UNIVERSITI PUTRA MALAYSIA

GENETIC TRANSFORMATION OF ORCHID *DENDROBIUM* SONIA-17 USING THE BIOLISTIC METHOD

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By

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Thesis Submitted in Fulfilment of the Requirement for the Degree of Doctor of Philosophy in the Faculty of Science and Environmental Studies
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DEDICATED TO:

Father (deceased), Mother, Brothers, and Sisters who always have faith in me.

Husband and daughters who are always there for me.

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

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June 2001

Chairman : Prof. Dr. Marziah Mahmood

Faculty : Science and Environmental Studies

The ever-changing tastes and preferences of orchid consumers initiated the need to create new and better varieties. Progress in molecular biology has allowed genetically well defined characteristics to be added to the gene pools, thereby increasing the potential for genetic improvement. However, such effort at creating a custom-made flower has yet to be realised in orchids. The present study aims at developing a genetic transformation system for the introduction of specific foreign genes into orchid.

Protocorm-like-bodies (PLBs) of orchid hybrid, *Dendrobium* Sonia-17, were established to be suitable target tissues for the introduction of foreign genes using the biolistic method. They were easily micropropagated *in vitro* that provided plenty of materials to work with and were a reliable source of potentially regenerable tissues.

The effect of blasting on the growth of the PLBs was evaluated by subjecting the PLBs to bombardment with uncoated gold microparticles. One month following bombardment, fresh weights gained by the PLBs were recorded. The results showed that bombarded PLBs had higher weight increments compared to non-bombarded treatments, indicating that subsequent lethal responses by the PLBs on antibiotic selections were mainly due to the selection pressure and not as a result of injuries inflicted during the bombardment.

The effectiveness of different selection agents (kanamycin, paromomycin, geniticin, hygromycin, and Basta) in inhibiting PLBs growth was evaluated. PLBs, after two weeks bombardment with uncoated gold microparticles only, were subjected to selection agents at concentrations ranging from 0 to 300 mg/L. The PLBs showed poor responses to kanamycin, paromomycin, and geneticin, but better sensitivity (at 25 mg/L) to hygromycin and Basta.

The physical and biological parameters affecting DNA delivery, based on the highest scorable transient GUS expression and minimal tissue dislocation upon impact, were optimised. The parameters tested were helium gas pressure, distance from macrocarrier to stopping screen, distance from stopping screen to target tissues, vacuum pressure, size gold microcarrier, presence of $CaCl_2$ and spermidine in DNA-microcarrier precipitation, number of bombardments, PLBs size, PLBs age, genotypes, DNA concentration, osmoticum type and concentration, duration of single PLBs in fresh medium prior bombardment, duration between post-

bombardment and GUS staining, duration between post-bombardment and selection, optimal PLBs size surviving selection pressure post-bombardment, and promoters. All the parameters tested had significant effects on DNA delivery except for PLBs age and osmoticum.

PLBs were transformed and selected using the above optimised physical and biological parameters. Resistant PLBs were subsequently regenerated into whole plants and the presence of the transgenes was verified by PCR, Dot and Southern Blot analyses.

Transformation of other orchid hybrids (*Dendrobium* Savin White, *Oncidium* Taka, and *Mokara* Chark Kuan) using the optimised transformation protocol established for *Dendrobium* Sonia-17 was also carried out. Presence of the transgene in transformed plantlets from each hybrid was verified by PCR analysis. Positive results were obtained for all hybrids except *Mokara* Chark Kuan. All PCR-positive transgenic plantlets were transferred onto medium supplemented with 20 μM BAP to induce i*n vitro* flowering. The result showed that only *Dendrobium* Sonia-17 produced the flower stalks after two to five months on culture.

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

TRANSFORMASI GENETIK ORKID *DENDROBIUM* SONIA-17 DENGAN MENGGUNAKAN KAEDAH BIOLISTIK

Oleh

JANNA ONG ABDULLAH @ ONG WEOI CHOO

Jun 2001

Pengerusi : Prof. Dr. Marziah Mahmood

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Citarasa dan keutamaan para pengguna orkid telah menggerakkan usaha untuk mencipta varieti yang baru dan lebih baik daripada yang sedia ada. Dalam masa yang sama, kemajuan dalam biologi molekul telah membolehkan sifat genetik yang tertentu diserapkan ke dalam himpunan gen yang lain, dan sekaligus meningkatkan potensi untuk perkembangan genetik berkenaan. Walaubagaimana pun, usaha mencipta sesuatu bunga seperti yang ditempah masih belum tercapai bagi tanaman orkid. Kajian ini dijalankan untuk mencipta satu sistem transformasi genetik untuk pemindahan gen asing tertentu ke dalam tanaman orkid.

Protocorm-like-bodies (PLBs) telah didapati sesuai untuk digunakan sebagai tisu sasaran dalam pemindahan gen asing dengan menggunakan kaedah biolistik. Melalui teknik kultur tisu, PLBs mudah dihasilkan dengan banyak dan juga sumber tetap tisu yang berpotensi untuk regenerasi.

Kajian terhadap kesan tembakan alat biolistik pada pertumbuhan PLBs telah dijalankan dengan penembakan pembawa mikro emas ke atas PLBs. Sebulan selepas tembakan, peningkatan berat basah PLBs direkodkan. Keputusan menunjukkan peningkatan berat basah adalah lebih tinggi bagi PLBs yang telah ditembak berbanding dengan PLBs yang tidak ditembak (kawalan) membuktikan bahawa tembakan alat biolistik ini tidak membawa kesan negatif pada pertumbuhan PLBs sebaliknya jika ada kesan negatif faktor-faktor yang lain seperti kesan agen perencat pertumbuhan adalah menjadi puncanya.

Penilaian terhadap keberkesanan agen perencat pertumbuhan PLBs (kanamisin, paromomisin, genitisin, higromisin, dan Basta) juga dilakukan. PLBs yang telah ditembak dengan pembawa mikro emas didedahkan kepada agen-agen tersebut pada kepekatan yang berbeza (0 hingga 300 mg/L) dua minggu selepas penembakan. Higromisin dan Basta menunjukkan kesan yang paling effektif dalam perencatan pertumbuhan PLBs iaitu pada kepekatan 25 mg/L.

Pengaruh faktor-faktor biologi dan fizikal dalam pemindahan DNA juga telah dioptimumkan berdasarkan kepada skor tertinggi ujian GUS dan kerosakan tisu semasa impak. Diantara faktor-faktor yang diuji adalah: tekanan gas helium, jarak diantara pembawa makro ke piring penghenti, jarak diantara piring penghenti ke tisu sasaran, tekanan hampagas, saiz pembawa mikro emas, kesan kalsium klorida dan spermidin terhadap "kemendapan" DNA dan pembawa mikro, bilangan tembakan, saiz PLBs, umur PLBs, genotip, kepekatan DNA, jenis dan kepekatan

bahan osmotik, jangkamasa PLBs disubkultur ke media segar sebelum tembakan, jangkamasa antara tembakan dan asei GUS, jangkamasa antara tembakan dan pendedahan pada agen-agen perencat pertumbuhan, saiz optimal PLBs yang berupaya untuk hidup dalam pendedahan agen-agen perencat pertumbuhan selepas tembakan, dan promoter-promoter. Setiap faktor-faktor yand dikaji diatas menunjukkan perbezaan yang jelas dalam pemindahan DNA kecuali umur PLBs dan bahan osmotik.

PLBs yang telah ditembak berdasarkan faktor-faktor fizikal dan biologikal yang dioptimumkan kemudiannya didedahkan kepada agen perencat pertumbuhan. PLBs yang rintang diasingkan dan ditumbuhkan menjadi pokok. Kehadiran gen dalam pokok-pokok demikian seterusnya dibuktikan melalui analisa PCR, Dot Blot, dan Southern Blot.

Transformasi hibrid-hibrid orkid yang lain (*Dendrobium* Savin White, *Oncidium* Taka dan *Mokara* Chark Kuan) juga telah dikaji dengan menggunakan protokol yang telah dioptimakan bagi hibrid *Dendrobium* Sonia-17. Kehadiran gen dalam pokok dari setiap hibrid telah dibuktikan melalui analisa PCR. Keputusan positif diperolehi dari semua hibrid kecuali *Mokara* Chark Kuan. Semua pokok transgenik kemudian disubkulturkan dalam media yang mengandungi 20 μM BAP untuk memangkinkan pertumbuhan tangkai bunga. Hanya *Dendrobium* Sonia-17 berjaya menghasilkan tangkai bunga selepas dua hingga lima bulan dalam subkultur.

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I certify that an Examination Committee met on 8th June 2001 to conduct the final examination of Janna Ong Abdullah @ Ong Weoi Choo on her Doctor of Philosophy thesis entitled "Genetic Transformation of Orchid *Dendrobium* Sonia-17 using the Biolistic Method" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommended that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy.

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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

JANNA ONG ABDULLAH @ ONG WEOI CHOO

Date 13th JUNE 2001

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*Act*1	promoter of rice actin 1 gene
*Adh*1	promoter of maize alcohol dehydrogenase 1 gene
ATP	adenosin triphosphate
bar	phosphinothricin acetyltransferase gene
Bp	base pairs
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
CaMV 35S	promoter of the cauliflower mosaic virus 35S gene
cpm	counts per minute
d	day
dNTP	deoxynicotinamide triphosphate
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
Emu	a recombinant promoter of the maize alcohol dehydrogenase gene that also contains enhancer elements from *Adh1* gene and *Agrobacterium*
ethanol	ethyl alcohol (100%)
GUS	β-glucuronidase
h	hour
Hg	mercury
Hyg^r	hygromycin resistant
hpt	hygromycin phosphotransferase gene
KAc	Potassium acetate
Kan^r	kanamycin resistant
kb	10^3 base pairs
KCl	potassium chloride
LB	Luria-Bertani (bacterial growth medium as described in Materials and Method)