UNIVERSITI PUTRA MALAYSIA

GENETIC TRANSFORMATION OF *Oncidium* SHARRY BABY USING BIOLISTIC METHOD

NG CHEA YEE

FBSB 2007 18

GENETIC TRANSFORMATION OF *Oncidium* SHARRY BABY USING BIOLISTIC METHOD

By

NG CHEA YEE

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

October 2007

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DEDICATED TO:

Father, Mother, Brother and Seong Ling who always have faith in me.

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

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October 2007

Chairman : Janna Ong Abdullah, PhD

Faculty : Biotechnology and Biomolecular Sciences

Orchid is one of the most important export commodities in Malaysia. To remain globally competitive the orchid industry needs to constantly improve the quality and variety of flowers exported. Genetic modification of orchids to create varieties will help to boost the orchid industry in Malaysia. The aim of this study was to develop an optimized transformation procedure using the PDS-He 1000 biolistic system for the introduction of potential genes of interest into *Oncidium* Sharry Baby protocorm-like-bodies (PLB). Determination of the minimal inhibitory concentration of hygromycin to select transformed PLB showed that 100% non-transformed PLB were killed at 5.0 μg/ml hygromycin. Optimization of the transformation parameters (time course of GFP transient expression in PLB, concentration of DNA, age of PLB, presence of spermidine and $CaCl_2$ in DNA-

microcarrier precipitation and duration of single PLB in fresh medium prior bombardment) were achieved by co-bombarding the PLB with the plasmids p35S, which carries a synthetic green fluorescence protein (*sgfp*) gene, and pSM-CHS, which carries the antisense chalcone synthase (CHS) and a hygromycin resistance (*hpt*II) genes. Optimized parameters were chosen based on GFP expression in transformed PLB and the number of survivals on hygromycin selection. The results showed that the highest GFP expression was observed in 4 weeks old PLB on the second day post-bombardment using 1.0μg DNA per bombardment. Pre-incubation of the PLB for 3 days on fresh medium prior to bombardment also enhanced GFP expression. Using spermidine alone in DNA-microcarriers precipitation process was shown to result in high GFP expression. PLB obtained from each of the parameters optimization step were subjected to 5.0 μg/ml hygromycin selection and the percentage of surviving plantlets was recorded. The highest number of survivals was obtained when 0.5 μg DNA per bombardment were used on 2 weeks old PLB which had undergone 1 day pre-treatment on fresh medium prior to the bombardment. The DNA was prepared with the presence of $CaCl_2$ in the DNA-microcarriers precipitation process. After 8 months of hygromycin selection, three types of plantlets, designated as Type A, Type B and Type C, were identified based on their growth morphology on the regeneration medium. Type A plantlet never attained more than one centimeter in height before tissue necrosis set in. Type B plantlet was dwarf-like when compared to normal plant. Type C showed normal growth

comparable to untransformed plantlet. Randomly picked leaves were viewed under Environmental Scanning Electron Microscopy (ESEM), which revealed a reduction in stomata number for Type A plantlet and altered shape of the guard cells. Type B showed a combination of both normal and abnormal stomata. Type C plantlet possessed normal stomata with typical guard cell shape. Subsequently, the presence of the transgenes (s*gfp, hpt*II and antisense CHS) in the genome of putative transformants were verified by polymerase chain reaction (PCR). Genomic DNA were obtained from randomly selected transformants and subjected to PCR analyses. About 81.3 % of Type A, 1.4 % of Type B, and 3.2 % of Type C transformants successfully showed amplification of the expected band size of CHS antisense gene. While 81.3 % of Type A, 76.1 % of Type B, and 83.9 % of Type C successfully amplified the expected band size of *hpt*II gene. About 56.3 % of Type A, 26.8 % of Type B, and 35.5 % of Type C amplified the expected band size of *sgfp* gene. An optimized transformation system has been established using biolistic method for *Oncidium* Sharry Baby PLB with 12.7%, 24.5% and 78.8 % transformation frequencies for antisense CHS, *sgfp* and *hpt*II genes, respectively. Hygromycin at 5.0 μg/ml is a good selective agent for *Oncidium* Sharry Baby transformation.

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

TRANSFORMASI GENETIK *Oncidium* SHARRY BABY DENGAN KAEDAH BIOLISTIK

Oleh

NG CHEA YEE

Oktober 2007

Pengerusi : Janna Ong Abdullah, PhD

Fakulti : Bioteknologi dan Sains Biomolekul

Bunga orkid merupakan salah satu comoditi eksport yang penting di Malaysia. Untuk bersaing di peringkat antarabangsa, industri orkid perlu meningkatkan kualiti dan variasi bunga yang akan dieksport. Modifikasi genetik orkid untuk menghasilkan lebih variasi yang akan membantu mengembangkan industri orkid di Malaysia. Tujuan kajian ini adalah untuk menghasilkan satu protocol transformasi yang optimum bagi pemindahan gen asing ke dalam jasad-berbentuk-protokom (PLB) *Oncidium* Sharry Baby dengan menggunakan Sistem PDS-He 1000. Penentuan kepekatan hygromicin yang terendah untuk memilih PLB yang telah ditransformasikan menunjuk bahawa 100% PLB yang tidak ditransformasikan dibunuh pada kepekatan hygromicin 5 μg/ml. Faktor-faktor yang mempengaruhi transformasi (jangka masa untuk pengkajian ekspresi

sementara GFP, kepekatan DNA, umur PLB, kehadiran spermidine dan $CaCl_2$ dalam pengendapan DNA-mikropembawa dan jangka masa bagi PLB tunggal di medium yang segar sebelum PLB ditembak) telah dioptimumkan melalui tembakan serentak (co-tembakan) dengan plasmid-plasmid p35S yang membawa gen *gfp* sintetik, dan p35-CHS yang membawa *antisense* bagi gen Chalcone synthase dan gen *hpt* II ke dalam PLB. Faktor-faktor yang dioptimumkan berdasarkan kepada ekspresi GFP dan bilangan pokok yang hidup pada medium hygromicin. Keputusan menunjuk bahawa ekspresi GFP yang tertinggi diperhati daripada 4 minggu tua PLB pada hari kedua selepas ditembak dengan 1.0 μg DNA /tembakan kepekatan. Tiga hari pre-kultur di medium yang segar juga meningkatkan ekspresi GFP. Spermidine sahaja digunakan dalam pengendapan DNA-pembawa mikro juga menghasilkan ekspresi GFP yang tinggi. Selepas lapan bulan pendedahan pada medium pemilihan, tiga jenis tumbuhan iaitu Jenis A, Jenis B dan Jenis C dikenalpastikan. Tumbuhan Jenis A tidak pernah tumbuh melebihi ketinggian satu sentimeter. Tumbuhan Jenis B lebih bantut jika dibanding dengan tumbuhan normal. Tumbuhan Jenis C menunjuk pertumbuhan yang normal sepertimana tumbuhan yang tidak ditransformasi. Daun-daun dipilih secara rawak dipermerhati dengan menggunakan *Environmental Scanning Electron Microscopy* (ESEM) menunjukkan bahawa bilangan stomata yang sedikit pada tumbuhan Jenis A, bentuk sel pengawalnya tidak normal. Tumbuhan Jenis B mempunyai stomata yang normal dan juga yang tidak normal. Tumbuhan Jenis

C mempunyai stomata dan sel pengawal yang normal. Kemudiannya, kehadiran gen asing (*sgfp,* hpt II dan *antisense* bagi CHS) dalam genom transforman putatif ditentukan melalui *polymerase chain reaction* (PCR). Kira-kira 81.3% Jenis A, 1.4% Jenis B, dan 3.2% Jenis C transforman berjaya mengamplifikasi saiz band CHS antisense gen. Manakala 81.3% Jenis A, 76.1% Jenis B, dan 83.9% Jenis C berjaya mengamplifikasi saiz band *hpt*II gen. 56.3% Jenis A, 26.8% Jenis B, dan 35.5% Jenis C berjaya mengamplifikasi saiz band *sgfp* gen. Satu sistem transformasi yang dioptimumkan telah dibentuk dengan mempunyai kekerapan transformasi 12.7%, 24.5% dan 78.8% bagi *antisense* CHS, *sgfp* dan *hpt* II masing-masing dengan kaedah biolistik bagi PLB *Oncidium* Sharry Baby. Hygromicin pada 5.0 μg/ml merupakan agen pemilihan yang baik bagi transformasi *Oncidium* Sharry Baby.

ACKNOWLEDGEMENTS

First and foremost, thank to god for thou guidance throughout this study. Without god, this work would have been impossible. I would also like to take this opportunity to express my deepest thank to the chairman of my supervisory committee, Dr. Janna Ong Abdullah for her valuable guidance, motivation, exhortation, and encouragement throughout the completion of the research. Deepest appreciate for her patience to guide me in study, research, completing thesis and encouragement that sustained me throughout this study. Thank are also expressed to supervisory members, Prof. Dr. Marziah Mahmood for helpful suggestions, assistance and precious guidance throughout this study. I am grateful to Dr. Nazir Basiran for his support, suggestion, encouragement and arrangement for me to use ESEM in MINT (Malaysia Institute of Nuclear Technology). Without their assistance and valuable contribution, this work would also have been impossible.

I would also like to express my heartiest appreciation and thanks to my beloved family's members. Thanks for all their understanding and support throughout the study. Thanks for all their love that sustained me throughout my research.

Last but not least, appreciations also go out to my ex- and present labmates (CY, Tee, Wilson, Sree, Shiao Wei, Li Ling, Rosli, Wai Sum and Chew) for their kind guidance, valuable assistance in helping to complete the research.

I certify that an Examination Committee has met on **30 october 2007** to conduct the final examination of Ng Chea Yee on his Master of Science thesis entitled "Genetic Transformation of *Oncidium* Sharry Baby" 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:

Sieo Chin Chin, PhD
Department of Microbiology
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Norihan Mohd. Saleh, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

Mohd. Puad Abdullah, PhD
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

Ruslan Abdullah, PhD
Guthrie Research Chemara
(External Examiner)

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

Date:

This thesis was 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 were as follows:

Janna Ong Abdullah, PhD
Lecturer
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

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

Nazir Basiran, PhD
Malaysian Institute for Nuclear Technology Research
(Member)

AINI IDERIS, PhD
Professor and Dean
School of Gradute Studies
Universiti Putra Malaysia

Date: 21 February 2008

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.

NG CHEA YEE

Date: 20 February 2008

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LIST OF ABBREVIATIONS

kb	10^3 base pairs
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
CaMV	*Cauliflower Mosaic Virus*
CHS	chalcone synthase
CsVMV	*Cassava Vein Mosaic Virus*
dNTP	deoxynicotinamide triphosphate
DsRed	red fluorescent protein
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ethanol	ethyl alcohol (100%)
gfp	green fluorescent protein
h	hour
GUS	β-glucuronidase
KAc	potassium acetate
KCl	potassium chloride
LB	Luria Bertani
luc	luciferase gene
mg	milligram
MgAc	magnesium acetate
$MgSO_4$	magnesium sulphate

min	minute
μl	microliter
mM	milli Molar
μm	micrometer
MS	Murashige and Skoog
NaCl	sodium chloride
NaOH	sodium hydroxide
NH_4Ac	ammonium acetate
nm	nanometer
npt II	neomycin phosphotransferase type II
OD	optical density
PCR	Polymerase Chain Reaction
p35S	35S-*sgfp*-TYG-nos GFP construct
PDS-1000/He	Helium-Powered-Driven-System-1000
PEG	Polyethylene Glycol
RNA	ribonucleic acid
rpm	rotation per minute
s	second
SDS	sodium dodecyl sulfate
TAE	40 mM Tris-Cl (pH 7.4), 20 mM sodium acetate, 1 mM EDTA
TE	10 mM Tris-Cl (pH 8.0), 1 mM EDTA
Ti	tumor induced
Tris	Tris[hydroxymethy]aminoethane

Tris-Cl	Tris-chloride
TUB-1	promoter of Tubulin-1
Ubi-1	promoter of maize ubiquitin-1 gene
USA	United States of America
UV	ultraviolet
vir	virulence gene
2x YT	2x Yeast-Tryptone

CHAPTER I

INTRODUCTION

The family *Orchidaceae* includes a number of commercially important species that are grown for cut flowers and potted plants. The fragrant types will provide additional commercial values. Most of these fragrant orchids are unique in Malaysia, but majority of them are dull in colour, so, it is not suitable for use as cut flower, especially when the orchid industries in Malaysia are facing great competition from Singapore. Therefore, there is substantial interest in the production and improvement of these commercially valuable plants. However, orchids usually have long juvenile periods and reproductive cycle (several years), which restricts genetic improvement using traditional sexual hybridization method. The application of modern genetic engineering techniques provides an effective alternative for orchid improvement of flower colour and morphology in order to increase the commercial value of orchid.

Chalcone synthase (CHS) represents the first enzymatic step in flavonoid biosynthesis and the most abundant enzyme of phenylpropanoid metabolism in plant cells. It is always the hope that a knockout of this gene using antisense technology will improve the quality of this plant. The antisense technology is based on blocking the information flow from DNA via RNA to protein by

introduction of a complementary RNA strand of the sequence of target mRNA. Duplex formation may impair mRNA maturation or translation. It may also lead to repair mRNA degradation and result in mimicking a mutation. Pale varieties can be produced using antisense technology as proven by Griesbach (1994) and Jorgensen (1995) in petunia.

Until recently, transgenic orchid plants have been reported for only a few genera of orchid such as *Brassia, Cattleya* and *Doritaenopsis* (Knapp et al., 2000), *Cymbidium* (Yang et al., 1999), and *Dendrobium* (Men et al., 2003b, Tee et al., 2003; Tee and Marziah, 2005). *Oncidium* (You et al., 2003) and *Phalaenopsis* (Anzai et al., 1996). All the former were transformed by bombardment, but there are some genera of orchid that were transformed by *Agrobacterium*-mediated such as *Dendrobium* (Yu et al., 2001) and *Phalaenopsis* (Belarmino and Mii, 2000). The first transgenic orchid plant was reported from *Dendrobium* and was generated using biolistic bombardment (Kuehnle and Sugii, 1992). Transformation was confirmed using kanamycin selection and polymerase chain reaction analysis. Chia et al. (1994) also transformed *Dendrobium* through biolistic bombardment and used a non-invasive selection system, firefly luciferase (*luc)* gene, as a reporter gene in the transformation. Tee et al. (2003) were the first to report the insertion of green fluorescent protein (gfp) gene into *Dendrobium* orchid as a reporter gene.

In this study, transformation of *Oncidium* Sharry Baby was carried out using the PDS-He 1000 system. Previously a Taiwanese group had successfully transformed *Oncidium* Sharry Baby with pCambia 1204 using *Agrobacterium*-mediated (Liau et al., 2003b). Their focus was on the expression of reporter gene (GUS gene) in transformed PLB. In this study, however, works were done on optimizing the transformation parameters by observing *gfp* expression in PLB, followed by subsequent transformation with gene having potential value for flower colour change. *Oncidium* Sharry Baby PLB were co-bombarded with p35SGFP, which carries a synthetic *gfp* gene as a reporter gene, and pSM-CHS, which carries the chalcone synthase antisense gene. Insertion of the CHS antisense gene into *Oncidium* Sharry Baby was carried out with the aim of altering the flower colour, which has not been studied before.

Earlier on, a preliminary study was conducted to set up a transformation system for *Oncidium* Sharry Baby using the *Agrobacterium*-mediated method. However, the study was not successful. Very low and inconsistent transformation frequencies were obtained. So, an alternative method was chosen, that is, using the biolistic method to transform *Oncidium* Sharry Baby.