



UNIVERSITI PUTRA MALAYSIA

**ESTABLISHMENT OF A BIOLISTIC-MEDIATED TRANSFORMATION
SYSTEM FOR AN INDIGENOUS FRAGRANT ORCHID, *PHALAENOPSIS
VIOLACEA***

CHEW YEE CHERN

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**ESTABLISHMENT OF A BIOLISTIC-MEDIATED TRANSFORMATION SYSTEM
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By

CHEW YEE CHERN

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

April 2006



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

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Chairman: Professor Maziah Mahmood, PhD

Faculty: Biotechnology and Biomolecular Sciences

Phalaenopsis, orchid recognised by its moth-like shape, orderly arranged flower, and long flower shelf-life, is one of the most important orchids grown for commercial production of cut flowers and potted plants. A study was carried out to develop the genetic transformation system for an indigenous fragrant orchid species - *Phalaenopsis violacea* Witte as this system is important for the development of novel orchid varieties with improved floriculture features and marketability.

Protocorm-like bodies (PLBs) were successfully induced from leaf segments of *in vitro* seedlings, culturing on ½ strength Murashige and Skoog (MS) medium containing auxin 2,4-D (0.2, 0.4, 0.6, 0.8 and 1.0 µM) and NAA (0.4 and 0.6 µM) in three months. The highest frequency of PLBs formation was scored at 53 % on ½ strength MS basal medium containing 0.8 µM 2,4-D. However, using ½ MS medium supplemented with each 0.4 and 0.6 µM auxin



NAA to induce PLBs, the frequency of PLBs induction was lower than 15 %. No PLBs induction observed when auxin picloram and dicamba were employed to leaf segments at a series of concentration examined (0.2, 0.4, 0.6, 0.8 and 1.0 μM). Among all the organic additives [banana homogenate (BN), tomato homogenate (TM), coconut water (CW) and taro homogenate (TR)] tested in various concentrations (range 10, 20, 30, and 40 % w/v or v/v) for PLBs multiplication, optimal proliferation of PLBs was achieved through culturing on $\frac{1}{2}$ MS medium supplemented with 10 % w/v BN. However, all the organic additives examined were found stimulated to PLBs growth in the concentration range of 5-10 % w/v or v/v compared to control treatment. Plant regeneration of PLBs was achieved in PGR-free $\frac{1}{2}$ MS basal medium (with or without 10% w/v BN).

The effectiveness of hygromycin in selecting transformed tissues has been investigated based on the minimal hygromycin level that capable to thoroughly inhibit and/or killed all the non-transformed tissues. *Phalaenopsis violacea* Witte PLBs had shown a high sensitive respond to hygromycin as a low concentration (4 mg/L) of hygromycin was sufficient to meet the requirements. Potential physical and biological parameters affecting DNA delivery into *Phalaenopsis violacea* Witte PLBs have been optimised. Green fluorescence protein (GFP) was served as the reporter system except in the study of 'Influence of co-bombardment plasmid DNA ratios on transient expression', both GFP and β -glucuronidase (GUS) detection were employed. Based on the optimised results, the ideal bombardment conditions were as followed: 6 cm target tissues distance, 1100 psi acceleration pressure, 1.0 μm gold particle size, 27 mmHg chamber vacuum pressure, 1 X bombardment time, spermidine for DNA

coating on gold particle, 72 hours post-bombardment incubation time, 2 µg of total plasmid amount and 2:1 as the ratio of plasmid DNA used.

Two putative transformed lines (recovered from hygromycin selection) were achieved from a total of 160 PLBs bombarded using the optimised transformation parameters, thus, 1.25 % transformation efficiency was obtained. However, the post-cultivation period after bombardment was found to be critical as the putative transformed PLBs were only produced with 30 post-cultivation days (indirect hygromycin selection), while selection without going through post-cultivation after bombardment (direct hygromycin selection) failed to produce any putative transformant. Pattern and behaviour of GFP expression along the path to regeneration were observed on line A putative transformant PLBs that recovered from hygromycin selection after 6-11 months of culture. Young cells or tissues showed strong green fluorescence while matured tissues gradually faded and lost their green fluorescence.

Polymerase chain reaction (PCR) results that referred to the presence of transgenes (*gfp*, *gusA*, and *hptII*) in line A putative transformant PLBs (including second and third generation clonal progenies) showed that 100 % over the 32 samples tested were positive. All the *gfp*, *gusA* and *hptII* genes were retained from first to second and to third generation of clonal progenies in the putative transformants. However, no *p35S::chs* transgene was detected in both putative transformants lines as believes incorporated DNA might be fragmented during or after the bombardment events. Subsequently, accomplishment in DNA sequencing double confirmed the presence of *gfp*, *gusA* and *hptII* transgenes in the putative transformed PLBs.



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

**PENUBUHAN SISTEM TRANSFORMASI GENETIK BIOLISTIK BAGI ORKID
BERAROMATIK TEMPATAN, *PHALAENOPSIS VIOLACEA***

Oleh

CHEW YEE CHERN

April 2006

Pengerusi: Profesor Maziah Mahmood, PhD

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Phalaenopsis, orkid yang terkenal dengan bentuk bunganya yang menyerupai rama-rama, susunan bunga yang teratur dan jangka hayat bungan yang panjang, merupakan salah satu daripada orkid yang penting, ditanam untuk dikomersialkan sebagai pakok tanaman atau bunga potong. Satu kajian telah dijalankan untuk membangunkan sistem transformasi genetik bagi orkid bau harum tempatan - *Phalaenopsis violacea* Witte, memandangkan sistem ini memainkan peranan yang penting untuk membangunkan orkid dengan rupa bentuk yang baru serta mempunyai nilai dagangan yang tinggi.

Protocorm-like-bodies (PLBs) telah berjaya dirangsang apabila potongan daun daripada anak pokok *in vitro* dikultur atas media Murashige dan Skoog (MS) berkekuatan separa yang mengandungi auksin 2,4-D (0.2, 0.4, 0.6, 0.8 dan 1.0 μM) dan NAA (0.4 dan 0.6 μM) dalam jangka masa 3 bulan. Frekuensi perangsangan PLBs adalah paling tinggi (53 %) pada

penggunaan media MS berkekuatan separa yang mengandung 0.8 μM kepekatan 2,4-D. Namun, perangsangan PLBs adalah tidak melebihi 15 % apabila auksin NAA dengan kepekatan 0.4 dan 0.6 μM masing-masing digunakan bersama media MS berkekuatan separa. Tiada perangsangan PLBs dapat diperhatikan apabila auksin picloram dan dicamba dalam lingkungan kepekatan tertentu (0.2, 0.4, 0.6, 0.8 dan 1.0 μM) digunakan ke atas potongan daun.

Di antara kesemua bahan tambahan organik [homogenasi pisang (BN), homogenasi tomato (TM), air kelapa (CW) dan homogenasi keladi (YM)] yang diuji dalam pelbagai kepekatan (10, 20, 30, dan 40 % w/v atau v/v) untuk proliferasi PLBs, proliferasi PLBs yang tertinggi telah tercapai melalui pangkulturan atas media MS berkekuatan separa dibekalkan dengan 10 % w/v BN. Walau bagaimanapun, semua jenis bahan tambahan organik yang diuji didapati merangsang pertumbuhan PLBs dalam lingkungan kepekatan 5-10 % w/v atau v/v berbanding dengan lawatan kawalan. PLBs telah berjaya ditukarkan kepada anak pokok apabila dikultur atas media kepekatan separa MS tanpa PGR (dengan atau tanpa tambahan 10 % w/v BN).

Keberkesanan *hygromycin* dalam pemilihan tisu yang transform telah dikaji berdasarkan paras minimal *hygromycin* yang berupaya untuk merencat dan/atau membunuh sel atau tisu yang tidak dapat ditransform secara keseluruhan. PLBs *Phalaenopsis violacea* Witte telah mempamerkan reaksi sensitif ke atas *hygromycin* dalam kajian ini memandangkan kepekatan *hygromycin* yang amat rendah (4 mg/L) sudah mencukupi untuk menepati syarat keperluan. Parameter fizikal dan biological yang berpotensi untuk memberi kesan kepada penghantaran

DNA ke dalam PLBs *Phalaenopsis violacea* Witte telah dibedilkan. *Green fluorescent protein* (GFP) telah digunakan sebagai sistem pelapor dalam semua eksperimen parameter pembedilan melainkan dalam ujian ‘kesan nisbah DNA plasmid *co-bombardment* ke atas ekspresi transien’, di mana kedua-dua GFP dan *β -glucuronidase* (GUS) telah digunakan. Berdasarkan kepada keputusan pembedilan, keadaan bedilan yang paling sempurna adalah seperti yang berikut: 6 cm jarak antara tisu sasaran dengan skrin penghenti, 1100 psi tekanan penambahan laju, 1.0 μm saiz pembawa mikro emas, 27 mmHg tekanan peti vakum, 1 X bilangan bedilan, spermidin digunakan sebagai bahan pengikatan DNA ke atas pembawa mikro emas, 72 jam pos-bedilan, 2 μg jumlah DNA plasmid dan 2:1 *co-bombardment* nisbah DNA plasmid.

Sebanyak dua barisan PLBs transforman putatif (yang pulih daripada ujian pemilihan *hygromycin*) telah berjaya diperoleh daripada sejumlah 160 PLBs yang dibedil dengan menggunakan parameter transformasi yang optimal. Dengan demikian, 1.25 % keberkesanan transformasi telah dicatatkan. Namun, tempoh pos-kultivasi selepas bedilan didapati kritikal memandangkan hanya melalui tempoh 30 hari pos-kultivasi, PLBs transforman putatif dihasilkan (pemilihan secara tidak langsung *hygromycin*); manakala pemilihan tanpa melalui tempoh pos-kultivasi selepas bedilan (pemilihan secara langsung *hygromycin*) telah gagal memberi sebarang PLBs transforman putatif. Corak dan sifat ekspresi GFP daripada PLBs transforman putatif (barisan A) yang telah dikultur atas media pemilihan selama 6-11 bulan secara berterusan di sepanjang proses regenerasi telah dikaji. Sel atau tisu yang muda memberi fluorescen hijau yang terang; manakala bagi tisu yang matang, fluorescen GFP menjadi semakin pudar dan hilang fluorescen hijau.

Keputusan *polymerase chain reaction* (PCR) yang ditentukan melalui pengesanan kehadiran transgen (*gfp*, *gus A* dan *hptII*) di dalam PLBs transforman putatif barisan A (termasuk klonal progeni PLBs generasi ke-2 dan ke-3) menunjukkan bahawa 100 % daripada sejumlah 32 sampel yang diuji adalah positif. Semua gen *gfp*, *gusA* dan *nptII* telah dikekalkan daripada klonal progeni generasi pertama kepada kedua dan daripada kedua kepada ketiga dalam PLBs transforman putatif. Walaupun demikian, tiada intergrasi *p35S::chs* transgen yang dapat dikesan dalam transforman barisan ini, maka dipercayai DNA telah terpotong pada masa atau selepas aktiviti pembedilan. Seterusnya, kejayaan dalam penjujukan DNA menguatkan lagi keputusan bagi kehadiran *gfp*, *gusA* dan *hptII* transgen di dalam PLBs transforman putatif.



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I certify that an Examination Committee has met on 12th April 2006 to conduct the final examination of Chew Yee Chern on his Master of Science thesis entitled “Establishment of a Biolistic-Mediated Transformation System for an Indigenous Fragrant Orchid, *Phalaenopsis violacea*” 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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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.

CHEW YEE CHERN

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LIST OF ABBREVIATIONS

2,4-D	2,4-dichlorophenoxy acetic acid
35S	promoter of the cauliflower mosaic virus gene
ACC	1-aminocyclopropane-1-carboxylic acid
ATP	adenosine triphosphate
BA	6-benzyladenine
<i>bar</i>	phosphinothricin acetyltransferase gene
Ca ²⁺	calcium ion
CaCl ₂	calcium chloride
CHS	chalcone synthase
DNA	deoxyribonucleic acid
DFR	dihydroflavonol-4-reductase
Dicamba	3,6-Dichloro-o-anisic acid
dNTP	deoxynicotinamide trisphosphate
EDTA	ethylenediaminetetraacetic acid
ethanol	ethyl alcohol
F3'5'H	flavonoid-3',5'-hydroxylase
FW	fresh weight
GC-MS	gas chromatography mass spectrometry
GFP	green fluorescent protein
GM	genetically modified
GMOs	genetically modified organisms
GUS	β-glucuronidase



h	hour
Hg	mercury
hpt	hygromycin phosphotransferase gene
<i>ipt</i>	isopentenyl transferase
ISAAA	the International Service for the Acquisition of Agri-biotech Applications
kb	kilo basepair
KCl	potassium chloride
KH_2PO_4	potassium dihydrogen phosphate
KNO_3	potasium nitrate
LUC	luciferase
MgCl_2	magnesium chloride
mgfp4	a modified version of GFP
Min	minute
mM	mili Molar
MS	Murashige and Skoog
N	nitrogen
NAA	naphthalene acetic acid
NaCl	sodium chloride
NaOH	sodium hydroxide
$(\text{NH}_4)_2\text{NO}_3$	ammonium nitrate
<i>nptII</i>	neomycin phosphotransferase type II
NTD	New Taiwan Dollar
P	phosphorus



p35S	35S- <i>sgfp</i> -TYG-nos GFP construct
PCR	polymerase chain reaction
PEG	polyethylene glycol
PGRs	plant growth regulators
Picloram	4-Amino-3,5,6-trichloropicolinic acid
PLBs	protocorm-like-bodies
psi	pound per square inch
PVP	polyvinyl pyrrolidone
Ri	root induce
RM	Ringgit Malaysia (Malaysia currency)
RNA	ribonucleic Acid
rpm	rotations per minute
SDS	sodium dodecyl sulfate
TAE	40 mM Tris-Cl (pH 7.4), 20mM sodium acetate, 1 mM EDTA
TDZ	thidiazuron
TE	10 mM Tris-Cl (pH 8.0), 1 mM EDTA
Ti	tumor induce
Tris	Tris[hydroxymethyl]aminoethane
Tris-Cl	Tris-chloride
Ubi1	maize ubiquitin 1 promoter
<i>uidA</i>	gene encodes GUS
USA	United States of America
UV	ultraviolet



<i>vir</i>	virulence gene
X-gluc	5-bromo-4-chloro-3-indoyl-glucuronide
μM	micro Molar



CHAPTER 1

INTRODUCTION

Few plants can create such an aura of mystique and grandeur as orchids. For centuries the intricate nature of orchids, for example, complex flora structure (Kurzweil, 2005), mycorrhiza reliant seed germination (Tim et al., 2002), pollination mechanism (Christensen, 1994) and resupination (Arditti, 2005) have enthralled many people including layman and scientists (Hew and Yong, 1997). Orchids are distributed all over the world except in the Polar Regions. The number of orchid genera cannot exactly be stated, but an estimate is that this number lies between 820 and 1042; meanwhile a conservative estimate for the total number of orchid species is about 25,000 known species worldwide (Vogel and Schuiteman, 2004). These make orchids (*Orchidaceae*) the largest family of flowering plants of the world. The Royal Horticultural Society of United Kingdom has 130,000 registered artificial hybrids, however, there is a huge unregistered hybrids available and more than 260,000 of the natural hybrids (Oakeley, 2004).

Knowing that Malaysia is one of the twelve mega biodiversity countries blessed as the home of 800 species, covering 120 genera of orchids (Teo, 1995), orchid industry has become an important contributor to Malaysia's economy. Malaysia floriculture industry has been given an important role under the support of National Agricultural Policy (NAP 1992 – 2010). The orchid industry in Malaysia has grown tremendously and the main destination of Malaysia fresh cut flowers orchids export was Singapore at 52 %, followed by Japan, 22 % and

