UNIVERSITI PUTRA MALAYSIA

BIOLISTIC TRANSFORMATION OF SELECTED ORCHID HYBRIDS FOR IMPROVED SHELF LIFE AND CLONING OF PARTICAL ACC OXIDASE GENE FROM ONCIDIUM GOWER RAMSEY

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By

MOHANA ANITA

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

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.....my richest gain, I count but loss
and lay it at your feet, O Lord.....

- Isaac Waats -

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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Chairperson: Associate Professor Saleh Kadzimin, PhD

Faculty: Agriculture

The aim of the project was to lengthen the shelf life of orchid flowers to get superior quality flowers. The strategy used was by retarding the internal ethylene biosynthesis pathway through transferring the ACC oxidase gene in the reverse orientation (antisense) into the orchid cells of *Dendrobium* Savin White and *Oncidium* Gower Ramsey. This is complimented by isolation of ACC oxidase gene fragments from *Oncidium* for future genetic manipulation.

A tissue culture system was established to provide plant materials for transformation work. Protocorm-like bodies (plbs) of *Dendrobium* and *Oncidium* were used to induce callus on half strength MS (Murashige and Skoog, 1962) medium. In *Dendrobium,* unwounded plbs or wounded plbs were tested to induce callus with Picloram (0, 0.6, 0.7, 0.8, 0.9 mg/L) in combination with Kinetin (0, 0.6, 0.7, 0.8, 0.9 mg/L). *Oncidium* callus was

induced with Picloram (0, 12, 20, 30, 40, 50 mg/L) or 2,4 Diphenoxyacetic acid (2,4-D) at concentrations of 0, 5, 10, 15, 20, 25 mg/L separately. The highest rate of *Dendrobium* callus (42%) was obtained using unwounded plbs with 0.9 mg/L Picloram combined with 0.8 mg/L Kinetin. Unwounded *Dendrobium* plbs produced the highest rate of callus (17%) with combinations of 0.8 mg/L Picloram and 0.7 mg/L Kinetin or 0.9 mg/L Picloram and 0.9 mg/L Kinetin. The most effective callus induction (43.3%) for *Oncidium* was obtained with 5mg/L of 2,4-D. Picloram at 50 mg/L had the highest rate of callus induction (36.7%). Histological observations revealed that callus cells were undifferentiated whereas plbs had distinctive meristematic areas. Regeneration of *Dendrobium* and *Oncidium* callus was successfully obtained.

Before transformation, a protocol was established for the selection of putative transgenic cells using hygromycin. Optimization of particle bombardment parameters (helium gas pressure and target/macrocarrier distance) was done with GUS assay. Helium pressure of 1100 psi (7580 kPa) with platform levels 1,3 or 1,4 was found suitable. ACC oxidase antisense construct (pPhACOAS1) was used for transformation and after hygromycin selection; one transgenic line of *Dendrobium* was obtained and regenerated. Confirmation of the transformed "lines" was done by Polymerase Chain Reaction (PCR) and Southern Blot.

ACC oxidase gene was isolated from pollinated *Oncidium* flowers. Physical changes during senescence of pollinated flowers were observed and ribonucleic acid (RNA) was isolated from various stages after pollination (0 hr, 18 hrs, 24 hrs, 36 hrs, 48 hrs, 72 hrs) and unpollinated flowers. ACC oxidase expression from the RNA samples was analyzed through Northern Blot and showed increased levels of expression over time. The Reverse-Transcription Polymerase Chain Reaction (RT-PCR) technique was used to isolate ACC oxidase gene fragments from the RNA samples and was successfully amplified from three stages (unpollinated, 18 hours and 48 hours after pollination). The gene fragments were then cloned into vectors, sequenced and characterized. The nucleic sequence and deduced amino acid sequence obtained from the three different stages had high homology with other ACC oxidase sequences in the Genebank. The analysis of the positive clones obtained showed two versions of ACC oxidase sequences (OncACO1 and OncACO2) which were successfully isolated.

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

TRANSFORMASI BIOLISTIK HIBRID ORKID UNTUK PEMANJANGAN HAYAT BUNGA DAN PENGKLONAN FRAGMEN SEPARA GEN ACC OKSIDA DARIPADA *ONCIDIUM* GOWER RAMSEY

Oleh

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Kajian-kajian telah dijalankan dengan tujuan untuk menghasilkan bunga orkid yang mempunyai jangka hayat bunga yang lebih lama dan berkualiti tinggi. Strategi yang digunakan ialah dengan merencatkan proses penghasilan etilena dalam bunga melalui pemindahan ACC oksida dalam susunan terbalik (antisense) ke dalam sel orkid *Dendrobium* Savin White dan *Oncidium* Gower Ramsey. Pemencilan gen ACC oksida daripada bunga *Oncidium* pula memainkan peranan yang sama penting dalam kerja-kerja manipulasi genetik.

Satu sistem kultur tisu telah dibentuk untuk membekalkan sumber eksplan. Protokom daripada *Dendrobium* dan *Oncidium* digunakan untuk induksi kalus di atas media MS (Murashige and Skoog, 1962) dalam separuh kekuatan. Bagi *Dendrobium,* kalus diinduksi dengan protokom atau

protokom yang dicederakan dengan kombinasi Picloram (0, 0.6, 0.7, 0.8, 0.9 mg/L) dan Kinetin (0, 0.6, 0.7, 0.8, 0.9 mg/L). Kalus *Oncidium* diinduksi dengan menggunakan Picloram (0, 12, 20, 30, 40, 50 mg/L) atau 2,4 diklorofenoksi (2,4-D) dalam kepekatan 0, 5, 10, 15, 20, 25 mg/L secara berasingan. Peratus penghasilan kalus *Dendrobium* yang terbanyak (42%) diperolehi dengan menggunakan protokom sebagai eksplan dengan kombinasi 0.9 mg/L Picloram dan 0.8 mg/L Kinetin. Protokom yang dicederakan menghasilkan kalus terbanyak (17%) dengan menggunakan kombinasi 0.8 mg/L Picloram dan 0.7 mg/L Kinetin atau 0.9 mg/L Picloram dan 0.9 mg/L Kinetin. Media yang mengandungi 5mg/L 2,4-D didapati paling sesuai untuk induksi kalus *Oncidium* (43.3%). Picloram pula menghasilkan peratus kalus yang terbanyak (36.7%) pada kepekatan 50 mg/L. Pemerhatian histologi menunjukkan sel-sel kalus berbeza antara satu dengan lain berbanding sel-sel protokom. Regenerasi kalus *Dendrobium* dan *Oncidium* juga berjaya diperolehi.

Satu protokol untuk pemilihan tisu transgenik dengan menggunakan antibiotik hygromycin juga telah dibentuk sebelum transformasi. Analisis GUS digunakan untuk mengoptimumkan parameter (tekanan gas helium dan jarak aras sasasaran/'macrocarrier') dalam 'particle bombardment'. Tekanan gas helium 1100 psi (7580 kPa) dengan kombinasi aras 1,3 dan 1,4 didapati sesuai. Konstruk antisense untuk gen ACC oksida (pPhACOAS1) di gunakan untuk transformasi dan selepas pemilihan dengan hygromycin;

kalus *Dendrobium* yang transgenik berjaya diperolehi dan dipindahkan ke media regenerasi untuk menghasilkan pokok. Transformasi untuk pokok trangenik yang dihasilkan daripada kalus dipastikan dengan menggunakan analisis molekul iaitu dengan menggunakan 'Polymerase Chain Reaction' dan 'Southern Blot'.

Gen ACC oksida dipencilkan daripada bunga *Oncidium* yang telah didebungakan. Perubahan fizikal yang dialami oleh bunga-bunga yang didebungakan telah diperhatikan dan pemencilan asid ribonukleik (RNA) dibuat pada pelbagai peringkat senesens selepas pendebungaan (0 jam, 18 jam, 24 jam, 36 jam, 48 jam, 72 jam) dan bunga tanpa pendebungaan. Ekspresi ACC oksida dalam pelbagai peringkat senesens dikaji dan didapati ekspresi yang semakin ketara dalam masa lebih lama selepas pendebungaan. Kaedah 'Reverse-Transcription-PCR' digunakan untuk memencilkan fragmen gen separa ACC oksida daripada sampel-sampel RNA. Produk RT-PCR telah berjaya diamplifikasikan daripada tiga tempoh masa (tanpa pendebungaan, 18 jam dan 48 jam selepas pendebungaan). Fragmen-fragmen gen separa ACC oksida yang diperolehi telah diklonkan ke dalam vektor dan dianalisis jujukan. Jujukan asid nukleik dan asid amino yang diperolehi daripada tiga peringkat itu mempunyai persamaan yang tinggi dengan jujukan ACC oksida yang lain di 'Genebank'. Analisis jujukan menunjukkan dua versi ACC oksida yang berbeza (OncACO1 and OncACO2) telah berjaya dipencilkan.

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The things you planned for us, No one can recount to you
Were I to speak of them, They would be too many to declare
– Psalms 40 : 5

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LIST OF ABBREVIATIONS

A_X	absorbance at X nm
ACC oxidase	1-aminocyclopropane-1-carboxylic acid
ANOVA	analysis of variance
BLAST	Basic Local Alignment Search Tool
bp	base pairs
$CaCl_2$	calcium chloride
cDNA	complementary DNA
CSIRO	Commonwealth Scientific and Industrial Research Organization
CTAB	cethyltriaminebromide
ddH_2O	distilled deionized water
DEPC	diethylpyrocarbonate
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNAase	deoxyribonuclease
dNTP	deoxynicotinamide triphosphate
EDTA	ethylenediaminetetraacetic acid
E. coli	*Escherichia coli*
ethanol	ethyl alcohol (100%)
FAA	formalin: acetic acid: absolute alcohol
GUS	β-glucuronidase
HCl	hydrochloric acid
hrs	hours

IPTG	isopropylthio-β-Dgalactoside
Kb	kilobase pairs for DNA, kilobases for RNA
KOH	potassium hydoxide
LB	Luria-Bertani (bacterial growth medium)
M	molarity
mM	millimolar
MARDI	Malaysian Agricultural Research and Development Institute
$MgCl_2$	magnesium chloride
MOPS	3-(N-morpholino) propanesulfonic acid
mRNA	messenger RNA
MS	Murashige and Skoog (tissue culture medium)
NaCl	sodium chloride
Na_2EDTA	disodium ethylenediaminetetraacetic acid
NaOH	sodium hydroxide
OD	optical density
OSM	medium with high osmolarity
PCR	Polymerase Chain Reaction
PDS 1000/He	helium powered driven system 1000
PEG	polyethylene glycol
pH	negative logarithm of hydrogen ion concentration [-log(H^+)]
plbs	protocorm-like bodies
psi	pound per square inch

RE	restriction enzyme
RT-PCR	Reverse Transcription Polymerase Chain Reaction
RNA	ribonucleic acid
RNAse	ribonuclease
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
SSC	150 mM NaCl, 15 mM sodium citrate (pH 7.0)
TAE	40 mM Tris-Cl (pH 7.4), 20 mM sodium acetate, 1 mM EDTA
TBA	Tertiary butyl alcohol
Tris	Tris[hydroxymethyl]aminoethane
Triton X-100	T-octylphenoxypoly-etoxyethanol
X-gluc	5-bromo-4-chloro-3-indoyl-glucuronide
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranosidase
VW	Vacin and Went (tissue culture medium)
v/v	volume for volume (volume in ml in a 100 ml total volume)
w/v	weight for volume (grams in a 100 ml volume)
2,4-D	2,4 dichlorophenoxyacetic acid

CHAPTER 1

INTRODUCTION

Orchidaceae is the largest family of flowering plants. It is estimated that 10 percent of all flowering plants are orchids (Yam, 1998). The diversity of the Orchidaceae family is absolutely magnificent and beyond imagination. This diversity and uniqueness of orchid has sparked off the interest of hobbyist, hobbyist-cum-commercial grower and also purely commercial growers. Therefore there is a demand for orchids both locally and overseas as orchid flowers sell readily and fetch lucrative returns (Fadelah *et al.*, 2001).

The Malaysian flower industry has developed into a very viable commercial enterprise. This trend is expected to continue in the future with higher standards of living of the local population and in developed countries. Malaysia has all the opportunities, including a conducive environment to exploit the floriculture industry (Zaharah and Noor Auni, 1994). Even though cultivation of orchids for fresh cut flowers in Malaysia began in the 1960s, it was not until in the eighties that commercial orchid production gained such popularity that Malaysia is now ranked as one of the well-known producers of these exotic blooms. Malaysian orchids are classified as tropical orchids and are now exported mainly to Japan, Singapore, the Netherlands, Taiwan, Europe and Australia. In Malaysia, the largest orchid production areas are mostly in Johor. The distribution of the rest of the