



UNIVERSITI PUTRA MALAYSIA

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

META SRITUA ARIEF

FSMB 1999 8

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OF TOMATO (*LYCOPERSICON ESCULENTUM* MILL.)
FOR THE DEVELOPMENT OF AN *AGROBACTERIUM*-
MEDIATED TRANSFORMATION SYSTEM**

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

May 1999



Dedicated

To My

Father (Dr. Sritua Arief)

And

Mother (Melanie Sritua Arief)



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TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS	iii
LIST OF TABLES	ix
LIST OF FIGURES	xii
LIST OF PLATES	xiv
LIST OF ABBREVIATIONS	xvi
ABSTRACT	xix
ABSTRAK	xxi
 CHAPTER	
I	
GENERAL INTRODUCTION	1
Plant Genetic Manipulation	1
Studies on Tomato and Tobacco	3
Justification of the Study	6
Objectives of the Study	7
 II	
LITERATURE REVIEW	8
General Characteristics of the Tomato Plant..	8
Taxonomy and Economical Value	8
Agronomy-related Problems	9
Some Molecular Studies on the Tomato Plant	10
The Role of the Tomato Plant in	
Molecular Studies	10
Studies on Pistil-specific Genes	11
Postulated Regulation of the <i>Chi2,1</i>	
Gene	13
Plant Tissue Culture	15
Principles of Tissue Culture	15
Previous <i>In vitro</i> Regeneration Works	
on Tomato	18
Agrobacterium-mediated Transformation (AMT)...	20
Principles of AMT	20
Previous AMT Works on Tomato	26
Methods of Confirming Transformation in	
Transgenic Plants	28
Histochemical-staining of the <i>GUS</i>	
Reporter Gene	28
Polymerase Chain Reaction (PCR)	
Amplification of Assayable and Selectable	
Marker Genes.....	29
Southern Hybridisation Analysis	29

	Characteristics of the Tobacco Plant	30
	Taxonomy and Economical Value.....	30
	The Role of the Tobacco Plant in Tissue Culture and Transformation Studies	31
III	IN VITRO SHOOT REGENERATION OF LOCAL TOMATO (<i>LYCOPERSICON ESCULENTUM</i> MILL.)	33
	Introduction	33
	Materials and Methods	35
	Explant Materials	35
	Chemicals	35
	Aseptic Germination of Seeds	35
	Culturing of Explants and Testing of Different Phytohormone Combinations	36
	Data Collection and Statistical Analysis	37
	Results and Discussion.....	39
	Effect of Cytokinin and Auxin Combinations on Callus Formation, Percentage of SPI and Percentage of SPE	40
	Effect of Cytokinins on Callus Formation, Percentage of SPI and Percentage of SPE	45
	Comparison between the Effect of BAP Alone and in Combination with IAA on the Shoot Regeneration Efficiency of Tomato Cotyledon Explants	49
	Comparison between the Effect of Kinetin Alone and in Combination with IAA on the Shoot Regeneration Efficiency of Tomato Cotyledon Explants	52
	Comparison among the Shoot Regeneration of Tomato Cotyledon Explants by the Applications of Zeatin in Different Concentrations.....	59
	Summary	64



IV	AGROBACTERIUM-MEDIATED TRANSFORMATION OF TOMATO AND TOBACCO	66
	Introduction	66
	Testing of the Susceptibility of Plant Species to AMT.....	66
	<i>Agrobacterium</i> -mediated Transformation Using Binary Vectors in Tomato and Tobacco	68
	Materials and Methods.....	71
	Explants Materials.....	71
	Explant Culture Medium and Chemicals.....	71
	<i>Agrobacterium</i> and <i>Escherichia coli</i> Strains...	71
	Bacterial Culture Media and Chemicals.....	72
	Plasmids: Information and Diagrams of pBY2.3, pBY4.1 and pBY6.1.....	73
	Aseptic Germination of Tomato and Tobacco Seeds.....	77
	Subculturing of Tomato and Tobacco Seedlings.....	77
	Growth of Bacterial Strains.....	78
	Infection of Seedlings.....	79
	Preliminary Experiments before Transformation of Tobacco Leaf Explants.....	79
	Transformation of Tomato Cotyledon and Tobacco Leaf Explants.....	85
	Analysis of Transformed Tomato Callus, Tobacco Shoot Primordia and Roots.....	89
	Minimal Inhibitory Concentration Assays of Kanamycin and Hygromycin.....	90
	Data Collection and Analysis.....	91
	Results and Discussion.....	92
	Testing of the Susceptibility of Local Tomato to AMT.....	92
	A Preliminary Study on the Developing of an AMT Protocol in Local Tomato.....	101
	Development of a Modified AMT Protocol in Tobacco.....	109
	Determination of Minimal Inhibitory Concentrations (MIC) of Kanamycin and Hygromycin in Tobacco Callus.....	112
	The Combined Effects of OD ₆₂₀ Levels (Cell Densities) and Acetosyringone Concentrations on the Regeneration of Co-cultivated Tobacco Leaf Explants.....	116

	The Combined Effects of Leaf Explant Treatment and Acetosyringone Treatment of <i>Agrobacterium</i> on the Transformation Efficiency of Tobacco Leaf Explants.....	120
	Summary.....	133
V	SUMMARY, CONCLUSION AND RECOMMENDATIONS.....	136
	Summary.....	136
	Conclusion and Recommendations.....	138
	REFERENCES	141
	APPENDICES	
A	MS Stock Solutions and Culture Media	149
B	Media for Bacterial Growth	153
C	Standard Solutions and DNA Isolation Solutions	155
D	GUS Histochemical Solutions	157
E	Additional Tables	159
	VITAE	171



LIST OF TABLES

Table		Page
1	Different Concentrations of Cytokinins used for Callus and Shoot Induction	36
2	Different Combinations of Cytokinin and Auxin used for Callus and Shoot Induction	37
3	Morphogenic Response of Tomato Cotyledon Explants (cultivars MTI and MTII) to Various Combinations of Cytokinins and Auxins	40
4	Morphogenic Response of Tomato Cotyledon Explants (cultivars MTI and MTII) to Various Concentrations of Cytokinins	46
5	Susceptibility of Local Tomato Seedling (cv.MTI) to Wild-type <i>Agrobacterium rhizogenes</i> Strains	93
6	Susceptibility of Local Tomato Seedling (cultivar MTI) to Wild-type <i>Agrobacterium tumefaciens</i> Strains.....	94
7	Cell Densities of <i>A. tumefaciens</i> Strains GV2260 and LBA4404 from OD Values of 0.1 to 0.4.....	117
8	Effects of GV2260 and LBA4404 Cell. Densities and Acetosyringone Concentrations on the Regeneration of Co-cultivated Leaf Explants	118
9	Effects of Leaf Explant Treatment and Acetosyringone Treatments of 10^8 GV2260 Cells on Transformation Frequencies in Tobacco Leaf Explants	122
10	Effects of Leaf Explant Treatment and Acetosyringone Treatments of 10^8 LBA4404 Cells on Transformation Frequencies in Tobacco Leaf Explants.....	126



11	Mean Values and Standard Error (±) for Effect of Various Cytokinin and Auxin Combinations on the Percentage of SPI of Tomato Cotyledon Explants (cv.MTI and cv.MTII).....	159
12	Mean Values and Standard Error (±) for Effect of Various Cytokinin and Auxin Combinations on the Percentage of SPE of Tomato Cotyledon Explants (cv.MTI and cv.MTII)	160
13	Mean Values and Standard Error (±) for Effect of Various Cytokinins on the Percentage of SPI of Tomato Cotyledon Explants (cv. MTI and cv.MTII).....	161
14	Mean Values and Standard Error (±) for Effect of Various Cytokinins on the Percentage of SPE of Tomato Cotyledon Explants (cv.MTI and cv.MTII).....	162
15	Mean Values and Standard Error (±) for Effect of BAP Alone and in Combination with IAA on the Mean SR of Tomato Cotyledon Explants (cv.MTI and cv.MTII).....	163
16	Mean Values and Standard Error (±) for Effect of BAP Alone and in Combination with IAA on the Mean Rate of SPI of Tomato Cotyledon Explants (cv.MTI and cv.MTII).....	163
17	Mean Values and Standard Error(±) for Effect of Kinetin Alone and in Combination with IAA on the Mean SR of Tomato Cotyledon Explants (cv.MTi and cv.MTII).....	164
18	Mean Values and Standard Error (±) for Effect of Various Kinetin and IAA Combinations on the Mean Rate of SPI of Tomato Cotyledon Explants (cv.MTI and cv.MTII).....	165
19	Mean Values and Standard Error (±) for Effect of 5 mg^l⁻¹ Kinetin Alone and in Combination with 1 mg^l⁻¹ IAA on the Mean Rate of SPE of Tomato Cotyledon Explants (cv.MTI and cv.MTII).....	165



20	Mean Values and Standard Error (\pm) for Effect of Various Zeatin Concentrations on the Mean SR of Tomato Cotyledon Explants (cv.MT1 and cv.MT11).....	166
21	Mean Values and Standard Error (\pm) for Effect of Various Zeatin Concentrations on the Mean Rate of SPE of Tomato Cotyledon Explants (cv.MT1 and cv.MT11).....	166
22	Mean Values for Infection Efficiency of Wild-type <i>Agrobacterium tumefaciens</i> Strains in Tomato Seedling.....	167
23	Mean Values for Infection Efficiency of Wild-type <i>Agrobacterium rhizogenes</i> Strains in Tomato Seedling.....	167
24	Mean Values and Standard Error (\pm) for Effect of Different Kanamycin Concentrations on the Percentage of Death in Non-transformed Tomato Callus.....	168
25	Mean Values and Standard Error (\pm) for Effect of Different Kanamycin Concentrations on the Percentage of Death in Non-transformed Tobacco Callus.....	169
26	Mean Values and Standard Error (\pm) for Effect of Different Hygromycin Concentrations on the Percentage of Death in Non-transformed Tobacco Callus.....	169
27	Mean Values and Standard Error (\pm) for Effect of Leaf Explant Treatment and Acetosyringone on the Mean Shoot Regeneration of Transformed Explants.....	170



LIST OF FIGURES

Figure		Page
1	Effect of Various Combinations of Cytokinin and Auxin on the Percentage of SPI of Local Tomato Cotyledon Explants (cv.MTI and cv.MTII)	43
2	Effect of Various Combinations of Cytokinin and Auxin on the Percentage of SPE of Local Tomato Cotyledon Explants (cv.MTI and cv.MTII)	44
3	Effect of Various Cytokinins on the Percentage of SPI of Local Tomato Cotyledon Explants (cv.MTI and cv.MTII).....	47
4	Effect of Various Cytokinins on the Percentage of SPE of Local Tomato Cotyledon Explants (cv.MTI and cv.MTII).....	48
5	Effect of BAP Alone and in Combination with IAA on the Mean SR of Local Tomato Cotyledon Explants (cv.MTI and cv.MTII).....	49
6	Effect of BAP Alone and in Combination with IAA on the Mean Rate of SPI of Local Tomato Cotyledon Explants (cv.MTI and cv.MTII)	50
7	Effect of Kinetin Alone and in Combination with IAA on the Mean SR of Local Tomato Cotyledon Explants (cv.MTI and cv.MTII).....	52
8	Effect of Various Kinetin and IAA Combinations on the Mean Rate of SPI of Local Tomato Cotyledon Explants (cv.MTI and cv.MTII)	55
9	Effect of 5 mg ⁻¹ Kinetin Alone and in Combination with 1 mg ⁻¹ IAA on the Mean Rate of SPE of Local Tomato Cotyledon Explants (cv.MTI and cv.MTII)	57
10	Effect of Various Zeatin Concentrations on the Mean SR of Local Tomato Cotyledon Explants (cv.MTI and cv.MTII).	61



11	Effect of Various Zeatin Concentrations on the Mean Rate of SPE of Local Tomato Cotyledon Explants	62
12	Restriction Map of p35SGUSINT	73
13	Plasmid Map of pCAMBIA 1301	75
14	Plasmid Map of pBI101	75
15	Plasmid Maps of pBY2.3, pBY4.1 and pBY6.1..	76
16	Infection Efficiency of Wild-type <i>Agrobacterium tumefaciens</i> Strains in Tomato Seedling	95
17	Infection Efficiency of Wild-type <i>Agrobacterium rhizogenes</i> Strains in Tomato Seedling	96
18	Flow-chart of Transformation Protocol Experimented in Cotyledon Explants of Local Tomato	102
19	Effect of Different Kanamycin Concentrations on the Percentage of Death in Non-transformed Tomato Callus (cultivars MTf and MTil)	106
20	Flow-chart of Transformation Protocol Experimented in Leaf Explants of Tobacco	111
21	Effect of Different Kanamycin Concentrations on the Percentage of Death in Non-transformed Tobacco Callus	112
22	Effect of Different Hygromycin Concentrations on the Percentage of Death in Non-transformed Tobacco Callus	114
23	Effect of Leaf Explant Treatment and Acetosyringone Treatment of 10^8 GV2260 Cells on the Mean SR of Transformed Explants	128
24	Effect of Leaf Explant Treatment and Acetosyringone Treatment of 10^6 LBA4404 Cells on the Mean SR of Transformed Explants	129



LIST OF PLATES

Plate		Page
1	Different Expression of the <i>Chi2;1</i> Gene in the Style of Tomato Pistil	5
2	Morphogenic Response of Tomato Cotyledon Explants to the Application of BAP and NAA	41
3	Callus Induced by 1 mg ^l ⁻¹ BAP + 1 mg ^l ⁻¹ 2,4-D in cv.MTII Cotyledon Explant	42
4	Callus Induced by 1 mg ^l ⁻¹ KIN + 1 mg ^l ⁻¹ NAA in cv .MTII Cotyledon Explant	42
5	Sturdy Shoot Primordia Induced by the Application of 2.5 mg ^l ⁻¹ BAP	51
6	Poor Proliferation of Shoot Primordia Induced by KIN + IAA (0.5 to 1 mg ^l ⁻¹)	53
7	Regeneration of Healthy Shoot Primordia in Tomato Cotyledon Explants	54
8	Different Degrees of Shoot Proliferation and Elongation in Tomato Cotyledon Explants Induced by Different Concentrations of Zeatin	60
9	Best Response of SPE in Cultivar MTI to the Application of 3 mg ^l ⁻¹ Zeatin	62
10	The Types of Crown Gall Tumours Induced by Wild-type <i>A. tumefaciens</i> Strains	97
11	Dense Hairy Root Formation Induced by Wild-type <i>A. rhizogenes</i> Strains	99
12	Morphology of Callus Generated After Co-cultivation and 2 Weeks Incubation on Carbenicillin-containing SRM	103
13	Transient <i>GUS</i> Expression in Sectioned Tomato (cv.MTII) Callus	104



14	The Majority of Necrotic Callus on Selection Medium	105
15	Response of Tomato Callus to SRM Supplemented with 100 and 200 mg ^l ⁻¹ Kanamycin	107
16	Survival and Death of Tobacco Callus on SRM containing Different Concentrations of Kanamycin	113
17	Survival and Death of Tobacco Callus on SRM containing Different Concentrations of Hygromycin	115
18	Development of Shoots from Tobacco Leaf Explants After 6 Weeks Co-cultivation with Untreated 10 ⁸ GV2260 Cells	123
19	Development of Shoots in Tobacco Leaf Explants After 6 Weeks Co-cultivation with Acetosyringone-treated (100 μM) 10 ⁸ GV2260 Cells	123
20	Plantlet Development from Treated Tobacco Leaf Explant After 6 Weeks Co-cultivation with Acetosyringone-treated (100 μM) 10 ⁸ GV2260 Cells	124
21	<i>GUS</i> Gene Expression in Sectioned Roots of Tobacco Plantlets Transformed with 10 ⁸ GV2260 Cells	124
22	Development of Shoot Primordia in Tobacco Leaf Explants after 6 Weeks Co-cultivation with 10 ⁸ LBA4404 Cells	127
23	Colonies of <i>A. tumefaciens</i> Strain GV2260 transformed with Different ChiP Fragments	132
24	Confirmation of Deleted ChiP Fragment Sizes from Transformed GV2260 Colonies by PCR Amplification	132



LIST OF ABBREVIATIONS

AMT	<i>Agrobacterium</i> -mediated transformation
ANOVA	Analysis of Variance
A	ampere
bp	base pair
BAP	6-Benzylaminopurine
35SCaMV	35S ribosomal subunit of the Cauliflower Mosaic Virus
ChiP	<i>Chi2;1</i> promoter
<i>chv</i>	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
<i>GUS</i>	β -glucuronidase gene
G	gram(s)
<i>HPT</i>	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
<i>NPT II</i>	Neomycin phosphotransferase gene
OD	Optical Density
<i>onc</i>	oncogenes

Ori	Origin of replication
% or Pct	percentage
PCR	Polymerase Chain Reaction
<i>rol</i>	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
<i>vir</i>	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

Abstract of thesis submitted to the Senate of Universiti Putra Malaysia in fulfillment of the requirements for the degree of Master of Science

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

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May 1999

Chairman: Dr. Harikrishna Kulavæerasingam

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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 mg^l⁻¹ ZEA and 5 mg^l⁻¹ KIN alone or in combination with 1 mg^l⁻¹ IAA, respectively in MS medium. The SR response induced by 3 mg^l⁻¹ ZEA in cv.MTI was more vigorous albeit slower than that induced by either 5 mg^l⁻¹ KIN alone or in combination with 1 mg^l⁻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 mg^l⁻¹) selection. A lower kanamycin level (50 to 80 mg^l⁻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 10⁸ and 10⁶, 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

Meta Sritua Arief

Mei 1999

Pengerusi: Dr. Harikrishna Kulaveerasingam

Fakulti: Sains Makanan dan Bioteknologi

Sejenis gen penting (*Chi2;1*) dari cDNA bunga betina tanaman tomat (*Gasser et al.*, 1989) menunjukkan pola ekspresi yang berbeza dalam tisu pindahan 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 pindahan serpihan sokongan gen *Chi2;1* (*ChiP*) kedalam tomat dan tembakau.

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



Hasil kajian mendapati beberapa penemuan berikut:

1. Eksplan kotiledon tomato tempatan (MTI dan MTII) menunjukkan kadar kecekapan tertinggi dalam pertumbuhan semula pucuk (berdasarkan jumlah purata pucuk dan purata pemanjangan pucuk) sebagai kesan penggunaan 3 mg l^{-1} zeatin dan 5 mg l^{-1} kinetin secara persendirian atau dengan 1 mg l^{-1} IAA di dalam medium MS. Pertumbuhan semula pucuk yang diberikan oleh 3 mg l^{-1} zeatin keatas cv.MTI adalah lebih banyak walaupun memerlukan waktu yang lebih lama berbanding dengan pertumbuhan semula pucuk yang diberikan oleh 5 mg l^{-1} kinetin secara persendirian atau dengan 1 mg l^{-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 saiz 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 mg l^{-1} . Kadar kanamycin yang lebih rendah (50 sampai 80 mg l^{-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 eksplan 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 memindahkan 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 (Beverdors, 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

