UNIVERSITI PUTRA MALAYSIA

DEVELOPMENT OF *IN VITRO* REGENERATION SYSTEM FOR *CAPSICUM ANNUUM* AND TRANSFORMATION WITH CUCUMBER MOSAIC VIRUS COAT PROTEIN GENE

PUA TEEN LEE

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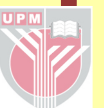

DEVELOPMENT OF *IN VITRO* REGENERATION SYSTEM FOR *CAPSICUM ANNUUM* AND TRANSFORMATION WITH CUCUMBER MOSAIC VIRUS COAT PROTEIN GENE

By

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DEVELOPMENT OF *IN VITRO* REGENERATION SYSTEM FOR *CAPSICUM ANNUUM* AND TRANSFORMATION WITH CUCUMBER MOSAIC VIRUS COAT PROTEIN GENE

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September 2007

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Chilli is one of the most important crops grown worldwide. It ranks as the most popular fruit vegetable and occupies the highest hectarage among the fruit vegetables in Malaysia. However, like any other crop of economic importance, chilli production is hampered by various virus diseases, especially Cucumber Mosaic Virus (CMV) disease. Genetic engineering seems to be the most important technique for the development of novel chilli cultivars with virus resistant property, since other conventional controls were highly ineffective. This study was carried out in three stages: first, to establish an efficient *in vitro* regeneration system for a local chilli cultivar Cilibangi-4 (CB4); second, to obtain a transforming vector containing Cucumber Mosaic Virus (CMV) Coat Protein (CP) gene; and third, development of transgenic chilli plants with CMV resistant.

The investigation was initiated to study the *in vitro* regenerative ability of seven local chilli cultivars (*Capsicum annum* cv. CB2, CB3, CB4, CB6, MC11, MC12 and Kulai) and to select the most responsive cultivar for subsequent experiments.

Explants (hypocotyls and cotyledonary leaves) were collected from 10-12 day-old seedlings and subjected to differentiation medium (DM). Genotypic differences for the *in vitro* regeneration ability were observed in this study. Of all the genotypes tested, cultivar CB4 was found to be the most responsive for both hypocotyls and cotyledonary leaves tested. Hence, subsequent experiments were carried out by using CB4.

BA and IAA concentrations have been optimised for DM. Five concentrations of both PGRs were tested, 5 mg/l(w/v) BA and 0.5 mg/l(w/v) IAA were only found to be the most suitable for bud induction. Up to 87.5% of the cultured hypocotyls formed buds in the induction medium and cotyledonary leaves with a lower percentage of 65%.

Effects of other cytokinins and auxins were investigated as well for bud induction in DM. Kinetin and zeatin were found to be less effective on bud induction compared to BA. While, Thidiazuron (TDZ) showed an extremely high percentage of bud formation, yet the buds induced were mostly stunted buds due to its high cytokinin activity. For auxins, Phenylacetic acid (PAA) was tested. PAA has shown some encouraging results among the concentrations tested. However, the buds seemed likely to form a rosette of distorted leaves and refused to develop further.

Elongation of shoot buds was examined. Treatment DMM (Buds were induced in DM and elongated on MS with 3% sucrose) was found to be the best among all the treatments applied. The leaves formed expanded as normal, shoots elongated well

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and the roots developed vigorously at the basal part of the explants. Shoots elongated were excised and allowed to root in PGR-free MS medium.

A construct of a plant expression cassette with CMV CP gene has been successfully cloned. The cloned CMV CP fragment was 655 bp and exhibited more than 90% similarity to those published CMV CP gene sequences.

The effectiveness of kanamycin in selecting transformed tissue has been investigated based on the minimal kanamycin concentration was that capable to thoroughly inhibit and/or kill all the non-transformed tissues. The minimal inhibitory concentrations of kanamycin were 100 and 200 mg/l(w/v) for cotyledonary leaves and hypocotyls, respectively.

Agrobacterium tumefaciens strain EHA105 harbouring the CMV CP construct was used to transform the chilli hypocotyls and cotyledonary leaf explants. The putative transformants were screened by subjecting to polymerase chain reaction (PCR) with specific primers. Unfortunately, no positive result was obtained.

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

KEMAJUAN TRANGENIK CILI (*CAPSICUM ANNUUM*) YANG MERINTANG PENYAKIT CUCUMBER MOSAIC VIRUS (CMV) MELALUI STRATEGI BERASALKAN VIRUS

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Cili merupakan salah satu tanaman penting yang telah ditanam secara meluas. Ia merupakan buah sayuran yang paling popular dan memenuhi kawasan tanaman yang terluas di antara buah sayuran yang lain di Malaysia. Tetapi, hasil tanaman cili selalu dihalang oleh pelbagai penyakit virus, terutamanya "*Cucumber Mosaic Virus*" (CMV). Kejuruteraan genetik dilihatkan sebagai teknik yang paling penting untuk kemajuan kultivar cili baru yang rintang terhadap virus memandangkan kawalan-kawalan konvensional tidak lagi berkesan. Kajian ini telah dijalankan dalam tiga peringkat: pertama, membangunkan satu sistem regenerasi yang berkesan bagi kultivar cili tempatan Cilibangi-4 (CB4); kedua, mendapatkan satu vektor transformasi yang membawa gen Kot Protein (CP) dari "*Cucumber Mosaic Virus*" (CMV); dan ketiga, pembangunan pokok cili trangenik yang rintang terhadap penyakit CMV.

Kajian bermula dengan mengkaji keupayaan regenerasi bagi tujuh kultivar cili tempatan (*Capsicum annum* cv. CB2, CB3, CB4, CB6, MC11, MC12 dan Kulai)

dan pemilikan kultivar yang paling responsif untuk eksperimen seterusnya. Eksplan (hipokotil dan daun kotiledon) telah dikumpulkan dari anak benih yang berumur 10–12 hari dan dikulturkan ke media pembezaan (DM). Perbezaan genotip dari segi keupayaan regenerasi *in vitro* telah diperhatikan. Antara kesemua genotip yang dikajikan, kultivar CB4 didapati paling responsif bagi kedua-dua eksplan yang digunakan. Kajian seterusnya telah dijalankan dengan menggunakan hanya kultivar CB4.

Kepekatan BA dan IAA bagi DM telah dioptimumkan. Antara lima kepekatan yang telah dikaji bagi kedua-dua BA dan IAA, 5 mg/l(b/i) BA dan 0.5 mg/l(b/i) IAA sahaja didapati paling sesuai untuk induksi tunas. Sebanyak 87.5% dari hipokotil yang telah dikulturkan menghasilkan tunas manakala bagi daun kotiledon pula adalah sebanyak 65%.

Kesan sitokinin dan auksin yang lain telah dikaji bagi induksi tunas dalam DM. Kinetin dan zeatin didapati kurang berkesan bagi induksi tunas berbanding dengan BA. Akan tetapi, Tidiazuron (TDZ) memberi peratusan yang tinggi dalam induksi tunas. Walau bagaimanapun, kebanyakan tunas yang telah dirangsang terbantut akibat dari aktiviti sitokinin TDZ yang tinggi. Bagi asid fenilasetik (PAA), antara pelbagai kepekatan yang telah dikajikan, PAA telah memberi keputusan yang menggalakkan. Tetapi, tunas yang telah dirangsang cenderong untuk membentuk daun ‘rosette’ dan terbantut.

Pemanjangan tunas juga telah dikaji. Rawatan DMM (tunas telah dirangsang dalam DM dan dipanjangan dalam MS dengan 3% sukrosa) telah didapati adalah yang

terbaik antara kesemua rawatan yang telah digunakan. Daun yang terbentuk berkembang dengan normal, pucuk telah memanjang dengan baik dan akar berkembang dengan lebat pada bahagian basal eksplan. Tunas yang memanjang telah dipotong dan dibiarkan berakar dalam medium MS tanpa PGR.

Satu kaset ekspresi tumbuhan dengan gen CMV CP telah berjaya diklonkan. Fragmen CMV CP yang telah diklonkan ialah 655 bp dan mempamerkan lebih daripada 90% kesamaan dengan jujukan gen CMV CP yang terdapat di dalam pangkalan data.

Keberkesanan kanamisin bagi pemilihan tisu yang ditransform telah dikaji berasaskan kepada paras minimum kanamisin yang dapat merencatkan dan / atau membunuh kesemua tisu-tisu yang tidak ditransform. Kepekatan perencatan minimum kanamisin yang didapati bagi kajian ini adalah 100 dan 200 mg/l(b/i) masing-masing bagi daun kotiledon dan hipokotil.

Bakteria *Agrobacterium tumefaciens*, strain EHA105, melabuhkan konstrak CMV CP telah digunakan untuk transformasi eksplan cili hipokotil dan daun kotilidon. Transforman putatif telah disaringkan dengan kaedah *polymerase chain reaction* (PCR) bersamaan dengan primer yang spesifik. Namun demikian, tiada sebarang keputusan positif diperolehi.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which 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.

PUA TEEN LEE

Date: 16/11/07

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LIST OF ABBREVIATIONS

%	percentage
°C	degree centigrade
µl	microliter
µg	microgram
2,4-D	2,4-dichlorophenoxyacetic acid
BA	benzylaminopurine
BH	banana homogenate
bp	base pair
CF	fruit homogenate
CIAP	calf intestinal alkaline phosphatase
CL	cotyledonary leaves
cm	centimeter
CMV CP	cucumber mosaic virus coat protein
CMV	cucumber mosaic virus
CN	corn homogenate
CP	coat protein
cv.	cultivar
DEPC	diethylpyrocarbonate
DM	differentiation medium
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside 5'-triphosphates
EDTA	ethylene diaminetetra acetic acid
EM	elongation medium

g	gram
GUS	β-glucuronidase
H	hypocotyl
hr	hour
IAA	indole-3-acetic acid
kb	kilo base
L	liter
LB	Luria and Bertani media
M	molar
mg/l	milligram per liter
min	minute
ml	milliliter
mM	millimolar
M-MuLV-Rt	Moloney murine leukemia virus reverse transcriptase
MS	Murashige and Skoog (1962)
MW	molecular weight
nm	nanometer
Npt II	Neomycin phosphotransferase II
OD	optical density
PAA	phenylacetic acid
PCR	polymerase chain reaction
PGRs	plant growth regulators
pmol/µl	pico mol per microliter
RNA	ribonuclease acid
rpm	rotations per minute

RT	reverse transcriptase
SDS	sodium dodecyl sulphate
SE	seedling extract
sec	second
TDZ	thidiazuron
TE	10 mM Tris-Cl (pH 8.0), 1mM EDTA
Ti	tumor inducing
U/µl	unit per microliter
v/v	volume over volume ratio
w/v	weight over volume ratio
YEB	yeast extracts broth
µm	micrometer

CHAPTER 1

INTRODUCTION

Chilli is one of the most important crops grown worldwide. Almost all the published papers, regarding chilli research, started their introduction with this statement (Agrawal et al., 1989). From this point of view, we can clearly understand that chilli is the 'supercrop' known by the world!

The *Capsicum* genus goes by an innumerable set of common names, such as chilli, chile, aji, pimiento, paprika, capsicum, and chilli pepper. There is a plethora of magnificently coloured and shaped chilli grown worldwide, and the uses are as diverse as the fruit types in the *Capsicum* genus. Chilli is consumed throughout the world. Chilli fruits are important items worldwide as ingredients of a wide variety of dishes in many countries, and also as the most used condiment and spice in the entire world. The chilli fruit characteristics such as shape, colour, form, pungency, flavor, nutrient and minerals, α and γ tocopherol, and antioxidant contents diversify the use of chilli not only as vegetable, but also as medicine and ornamental crop.

Capsaicin and dihydrocapsaicin, the substances responsible for pungency of chillies have been found to exhibit antimicrobial properties, physiological and pharmacological effects (Dorantes et al., 2000; Govindarajan and Sathyanarayana, 1991). Dorantes et al. (2000) reported that cinnamic and *m*-coumaric acids found in chilli extracts contributed to the inhibition of the four important food borne pathogens (*Listeria monocytogenes*, *Salmonella typhimurium*, *Bacillus cereus* and *Staphyloccoccus aureus*). Capsaicin is also widely used for elaboration of different

pharmaceutical products including pads for relieving muscular pains, rubefascient creams, and products for alleviating the pain of diseases like arthritis (Ochoa-Alejo and Ramirez-Malagon, 2001). Generally, about ten research papers a month are published on the medicinal use of chilli (Bosland and Votava, 2000).

Capsicum spp. belongs to the *Solanaceae* family, a large tropical family that includes tomato, potato, tobacco and petunia. There are about 20 to 30 species in the genus. However, there are only 5 species that have been domesticated and are currently cultivated: *C. annuum* Linné, *C. baccatum* Linné, *C. chinense* Jacquin, *C. frutescens* Linné and *C. pubescens* Ruiz & Pavon (Greenleaf, 1986). The five cultivated species are derived from different ancestral stocks found in three distinct centres of origin. Mexico is the primary centre for *C. annuum*, followed with Guatemala a secondary centre; Amazonia for *C. chinense* and *C. frutescens*; Peru and Bolivia for *C. baccatum* and *C. pubescens* (Greenleaf, 1986).

The most extensively cultivated species of *Capsicum* is *Capsicum annuum* L. With the exception Tabasco (*C. frutescens*), all economically important chilli cultivars (hot and sweet) belong to the genus and species *Capsicum annuum* (Sundstrom, 2002). The world production of *Capsicum* fruits was 24,988,194 metric tons in 2005 (FAO, 2005). Today, they have been widely grown in many countries of the Asian region, and rank at or near the top in terms of growing area. By the year 2000, sixty percent of this crop was produced in Asia. The world leader in chilli (*Capsicum annuum*) cultivation is India, with 900,000 ha annually followed by China and Indonesia (Berke and Shieh, 2000).

In Malaysia, chilli forms an integral part of the local cuisine such as sambal, curry, spicy pickles, chilli powder, chilli sauces etc. Therefore, chilli production has gained a tremendous market value. It ranks as the most popular fruit vegetable and occupies the highest hectarage among the fruit vegetables in this country (Ong et al., 1979; Berke and Shieh, 2000). According to FAMA statistical analysis, in year 2004, the estimated chilli production was 163,822 metric tonnes. Nevertheless, the production potential of chilli does not reach targets required to meet the local market needs of 188,891 metric tonnes. As a result, 25,069 metric tonnes of chilli was being imported.

In many regions of the world, like any other crops of economic importance, chilli production is hampered by pest and diseases. The Asian Vegetable Research and Development Center (AVRDC) has conducted numerous pest and disease surveys in Asia. The major insects and pests that attack chillis are white flies, thrips, mites and aphids, which cause extensive losses in the yield and quality of chilli. Chilli is also susceptible to phytopathogenic fungi, bacteria and viruses (Berke and Shieh, 2000). The most serious diseases are caused by viruses, sometimes causing whole field to be abandoned prior to harvest.

Chilli plants are susceptible to several viruses including cucumber mosaic virus (CMV), chilli venal mottle virus (CVMV), tobacco leaf curl virus (TLCV), tobacco mosaic virus (TMV), and tomato spotted wilt virus (TSWV). Among these, CMV and CVMV were found to be the major viral pathogens in Malaysia, which cause the severest damage and most important, economic losses in chilli pepper production (Inon et al., 1999; Ong et al., 1979; Fujisawa et al., 1986; Mohamad Roff and Ong,

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