesis entitled "*Agrobacterium*-Mediated Transformation of Wild 'Senduduk' (*Melastoma malabathricum*) and Blue 'Senduduk' (*Tibouchina semidecandra*) with Sense and Antisense Dihydroflavonol-4-reductase 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:

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Date: 9 August 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.

_____

WILSON YONG THAU LYM

Date: 27 June 2007

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LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
B5	basal media for tissue culture (Gamborg *et al.*, 1968)
BAP	6-benzylaminopurine
bp	base pair
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
CAB	chlorophyll a/b binding protein promoter
CaMV 35S	cauliflower mosaic virus 35S promoter
CAT	chloramphenicol acetyl transferase
cDNA	complementary DNA
cfu mL^{-1}	colony forming units per millilitre
CHS	chalcone synthase
CO_2	carbon dioxide
CTAB	hexadecyltrimethylammonium bromide
dATP	deoxyadenosine triphosphate
DFR	dihydroflavonol-4-reductase
DNA	deoxyribonucleic acid
dNTP	deoxynicotinamide triphosphate
DTT	dithiothreitol
E12Ω	revised *CaMV* 35S promoter
EDTA	ethylenediaminetetraacetic acid
EtBr	ethidium bromide

Fe-EDTA	iron-EDTA
ethanol	ethyl alcohol (100%)
GFP	green fluorescent protein
GUS	β-glucuronidase
*gus*A	β-glucuronidase gene
h	hour
HCl	hydrochloric acid
hpt	hygromycin phosphotransferase gene
*hpt*II	hygromycin phosphotransferase II gene
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
KAc	potassium acetate
Kb	kilobase
KCl	potassium chloride
LB	Luria Bertani
LiCl	lithium chloride
LUC	luciferase
luc	luciferase gene
M	molarity
MARDI	Malaysian Agricultural Research and Development Institute
MgAc	magnesium acetate
$MgCl_2$	magnesium chloride
mgfp5	modified version of GFP gene

min	minute
mRNA	messenger RNA
MS	basal media for tissue culture (Murashige and Skoog, 1962)
N	normality
N6	basal media for tissue culture (Chu *et al.*, 1975)
NAA	naphthaleneacetic acid
NaCl	sodium chloride
NaH_2PO_4	sodium dihydrogen phosphate
Na_2HPO_4	disodium hydrogen phosphate
NaOH	sodium hydroxide
Na_2SO_3	sodium sulphate
NCBI	National Center for Biotechnology Information
NLB	200 mM Tris-Cl, 50 mM EDTA, 2 M NaCl, 2% CTAB
NN	basal media for tissue culture (Nitsch and Nitsch, 1969)
NO_3^-	nitrate ion
NPTII	neomycin phosphotransferase II
*npt*II	neomycin phosphotransferase II gene
OD_{600nm}	optical density in 600 nm
PCR	polymerase chain reaction
PEG	polyethylene glycol
RNA	ribonucleic acid
RNaseA	ribonuclease A
ROT3	rotundifolia 3
rpm	revolutions per minute