UNIVERSITI PUTRA MALAYSIA

STUDIES ON *IN VITRO* SHOOT REGENERATION OF TOMATO (*LYCOPERSICON ESCULENTUM* MILL.) FOR THE DEVELOPMENT OF AN AGROBACTERIUM – MEDIATED TRANSFORMATION SYSTEM

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By

META SRITUA ARIEF

Thesis Submitted in Fulfillment of the Requirements for the Degree of Master of Science in the Faculty of Food Science and Biotechnology, Universiti Putra Malaysia

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Dedicated

To My

Father (Dr. Sritua Arief)

And

Mother (Melanie Sritua Arief)

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LIST OF ABBREVIATIONS

AMT	*Agrobacterium*-mediated transformation
ANOVA	Analysis of Variance
A	ampere
bp	base pair
BAP	6-Benzylaminopurine
35SCaMV	35S ribosomal subunit of the Cauliflower Mosaic Virus
ChiP	*Chi2;1* promoter
chv	chromosomal genes
CIM	Callus Induction Medium
cv	cultivar(s)
°	degrees
DNA	deoxyribonucleic acid
2,4-D	2,4-Dichlorophenoxyacetic acid
DMSO	Dimethyl sulfoxide
DMRT	Duncan's Multiple Range Test
EtBr	Etidium bromide
GUS	β-glucuronidase gene
G	gram(s)
HPT	Hygromycin phosphotransferase gene
h	hour(s)

IAA	Indole-3-acetic acid
INT	intron
kb	kilobase
KIN/kinetin	6-(furfuryl-amino)-purine
LB	Luria Bertani
M	molar
MCS	Multiple Cloning Site
MIC	Minimal Inhibitory Concentration
Mm	millimolar
µM	micromolar, 10^{-6} M or 10^{-3} mM
µl	microlitre, 10^{-6} l or 10^{-3} ml
mg	milligram(s)
µg	microgram(s), 10^{-6} g or 10^{-3} mg
µm	micrometre, 10^{-6} m or 10^{-3} mm
MS	Murashige and Skoog medium/media
MSO	hormoneless Murashige and Skoog medium (full strength)
NAA	Napthaleneacetic acid
nm	nanometre, 10^{-9} m or 10^{-6} mm
Nos ter	Nopaline synthase gene terminator
NPT II	Neomycin phosphotransferase gene
OD	Optical Density
onc	oncogenes

Ori	Origin of replication
% or Pct	percentage
PCR	Polymerase Chain Reaction
rol	auxin-synthesising genes
rpm	revolution per minute
SDS	Sodium Dodecyl Sulphate
SRM	Shoot Regeneration Medium/Media
SR	shoot regeneration
SPI	shoot primordia induction
SPE	shoot primordia elongation
UV	ultra violet
vir	virulence genes
V	volts
v/v	volume per volume
w/v	weight per volume
X-gluc	5-bromo-4-chloro-3-indoly-glucuronide
ZEA/zeatin	6-(4-hydroxy-3-methylbut-2-enylamino)-purine

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By

Meta Sritua Arief

May 1999

Chairman: Dr. Harikrishna Kulaveerasingam

Faculty: Food Science and Biotechnology

A potentially important gene (*Chi2;1*) isolated from a tomato pistil cDNA library (Gasser *et al.*, 1989), showed a differential expression pattern in the transmitting tissue of the style. This finding led to the hypothesis that a gradient of promoter-binding transcription factors controlled this expression. The isolation of these transcription factors requires the identification of such promoter elements via the transformation of deleted *Chi 2;1* promoter (ChiP) fragments into tomato and tobacco.

This study forms the basis for the above requirement. It focuses on the development of *in vitro* shoot regeneration (SR) in local tomato and *Agrobacterium*-mediated transformation (AMT) protocols in tomato and tobacco. The results of this study revealed the following findings:

1. Cotyledon explants of local tomato (MTI and MTII) exhibited the highest shoot regeneration (SR) efficiency (mean shoot production and mean shoot primordia elongation) in response to applications of

3 mgl^{-1} ZEA and 5 mgl^{-1} KIN alone or in combination with 1 mgl^{-1} IAA, respectively in MS medium. The SR response induced by 3 mgl^{-1} ZEA in cv.MTI was more vigorous albeit slower than that induced by either 5 mgl^{-1} KIN alone or in combination with 1 mgl^{-1} IAA in cv.MTII.

2. Infection of local tomato seedlings with wild-type *A. tumefaciens* and *A. rhizogenes* strains resulted in the induction of crown gall tumours and hairy roots of various size and length, respectively. The infection of cotyledon explants with *A. tumefaciens* strain GV2260 (a binary vector system with a *GUS* reporter gene and kanamycin-resistant gene), induced *GUS*-expressing callus which failed to regenerate shoots and eventually died under kanamycin (100 mgl^{-1}) selection. A lower kanamycin level (50 to 80 mgl^{-1}) is recommended to allow the survival and shoot regeneration of transformed callus.

3. The utilisation of two *A. tumefaciens* strains, GV2260 and LBA4404 (binary vectors) in the infection of tobacco leaf explants showed that only specific cell densities of 10^8 and 10^9, respectively were conducive to the regeneration of transformed shoots. Pre-culturing, infection with acetosyringone-treated bacterial cells, and induction of callus and shoot primordia before antibiotic selection, enhanced a significantly higher percentage and SR of *GUS*-positive explants in the treated explants than in the control explants. As strain GV2260 was more efficient than strain LBA4404, the conditions discovered using strain GV2260 were used in the transformation of several deleted ChiP fragments into tobacco.

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

KAJIAN PERTUMBUHAN SEMULA PUCUK TOMATO TEMPATAN (*LYCOPERSICON ESCULENTUM* MILL.) SECARA *IN VITRO* UNTUK PERKEMBANGAN SISTEM PEMINDAHAN GEN SECARA *AGROBACTERIUM*

Oleh

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Mei 1999

Pengerusi: Dr. Harikrishna Kulaveerasingam

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Sejenis gen penting (*Chi2;1*) dari cDNA bunga betina tanaman tomato (Gasser *et al.*,1989) menunjukkan pola ekspresi yang berbeza dalam tisu pemindahan bunga. Penemuan ini menyebabkan terbentuknya suatu hipotesis yaitu sekumpulan faktor transkripsi yang mengikat pada elemen-elemen sokongan gen khas, mengawal pola ekspresi gen ini. Penemuan faktor-faktor transkripsi ini memerlukan pengenalpastian elemen-elemen berkenaan melalui pemindahan serpihan sokongan gen *Chi2;1* (ChiP) kedalam tomato dan tembakau.

Kajian ini menerangkan tatacara untuk keperluan diatas. Ianya menumpukan kepada perkembangan pertumbuhan semula pucuk tomato tempatan secara *in vitro*. Ianya juga menumpukan kepada tatacara pemindahan gen secara *Agrobacterium* kedalam tomato dan tembakau.

Hasil kajian mendapati beberapa penemuan berikut:

1. Eksplan kotiledon tomato tempatan (MTI dan MTII) menunjukkan kadar kecekapan tertinggi dalam pertumbuhan semula pucuk (berasaskan jumlah purata pucuk dan purata pemanjangan pucuk) sebagai kesan penggunaan 3 mgl^{-1} zeatin dan 5 mgl^{-1} kinetin secara persendirian atau dengan 1mgl^{-1} IAA di dalam medium MS. Pertumbuhan semula pucuk yang diberikan oleh 3 mgl^{-1} zeatin keatas cv.MTI adalah lebih banyak walaupun memerlukan waktu yang lebih lama berbanding dengan pertumbuhan semula pucuk yang diberikan oleh 5 mgl^{-1} kinetin secara persendirian atau dengan 1 mgl^{-1} IAA keatas cv.MTII.
2. Infeksi semaian tomato tempatan oleh jenis *A.tumefaciens* dan *A.rhizogenes* semulajadi mengakibatkan wujudnya barah dan akar berbulu yang berbeza sais dan jumlah. Infeksi eksplan kotiledon dengan *A.tumefaciens* jenis GV2260 (vektor binari dengan gen pelapor *GUS* dan gen tahan kanamycin), menghasilkan kalus yang mengekspresikan *GUS*. Kalus ini kemudiannya gagal untuk melakukan pertumbuhan semula pucuk dan mati disebabkan oleh kanamycin dalam kadar 100 mgl^{-1}. Kadar kanamycin yang lebih rendah (50 sampai 80 mgl^{-1}) dicadangkan bagi membolehkan kalus yang mengalami pemindahan gen, melakukan pertumbuhan semula pucuk dan bertahan hidup.
3. Penggunaan dua jenis bakteri *A. tumefaciens*, GV2260 dan LBA4404 (vektor binari) dalam memindahkan gen kedalam eksplan daun tembakau menunjukkan bahawa hanya ketumpatan sel tertentu (10^8 dan 10^6) yang baik untuk berlakunya pertumbuhan semula pucuk dari

ekspan. Inkubasi awal, infeksi dengan sel *Agrobacterium* yang telah diberi acetosyringone, dan induksi kalus serta pucuk sebelum seleksi oleh antibiotik), meningkatkan persentase ekspian dan jumlah purata pucuk yang mengekspresikan gen *GUS*. Memandangkan bakteri jenis GV2260 lebih cekap daripada jenis LB4404, tatacara yang menggunakan jenis GV2260 ini digunakan untuk memindahan serpihan ChiP kedalam tembakau.

CHAPTER I

GENERAL INTRODUCTION

Plant Genetic Manipulation

Plants have a unique ability to capture light energy from the sun and use it to convert physical elements from the earth into biological energy and many biological products. In this way, plants satisfy either directly or indirectly the nutritional requirements of human beings. In addition, plants provide oxygen, fibres, flowers, aromas and therapeutic medicines. Plant cultivation is therefore a very important practice in human life. Over the past decade, plant cultivation has made use of various technologies, including plant breeding, which involves the crossing of plant species to protect and select desirable traits. Breeders have developed sophisticated crossing schemes which increased the yields of crops such as rice, wheat and maize. However, plant breeding is slow, time-consuming and labourious. It also requires the utilisation of large land areas; a present rarity in many developing countries where land area is shrinking due to overpopulation (Beversdorf, 1993).

The introduction of Genetic Engineering (recombinant DNA and gene transfer) techniques as well as plant cell and tissue culture techniques in the 1970s, have provided an attractive alternative to conventional plant breeding. From 1982 onwards, when the first single gene was successfully transferred, progress has been rapid. At present, important crops such as