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CONSTRUCTION OF A TRANSFORMATION VECTOR CONTAINING CUCUMBER MOSAIC VIRUS (CMV) COAT PROTEIN (CP) GENE AND DEVELOPMENT OF AN AGROBACTERIUMTRANSFORMATION PROCEDURE FOR CHILLI

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By

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Master of Science

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May 2003

Chairman : Professor Marziah Mahmood, Ph.D.

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Chilli (*Capsicum annuum* L.) is one of the major vegetable and spice crops grown widely, yet it suffers great losses due to infection by various viruses, including Cucumber Mosaic Virus (CMV). The conventional breeding of chilli hybrids for improved disease resistance is a long process with problems of interspecific incompatibility. The objectives of the study were to construct a transforming vector containing Cucumber Mosaic Virus (CMV) Coat Protein (CP) and development of an *Agrobacterium*-transformation procedure which can offer solutions to these problems.

This study was approached primarily from the molecular aspect which the construct of a CMV CP has been successfully cloned. The cloned fragments were 655 bp and exhibited more than 90% similarity to those published CMV CP gene sequences.

SDS-PAGE analysis showed the size of the expressed coat protein to be similar to the predicted size of 30 kD based on the DNA sequences. Western blot analysis was also conducted to confirm the bacterial expressed coat proteins. All these verified that the cloned genes were translatable CMV CP genes.

A protocol for regeneration and genetic transformation was established for chilli. This study showed that GUS reporter gene can be transformed into chilli by using *Agrobacterium*-mediated techniques. Using *Agrobacterium* AGL0 with 24 hours co-cultivation was found to be the best way for chilli transformation as it showed a 100% success rate in the tested explants. However, *Agrobacterium* EHA 105 can also be used for chilli transformation with a shorter period, found to be 3 hours, co-cultivation period of 3 hours to achieve an 87.5% success rate.

Methods that permit normal shoot development have generally been found on regenerated decapitated seedlings. Formation of buds and elongated shoots occurred successfully by injuring the apical region of hypocotyls tissues of seedlings during curved stages. The best responses of regenerative ability showed shoot-like structures induced at the wounded sites on Murashige and Skoog's (MSD) medium supplemented with 2 mg/L Benzyladenine (BAP) and 0.25 mg/L Indole-3-acetic (IAA). Direct shoot elongation appeared after transferring to hormone free MSD medium. These elongated shoots were excised and rooted on MSD medium with 0.05 mg/L IAA and until development into plants.

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

PEMBINAAN VEKTOR TRANSFORMASI YANG MENGANDUNGI GEN CUCUMBER MOSAIC VIRUS (CMV) KOT PROTEIN (CP) DAN KEMAJUAN KAEDAH *AGROBACTERIUM*-TRANSFORMASI UNTUK CILI

By

LIM JEE HIAN

Mei 2003

Pengerusi : Profesor Marziah Mahmood, Ph.D.

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Cili (*Capsicum annuum* L.) merupakan salah satu sayuran utama dan tanaman rempah-ratus yang ditanam secara meluas, tetapi ia mengalami kerugian yang besar disebabkan rentan kepada berbagai jenis infeksi virus, termasuk Cucumber Mosaic Virus (CMV). Pembiakbakaan secara konvensional cili untuk memperbaiki rentan terhadap serangan penyakit memakan masa yang lama dengan wujud masalah interspesifik ketidak sepadanan. Objektif kajian ini ialah pembentukkan satu vektor transformasi yang mengandungi gen Cucumber Mosaic Virus (CMV) Kot Protein (CP) serta memajukan kaedah *Agrobacterium*-transformasi untuk penyelesaian masalah tersebut.

Kajian ini dimulakan dengan pembentukkan casette gen CMV CP yang telah berjaya diklonkan. Fragmen yang diklonkan ialah 655 bp dan mempamerkan lebih daripada 90% persamaan antara rangkaian CMV CP yang telah diterbitkan. Analisis SDS-

PAGE mempamerkan saiz kot protein yang diekspresikan adalah sama dengan saiz ramalan 30 kD berdasarkan rangkaian DNA. Analisis "Western" juga dijalankan untuk mengesahkan pengekspresan kot protein. Kesemua ini membuktikan bahawa gen yang diklon merupakan gen CMV CP yang berupaya diterjemahkan.

Suatu protokol untuk penjanaan dan transformasi genetik telah dibentuk untuk cili. Kajian ini menunjukkan gen penyaringan GUS berjaya tertransformasi ke cili dengan kaedah berperantarakan *Agrobacterium*. Penggunaan *Agrobacterium* AGL0 dengan 24 jam kokultivasi didapati adalah cara terbaik untuk transformasi cili dengan 100% kadar kejayaan dalam eksplan yang diuji. Walau bagaimanapun, *Agrobacterium* EHA 105 juga boleh digunakan dengan 3 jam kokultivasi untuk mencapai 87.5% kadar kejayaan.

Kaedah penjanaan anak benih "decapitated" membolehkan perkembangan pucuk kelihatan normal. Penghasilan tunas dan pemanjangan pucuk terbentuk pada bahagian apical tisu hipokotil, anak benih melengkung yang dilukakan. Keupayaan penjanaan struktur "shoot-like" yang terbaik dijumpai pada bahagian luka anak benih di atas media Murashige and Skoog's (MSD) dengan tambahan 2 mg/L Benzyladenine (BAP) dan 0.25 mg/L Indole-3-acetic (IAA). Pemanjangan pucuk secara langsung kelihatan selepas pemindahan di atas media MSD tanpa hormon. Anak pucuk yang memanjang akan dipotong dan dipindahkan ke media pengakaran dengan tambahan 0.05 mg/L IAA dan sehingga menjadi tumbuhan.

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Last but not least, the author would like to express his heartiest appreciation and thanks to his beloved family's members. Thanks for all theirs understanding and support throughout the studies. Thanks for all theirs love that sustained author throughout his research.

I certify that an Examination committee met on 8th May 2003 to conduct the final examination of Lim Jee Hian on his Master of Science thesis entitled "Construction of a Transformation Vector Containing Cucumber Mosaic Virus (CMV) Coat Protein (CP) Gene and Development of an *Agrobacterium*-Transfomation Procedure for Chilli" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulation 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are 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.

LIM JEE HIAN

Date: 18 JUL 2003

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ABBREVIATION

µL	micro liter
µM	micro molar
µg/mL	microgram per milliliter
BAP	benzylaminopurine
bp	base pair
cm	centimeter
CMV	Cucumber Mosaic Virus
DNA	deoxyribonucleic acid
DNTP	deoxynucleoside 5'-triphosphates
EDTA	ethylene diaminetetra acetic acid
g	gram
GUS	β-glucuronidase
HCl	hydrochloric acid
IAA	indole-3-acetic acid
IPTG	Isopropyl β-D-Thiogalactopyronidase
kb	kilo base
kg	kilogram
LB	Luria and Bertani media
M	molar
$MgCl_2$	magnesium chloride
mg/L	miligram per liter

$MgSO_4$	Magnesium sulphate
min	minute
mL	milliliter
mM	millimolar
MS	Murashige and Skoog (1962)
MSD	Murashige and Skoog with Dextrose
MW	molecular weight
NaCl	sodium chloride
NaOH	sodium hydroxide
NptII	Neomycin phosphotransferase II
°C	degree centigrade
%	percentage
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
ppm	part per million
RNA	ribonuclease acid
SDS	Sodium dodecyl sulphate
TDZ	thidiazuron
TEMED	tetramethyl-ethylenediamine
Ti	tumor inducing
UV	ultra violet

v/v	volume over volume ratio
var.	variety
w/v	weight over volume ratio
X-Gal	5-Bromo-4-chloro-3-indolyl-β-D-galactoside
YEB	yeast extract broth

CHAPTER 1

INTRODUCTION

1.1 Distribution

Chilli (*Capsicum spp.*) is an important nontuberous vegetable of the family *Solanaceae* besides tomato and potato. It is grown worldwide. Through its important contribution to nutrition and the economy, chilli becomes very closely associated with the Malaysian people and inextricably woven into their lives. Today this important cultivated plant is a source of income for many thousands of farmers. Since cultivation of chilli, people have noted the exceptional flavours, aroma and colourings, as well as its excellent spice quality. As a rich source of vitamins, it has become a popular and widely used spice throughout the world.

1.2 Economic Importance

Chilli is consumed throughout the world. Forty per cent of this crop is produced in Asia, and India is the leading producer in area and production. Greenleaf (1986) has reviewed the economic importance of this crop. In Malaysia, chilli ranks as the most popular fruit vegetable and occupies the highest hectarage among the fruit vegetables in this country (Ong et al., 1979, Inon et al., 1999). *Capsicum annuum* and *Capsicum frutescens* are the two main types of chilli cultivated in Malaysia. Between the two, *C. annuum* is more important in terms of economic and area cultivated. Recent report indicated that over 3,055 ha were planted with this crop, with a production of 21,890 tonnes chilli per year (Inon et al., 1999)

1.3 Production Related Problem

Although chilli is economically important it is highly susceptible to microbial diseases especially viral diseases, resulting in significantly reduction of crop yields and sometimes even complete crop failure. Five types of viruses have been reported in infecting chilli in Malaysia, namely Cucumber Mosaic Virus (CMV), Chilli Venial Mottle Virus (CVMV), Tobacco Mosaic Virus (TMV), Tomato Spotted Wilt Virus (TSMV), and Tobacco Leaf Curl Virus (TLCV) (Ong et al., 1979; Fujisawa et al., 1986; Mohamad Roff and Ong, 1992). Among these, CMV and CVMV were found to be the most common viruses infecting and damaging chilli in Malaysia which results in the losses of yield ranges from 10%-15% if infection came in late and may reach up to 60% if plants were infected at early stage (Ong et al., 1979). As a result, production of chilli is insufficient to meet the local consumption, and approximately RM30 million worth of chilli is imported annually (Ong et al., 1979).

The use of pesticides to control insect vector populations implicated in transmission of the virus represents an important tool to limit the incidence of viral disease outbreaks. However, none of these non-genetic control measures is likely to provide the long term answer to combat viral diseases, because of their expense and their sometimes questionable effectiveness and reliability. Moreover, current concerns about pollution and food safety are forcing hazardous pesticides out of the market.

Since pesticides are progressively abandoned, there is a growing urgency for the development of alternative methods to control viral diseases. Breeding for virus

resistance generally provides the best prospect for virus control in the long term. In the past, the introgression of genetic sources for host plant resistance that are naturally present within the gene pool of the crop involved, has been successfully applied to develop virus resistant crop cultivars for a considerable number of agronomically important crops (Hoisington et al., 1999).

Conventional breeding programs for viral resistance genes in local chilli varieties have been conducted (Ong et al., 1979), only a small group of genotypes were found to be resistant and they do not possess resistance to both CMV and CVMV. The limitations of conventional breeding and routine culture practices urge the need for the development of alternative forms of virus control that can be fully integrated within traditional methods.

This whole concept is an interesting trend. It started with the intention of Takebe in Japan to facilitate the infection of plants cells (tobacco) by plant viruses. A protoplast-to-plant system was achieved (Takebe et al., 1971), and the virus infected the protoplasts and replicated in them. Due to vast progress in molecular methodologies and fruitful investigations in several plant molecular-biology laboratories, especially on the biology and molecular genetics of the plant pathogen *Agrobacterium* spp.- did genetic transformation of plants become a reality.

In recent years, a lot of progress has been achieved on the understanding of plant virus genome organisation and functions (Martin et al., 1997; Parniske et al., 1998; Speulman et al., 1999). This knowledge, together with the virus derived resistance strategies, has been used for developing virus resistant cultivars.

Various DNA sequences (coding for the coat protein, movement protein, the polymerase gene as well as non-coding sequences) have been introduced, through genetic engineering into plant genomes in order to create viral resistance varsity. In some instances, high levels of resistance which justify for commercial applications have been successfully obtained (Comai et al., 1985; Powell et al., 1986; Gerlach et al.,1987; Hider et al.,1987). The viral coat protein (CP) gene, however, has thus far been more widely applied than any other viral sequence (Powell et al., 1986; Beachy et al., 1990). Beachy et al. (1990) reported that the transformation of tomato with the CP gene from Cucumber Mosaic Virus was shown to generate high levels of protection to CMV infection in tomato hybrids, not only when challenged by mechanical inoculation, but also when exposed to repeated inoculation by viruliferous aphids in open field.

1.4 Aims of This Thesis

It is foreseen that similar strategies can, therefore, be utilised to give coat protein-mediated protection against CMV diseases in chilli. The objectives of this research are

(a) to obtain a full-length translatable Coat Protein(CP) gene of a local strain of chilli infected CMV in bacteria and sub-clone it into plant expression cassette

(b) to develop and optimise an efficient *Agrobacterium* transformation protocols using GUS reporter gene as well as CMV CP gene

(c) to develop plant regeneration system for chilli.

CHAPTER 2

LITERATURE REVIEW

2.1 Properties of Cucumber Mosaic Virus (CMV)

CMV (Figure 1) is an isometric particle, about 30 nm in diameter and consists of 180 subunits in pentamer-hexamer clusters. The molecular weight of the particle is about 5.8-6.7 x 10^6 daltons and has a tripartite genome composed of four RNA species, three genomic and one subgenomic RNA (Peden and Symons, 1973). RNAs 1 (3.3 kb) and RNAs 2 (3.0 kb) encode proteins with molecular weights of 111 kDa and 97 kDa respectively, both of which are essential for virus replication (Nitta et al., 1988; Matthew, 1992). RNA 3 (2.2 kb) encodes for the viral coat protein with a mass of about 24 kDa (Daniels and Campbell, 1992). An additional protein with molecular weight of about 30 kDa, encoded by subgenomic RNA 3, is involved in the cell to cell movement of the virus (Davies and Symons, 1988).

CMV exists as a collection of strains with overlapping host ranges encompassing over 800 plant species (Douine et al., 1979; Kaper and Waterworth, 1981; Palukaitis et al., 1992). CMV exists as a variety of isolates which can be divided into two major serological subgroups defined as DTL and ToRS (Francki et al., 1979). DTL commonly induced more severe symptoms and occurred predominantly in the tropics and subtropics, whereas the ToRs isolates are prevalent in the temperate regions. Most of the approaches for virus detection could be used for characterization and relationship study of a virus. A number of methods and techniques which including biologically, serologically or physically approaches have been used to characterize CMV isolates.

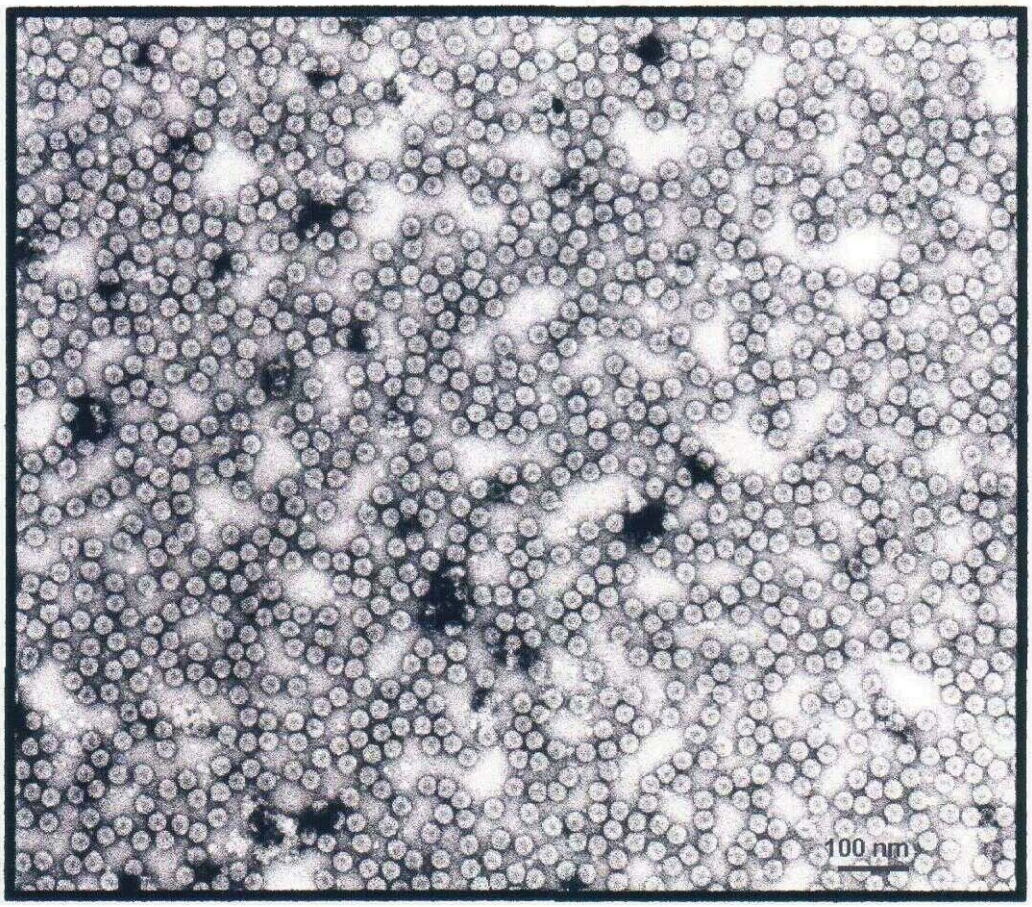

Figure 1: Electron microscopy of Cucumber Mosaic Virus (CMV).