OPTIMISATION OF GENETIC TRANSFORMATION PARAMETERS THROUGH MICROPROJECTILE BOMBARDMENT USING GREEN FLUORESCENT PROTEIN REPORTER SYSTEM IN *DENDROBIUM* 'SONIA 17' CALLUS

By

TEE CHONG SIANG

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

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DEDICATED TO:

A-PA, A-MA, BROTHERS AND SISTERS,

ALSO NOT FORGETING TEACHERS AND FRIENDS

WHO ALWAYS HAVE CONFIDENCE IN ME AND THEIR FAITH AND CEASELESS SUPPORTS THAT GUILDING ME GONE THROUGH ALL THE OBSTACLES IN THE LIFE.

Abstract of thesis submitted to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the Degree of Doctor of Philosophy

OPTIMISATION OF GENETIC TRANSFORMATION PARAMETERS THROUGH MICROPROJECTILE BOMBARDMENT USING GREEN FLUORESCENT PROTEIN REPORTER SYSTEM IN *DENDROBIUM* 'SONIA 17' CALLUS

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Chairman: Professor Maziah Mahmood, Ph.D

Faculty: Biotechnology and Biomolecular Sciences

Dendrobium 'Sonia 17' was used for this study as it is one of the common *Dendrobium* hybrids grown for cut-flower market in Malaysia. Different concentrations of auxins (picloram, dicamba and 2,4-D) were investigated for the effectiveness of inducing callus. Callus was successfully induced in the dark in all types of media studied, however, 2,4-D was ineffective in callus induction. Calluses induced from the protocorm-like-bodies (PLBs) were used as the potential target tissues for genetic transformation. Three types of morphologically distinct callus were identified (types A, B and C) from 50 μM picloram-containing half strength Murashige and Skoog (MS) medium (type C callus) and phytohormone-free half strength MS medium (types A and B callus). Morphologically, type A callus is a mixture of compact and friable tissues, type B callus is comprised of nodal shape tissues and type C callus has a more variable shape. Regeneration studies of these

calluses were carried out to examine the effect of different cytokinins concentrations (zeatin, kinetin, BA) on the plant regeneration frequency. It was found that *Dendrobium* 'Sonia 17' plant regeneration for callus was a very slow process, after four months, and the regeneration frequency was approximately 28 % (type A callus), 20 % (type B callus) and 12 % (type C callus) in the phytohormone-free medium.

Different hygromycin concentrations were used to investigate the sensitivity of different potential target tissues. The determined hygromycin concentrations that could effectively inhibit or kill the tissues were determined at 25 mg/L for type A callus, 20 mg/L for type B callus, 10 mg/L for type C callus and 25 mg/L for PLBs.

In investigating the genetic transformation system for *Dendrobium* 'Sonia 17', the green fluorescent protein (GFP) was chosen as the reporter system. The GFP transient expression characteristics were observed and the highest GFP transient expression in all the explants types bombarded and plasmid types examined was on day two post-bombardment. Based on the results obtained and observations carried out, types A and B callus were chosen as the potential target tissues and the 35S-SGFP-TYG-nos GFP plasmid was chosen and used for the genetic transformation study of *Dendrobium* 'Sonia 17'.

Both GFP and β-glucuronidase (GUS) reporter systems were used to optimised the bombarment parameters to increase the reliability and accuracy of the co-

transformation system. Expressions of GFP and GUS genes were both driven by 35S promoter from two different plasmids, p35S and pSMDFR respectively. Bombardment parameters was optimised for both type A callus (1100 psi, 6 cm target distance, 1.0-μm gold particle size, 0.4 μg plasmid DNA per bombardment, 1:1 co-bombardment plasmid DNA ratio and two days pre-bombardment sub-culture duration) and type B callus (650 psi, 6 cm target distance, 1.0-μm gold particle size, 0.4 μg plasmid DNA per bombardment, 1:1 co-bombardment plasmid DNA ratio and two days pre-bombardment sub-culture duration). In addition, GFP was observed to have higher expression frequency in both types A and B callus in all the parameters investigated compared to the GUS system indicating the GFP system could be used as a reporter system in this co-bombardment study. The non-destructive and rapid GFP monitoring system is a better reporter system than the conventional GUS reporter system.

The bombarded callus tissues were transferred to the hygromycin-containing selection medium. After a month of culturing in the selection medium, only the putative transformed tissues were able to survive and proliferate. Four putative transformant lines were isolated and the transformation efficiency was 1.7 %. At the same time GFP and GUS were used to investigate the expression of the genes. All putative transformed lines were subjected to the molecular analyses. Insertion of the transgenes (*gfp, gusA and hptII* genes) into the genome was confirmed using (polymerase chain reaction) PCR, Southern blot and DNA sequencing. PCR analysis showed co-transformation frequency of the un-linked genes from different

plasmids was 66 %. Besides, GFP was used to monitor the expression patterns of the transformed tissues and the transformed lines were able to multiply and regenerate into plantlets.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

KAJIAN MENGOPTIMUMKAN PARAMETER TRANSFORMASI GENETIK MELALUI MIKROPROJEKTIL BEDILAN DENGAN MENGGUNAKAN SISTEM PELAPOR *GREEN FLUOROSCENT PROTEIN* UNTUK KALUS *DENDROBIUM* 'SONIA 17'

Oleh

TEE CHONG SIANG

September 2004

Pengerusi: **Profesor Maziah Mahmood, Ph.D.**

Fakulti: **Bioteknologi dan Sains Biomolekul**

Dendrobium 'Sonia 17' dipilih sebagai bahan penyelidikan kerana ia merupakan salah satu *Dendrobium* hibrid yang biasa ditanam untuk industri bunga potong di Malaysia. Pelbagai jenis auxin (picloram, dicamba dan 2,4-D) dengan kepekatan yang berlainan telah digunakan untuk menguji keberkesanan untuk induksi kalus. Kalus boleh dihasilkan di semua jenis media yang digunakan dalam keadaan gelap, walaubagaimanapun, 2,4-D adalah tidak sesuai untuk induksi kalus. Kalus yang didapati daripada *protocorm-like-bodies* (PLBs) digunakan sebagai tisu sasaran untuk transformasi genetik. Terdapat tiga jenis kalus (jenis A, B dan C) yang berlainan morfologi dikenalpasti dari kepekatan separa Murashige dan Skoog (MS) media yang mengandungi 50 μM picloram (kalus jenis C) dan juga media tanpa fitohormon (kalus jenis A dan B). Dari segi morfologi, kalus jenis A terdiri dari

campuran struktur tisu yang padat dan longer, kalus jenis B terdiri dari tisu yang berbentuk nodul dan kalus jenis C mempunyai bentuk tisu yang tidak tetap. Penyelidikan regenerasi kalus-kalus ini telah dijalankan untuk mengetahui kesan pelbagai sitokinin (zeatin, kinetin dan BA) dengan kepekatan berlainan kepada frekuensi regenerasi pokok. Didapati regenerasi kalus *Dendrobium* 'Sonia 17' adalah proses yang lambat, selepas 4 bulan, dan frekuensi regenerasi adalah 28 % (kalus jenis A), 20 % (kalus jenis B) and 12 % (kalus jenis C) dalam media tanpa fitohormon. Keupayaan regenerasi bagi kalus-kalus ini pada media tanpa fitohormon merupakan satu kelebihan untuk mengurangkan kejadian variasi somaklonal.

Kepekatan *hygromycin* yang berlainan telah digunakan untuk menyelidik kerentanan pelbagai tisu sasaran. Kepekatan *hygromycin* yang berkesan untuk membunuh berlainan jenis tisu sasaran adalah 25 mg/L untuk kalus A, 20 mg/L untuk kalus B, 10 mg/L untuk kalus C dan 25 mg/L untuk PLBs.

Dalam penyiasatan transformasi genetik sistem bagi *Dendrobium* 'Sonia 17', *green fluorescent protein* (GFP) telah dipilih sebagai sistem pelapor. Ciri-ciri ekspressi sementara GFP telah diikuti dan direkod untuk semua tisu dan plasmid GFP yang disiasat. Didapati hari kedua selepas bedilan menunjukkan ekspressi GFP yang paling tinggi bagi semua jenis tisu yang telah dibedil dengan semua jenis plasmid DNA. Berdasarkan keputusan dan pemerhatian yang diperolehi, kalus jenis A dan B telah dipilih sebagai tisu sasaran yang berpotensi dan 35S-SGFP-TYG-nos plasmid

GFP telah dipilih dan digunakan untuk kajian transformasi genetik *Dendrobium* 'Sonia 17'.

Kedua-dua sistem pelapor, GFP dan *β-glucuronidase* (GUS) sistem, telah digunakan dalam kajian parameter pembedilan supaya keputusan lebih tepat bagi sistem *co-bombardment*. Eskpressi gen GFP dan GUS yang terletak dalam plasmid p35S dan plasmid pSMDFR adalah dikawal oleh *promoter* 35S. Parameter pembedilan telah dikaji untuk kalus jenis A (1100 psi, 6 cm jarak antara tisu sasaran dengan skrin penghenti, 1.0-μm saiz pembawa mikro emas dan 0.4 μg plasmid DNA per bedilan, 1:1 *co-bombardment* nisbah plasmid DNA, dua hari subkultur sebelum pembedilan) dan B (650 psi, 6 cm jarak antara tisu sasaran dengan skrin penghenti, 1.0-μm saiz pembawa mikro emas dan 0.4 μg plasmid DNA per bedilan, 1:1 *co-bombardment* nisbah plasmid DNA, dua hari subkultur sebelum pembedilan. Tambahan lagi, GFP telah diperhatikan mempunyai frekuensi ekspresi yang lebih tinggi bagi kedua-dua jenis kalus A dan B dalam semua parameter yang disiasat dibandingkan dengan sistem GUS menunjukkan sistem GFP boleh digunakan sebagai sistem pelapor tunggal dalam pengajian pembedilan bersama ini. Sistem GFP yang tidak memusnah dan cepat jelasnya adalah sistem pelapor yang lebih baik dari sistem GUS yang lama.

Tisu kalus yang telah dibedil telah dipindahkan ke media pemilihan yang mempunyai hygromycin. Selepas satu bulan dikulturkan pada media pemilihan, hanya tisu yang kemungkinan telah ditransformasikan akan hidup dan bercambah. Empat potensi transforman berjaya diperolehi dengan frekuensi transformasi 1.7 %.

Pada masa yang sama, GFP dan GUS telah digunakan untuk mengkaji pengekspreskan gen. Analisa molekul telah dijalankan bagi semua tisu yang telah ditransformasikan. Kehadiran transgen (*gfp, gusA and hptII* gen) dalam genom telah dibuktikan dengan menggunakan analisis *polymerase chain reaction* (PCR), *Southern blot* dan jujukan DNA. Analisis PCR mendapati frekuensi co-transformasi untuk gen dari plasmid berlainan adalah 66 %. Tambahan lagi, GFP telah digunakan untuk memerhati corak eskpressi tisu yang telah ditransformasikan dan transforman didapati boleh membiak dan regenerasi menjadi pokok.

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I certify that an Examination Committee met on 21st September 2004 to conduct the final examination of Tee Chong Siang on his Doctor of Philosophy thesis entitled "Optimisation of Genetic Transformation Parameters Through Microprojectile Bombardment Using Green Fluorescent Protein Reporter System in *Dendrobium* 'Sonia 17' Callus" 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:

Mohd Arif Syed, Ph.D,
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Nor'Aini Mohd Fadzillah, Ph.D.
Associate Professor
Faculty of Science and Environmental Studies
Universiti Putra Malaysia
(Member)

Janna Ong Abdullah, Ph.D.
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

Masahiro Mii , Ph.D.
Professor,
Faculty of Horticulture
Chiba University
(Independent Examiner)

_____
GULAM RUSUL RAHMAT ALI, Ph.D.
Professor/ Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

This thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee are as follows:

Maziah Mahmood, Ph.D.
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Tan Chon Seng, Ph.D.
Malaysian Agricultural Research and Development Institute (MARDI)
Serdang, Selangor
(Member)

Mohd Puad Abdullah, Ph.D.
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

_____
AINI IDERIS, Ph.D.
Professor/ Dean
School of Graduate School
Universiti Putra Malaysia

Date:

DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations that 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.

TEE CHONG SIANG

Date:

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