UNIVERSITI PUTRA MALAYSIA

TISSUE CULTURE AND *AGROBACTERIUM*-MEDIATED TRANSFORMATION ON *ROSA HYBRIDA* L. 'CHRISTIAN DIOR'

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TISSUE CULTURE AND *AGROBACTERIUM*-MEDIATED TRANSFORMATION ON *ROSA HYBRIDA* L. 'CHRISTIAN DIOR'

By

KONG SWEE LAN

Thesis Submitted in Fulfilment of the Requirements for the Degree of Master of Science in the Faculty of Science and Environmental Studies, Universiti Pertanian Malaysia.

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%	percentage
ABA	abscissic acid
BA	6-benzylaminopurine
bp	base pair
CAT	chloramphenicol acetyltransferase
CH	casein hydrolysate
cv.	cultivar
dH_2O	deionised water
2,4-D	2,4-dichlorophenoxyacetic Acid
GA_3	giberellic acid
GUS	ß-glucuronidase enzyme
h	hour
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
kbp	kilobase pair
kinetin	6-furfurylaminopurine
LUX	luciferase enzyme
min	minute
MS	Murashige and Skoog
MU	4-methylumbelliferone
MUG	4-methylumbelliferyl-ß-D-glucuronide
NAA	napthaleneacetic acid

*npt*II	neomycin phosphotransferase
PGR	plant growth regulator
s	second
TDZ	thidiazuron
v/v	volume/volume
w/v	weight/volume
X-Gluc	5-bromo-4-chloro3-indoyl-ß-D-glucuronic acid

Abstract of thesis submitted to the Senate of Universiti Pertanian Malaysia in fulfilment of the requirements for the degree of Master of Science.

TISSUE CULTURE AND AGROBACTERIUM-MEDIATED TRANSFORMATION ON ROSA HYBRIDA L. 'CHRISTIAN DIOR'

By

KONG SWEE LAN

January 1997

Chairman: Professor Marziah Mahmood, Ph.D.

Faculty: Science and Environmental Studies

Callus induction from leaf explants of *Rosa hybrida* L. 'Christian Dior' was established. Two types of auxins (2.4-D and NAA) at three concentrations (4.5, 9.0 and 13.5 μM) and two types of cytokinins (kinetin and BA) at three concentrations (2.3, 4.7 and 9.3 μM) were used in a 3 X 4 experimental design. The best caulogenesis and callus maintenance medium was 1/2 MS + 30 g/L sucrose + 9.0 μM NAA + 2.3 μM BA cultured in the dark. Several attempts were carried out to induce shoot organogenesis and/or somatic embryogenesis from callus. None of the media tested induced shoots or somatic embryos. However, there were differences observed for callus growth on the medium tested. Combination of cytokinins (BA and TDZ) at 5.0-10.0 μM with auxins (NAA and IBA) at 1.0-2.0 μM, generally promoted callus proliferation. Callus cultured on cytokinin only showed occurence of browning.

Amino acids proline and glutamine also enhanced callus proliferation. Observations under the stereo microscope revealed that the callus was globular. Medium without ammonium ion enhanced callus proliferation. TDZ was found to be the better cytokinin for callus proliferation. Observation of cells taken from callus cultured on the various media revealed that there was no meristematic primordias.

Agrobacterium-mediated transformation of *R. hybrida* L. 'Christian Dior' callus produced three transgenic callus lines that were confirmed by dot blot and Southern hybridisation assays. Leaf-derived callus was transformed with *Agrobacterium tumefaciens* LBA4404 carrying plasmid pBI121 which contained the ß-glucuronidase (GUS) gene and *npt*II gene coding for kanamycin resistance. The best condition for infection of callus tissues with *A. tumefaciens* was exposure to 1 X 10^9 cells/ml for 5 minutes. At this condition, GUS activity was found to be the highest (6.39 $\pm$ 0.01 pmol MU/h/μg protein). It was also found that at low *Agrobacterium* concentration, increasing the exposure period increased GUS transient activity, and at high *Agrobacterium* concentration, increasing exposure period reduced GUS transient activity. Transformation efficiency improved when callus was cultured for 2 weeks on medium without kanamycin (but with 500 μg/ml carbenicillin) before transferring the callus to medium with 300 μg/ml kanamycin + 500 μg/ml carbenicillin for selection. Carbenicillin served to eliminate *Agrobaterium*. Kanamycin was also found to be unsuitable for used as a selective marker.

Abstrak tesis yang telah dikemukakan kepada Senat Universiti Pertanian Malaysia sebagai memenuhi syarat untuk Ijazah Master Sains

KULTUR TISU DAN TRANSFORMASI-*AGROBACTERIUM* KE ATAS ROSA HYBRIDA L. 'CHRISTIAN DIOR'

oleh

KONG SWEE LAN

Januari 1997

Pengerusi : Profesor Marziah Mahmood, Ph.D.

Fakulti : Sains dan Pengajian Alam Sekitar

Kalus dari eksplan daun pokok *Rosa hybrida* L. 'Christian Dior' berjaya dihasilkan. Dua jenis auksin (2,4-D dan NAA) pada tiga kepekatan (4.5, 9.0 dan 13.5 μM) bersama-sama dengan dua jenis sitokinin (kinetin dan BA) pada tiga kepekatan (2.3, 4.7 dan 9.3 μM) digunakan dalam eksperimen bercorak 3 X 4. Media untuk pengkalusan dan untuk subkultur yang terbaik ialah 1/2 MS + 30 g/L sukrosa + 9.0 μM NAA + 2.3 μM BA dan dikultur dalam gelap. Beberapa percubaan dilakukan untuk mengaruh regenerasi pucuk dan/atau embrio somatik. Media yang dipilih tidak berjaya mengaruhkan pucuk atau embrio somatik. Walaubagaimanapun, media yang dipilih itu mempengaruhi kadar pertumbuhan kalus. Kombinasi sitokinin (BA dan TDZ) pada kepekatan 5.0-10.0 μM bersama-sama dengan auksin (NAA dan IBA) pada kepekatan 1.0-2.0 μM mengaruh pertumbuhan kalus pada kadar yang lebih cepat. Kalus yang dikultur dalam media yang mengandungi sitokinin sahaja mudah menjadi perang.

Asid amino prolina and glutamina juga mengaruh kadar pertumbuhan kalus yang lebih pesat. Pemerhatian di bawah mikroskop stereo menunjukkan bentuk kalus yang bulat (globular). Media tanpa ion amonia juga mengaruh kadar pertumbuhan kalus yang lebih pesat. TDZ lebih berkesan dalam mempercepatkan kadar pertumbuhan kalus. Sel-sel yang diambil dari kalus yang dikultur dalam media yang dipilih nenunjukkan ketidakhadiran primordia meristem.

Transformasi kalus *R. hybrida* L. 'Christian Dior' melalui *Agrobacterium* berjaya menghasilkan tiga kalus transgenik yang telah dibuktikan melalui kaedah hibridisasi 'dot blot' dan 'Southern blot'. Transformasi dilakukan melalui *Agrobacterium tumefaciens* LBA4404 yang membawa plasmid pBI121 yang mengandungi gen ß-glucuronidase (GUS) dan gen *npt*II yang mengkodkan kerentangan terhadap kanamycin. Keadaan terbaik untuk menjangkiti kalus dengan *Agrobacterium* ialah pada kepekatan 1 X 10^9 sel/ml dan pendedahan selama 5 minit. Pada keadaan begini, aktiviti transien GUS adalah paling tinggi iaitu 6.39 $\pm$ 0.01 pmol MU/jam/μg protein. Pada kepekatan *Agrobacterium* yang rendah, melanjutkan tempoh pendedahan kepada *Agrobacterium* mengakibatkan aktiviti transien GUS meningkat. Pada kepekatan *Agrobacterium* yang tinggi, melanjutkan tempoh pendedahan mengakibatkan aktiviti transien GUS menurun. Kadar transformasi bertambah baik jika kalus yang dijangkiti *Agrobacterium* dikultur selama 2 minggu pada media yang tidak mengandungi kanamycin tetapi mengandungi 500 μg/ml carbenicillin sebelum dipindahkan ke media yang

mengandungi 300 μg/ml kanamycin + 500 μg/ml carbenicillin untuk pemilihan. Carbenicillin adalah untuk membunuh *Agrobacterium*. Kanamycin juga didapati tidak sesuai digunakan sebagai petanda pemilihan (selective marker).

CHAPTER 1

INTRODUCTION

Plant Improvement *Via* Genetic Manipulation

Improvement of plants through classical breeding is a long and tedious process. Biotechnology has introduced gene transformation as an alternative method for plant improvement. Basically, transformation provides the opportunity to introduce any gene from any source into plant cells (Fisk and Dandekar, 1993). Three key components which must be considered when developing such a transformation system (Schuerman and Dandekar, 1991; Songstad et al., 1995) are:

1) recombinant DNA manipulation to obtain and generate an appropriate transgene containing the gene of interest and functional reporter gene constructs, and proper selection conditions to select transgenic events,

2) a plant tissue culture system to regenerate whole plantlets from various cells or tissues, and

3) a DNA delivery system to deliver the transgene into plant cells or tissues that minimizes damage but permits stable integration of the transgene into the recipient genome and allows for sustained cell proliferation to eventually regenerate whole plantlets.

Recombinant DNA technology has progressed tremendously and routine DNA manipulation is possible while plant tissue culture has to be established for each individual plant species using established basic techniques. Several DNA delivery systems are currently being widely used and are generally divided into two categories (Fisk and Dandeker, 1993; Songstad et al., 1995) :-

1) Indirect DNA uptake - *Agrobacterium*-mediated transformation
2) Direct DNA uptake:
    a) protoplast-mediated transformation
    b) microprojectile bombardment

Agrobacterium-mediated transformation captures on the natural transformation abilities of the soil bacteria, *A. tumefaciens* and *A. rhizogenes*. Protoplast-mediated transformation requires the isolation of protoplasts and delivery of DNA using either high voltage electroporation or polyethylene glycol (PEG) permeabilisation. Microprojectile bombardment involves the delivery of high velocity DNA-coated microparticles into plant tissues.

Improvement of Rose (*Rosa sp.*)

Rose (*Rosa sp.*) is the world's most important ornamental crop (Robinson and Firoozabady, 1993) and has earned the nickname 'Queen of Flowers' (Genders, 1965). Plants bearing traits such as disease, pest and herbicide resistance are often sought after. Other traits such as flower colour, flower morphology, and extended vase life are also important characters in ornamental breeding (Mol et al., 1989).

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Conventional breeding method for rose improvement may take up to eight years and is difficult to accomplish due to its high degree of heterozygosity and long generation time. Genetic breeding through plant transformation presents an alternative method to classical breeding. To date, there has been only one report on rose transformation (Firoozabady et al., 1994), while there are several reports on rose tissue culture system (Burger et al., 1990; Rout et al., 1991; Kunitake et al., 1993; Hsia and Korban, 1996). Generally, regeneration of rose is genotype dependent with a low rate of regeneration (Hsia and Korban, 1996; Van der Salm et al., 1996).

Objectives

The objectives of this project are:-

a) to establish a regeneration system from leaf tissues of a locally grown rose, *R. hybrida* L. 'Christian Dior' through tissue culture techniques, and

b) to establish a protocol for *Agrobacterium*-mediated transformation of rose callus.

For the first objective, procedures were established for callus induction from leaf tissues. Subsequently, attempts were made to induce shoot organogenesis and somatic embryogenesis from callus tissues.

The second objective concentrated on the establishment of *Agrobacterium*-mediated transformation using callus tissues. The binary vector

plasmid pBI121 (Jefferson et al., 1987) was first mobilised into *A. tumefaciens* strain LBA4404 *via* electroporation. This plasmid contains a selectable neomycin phosphotransferase II (*npt*II) gene which confers kanamycin resistance and a scorable ß-glucuronidase (GUS) gene (*uid*A). Transient expression of the GUS enzyme was carried out by flourometry assay and histochemical staining. The isolation and labelling of a 3 kb *Eco*RI/*Hind*III restriction enzyme digest fragment of the pBI 121 plasmid was firstly carried out to serve as a probe for stable transformation assay by dot blot and Southern blot hybridisation.

CHAPTER II

LITERATURE REVIEW

The Family *Rosaceae*

Rosaceae, also known as the Rose Family is classified under the order Rosales. Its distribution is cosmopolitan, but occurs mainly in the Northern region (Rendle, 1959; Jones and Luchsinger, 1986). Members of *Rosaceae* are mostly trees, shrubs, and herbs with alternate, simple or compound leaves which often have prominent stipules (Porter, 1967). Under taxonomic classification of Rendle (1959) and Lawrence (1960) *Rosaceae* is grouped into six subfamilies which are *Spiraeoideae*, *Pomoideae*, *Prunoideae*, *Chrysobalanoideae*, *Neuradoideae* and *Rosoideaea*, but Jones and Luchsinger (1986) described only four subfamilies which are *Spiraeoideae*, *Maloideae*, *Rosoideae*, and *Prunoideae*.

About 100 genera and 3000 species have been classified under the *Rosaceae* family (Porter, 1967; Jones and Luchsinger, 1986). Among the more important genera are the fruit producing members such as apple (*Malus*), pear (*Pyrus*), quince (*Cydonia*), cherry, plum, prune, peach, nectarine, apricot, almond (*spp. Prunus*), strawberry (*Fragaria*), raspberry, blackberry (*spp. Rubus*), and loquat