



**UNIVERSITI PUTRA MALAYSIA**

**DIFFERENTIATION AND GENETIC STUDIES  
OF SEVERAL ISOLATES OF  
CUCUMBER MOSAIC VIRUS**

**OMAR MUSSA EL-SANOUSI**

**FSAS 1997 17**

**DIFFERENTIATION AND GENETIC STUDIES  
OF SEVERAL ISOLATES OF  
CUCUMBER MOSAIC VIRUS**

**BY**

**OMAR MUSSA EL-SANOUSI**

**Dissertation Submitted in Fulfilment of the Requirements  
for the Degree of Doctor of Philosophy in the  
Faculty of Science and Environmental Studies,  
Universiti Putra Malaysia.**

**June 1997**



## ACKNOWLEDGEMENT

In the name of ALLAH, Most Gracious, Most Merciful.

Praise be to ALLAH, the Cherisher and Sustainer of the worlds, May He bless and grant peace to our Lord Muhammad who was sent by Him to save these worlds. My deepest gratitude and love to my father, my mother, my wife and other family members who constantly pray for my success.

I express sincere appreciation and deep gratitude to Assoc. Prof Dr. Norani Abdul-Samad, chairman of my supervisory committee, for her wise counselling, guidance, support and encouragement throughout the entire graduate programme. Grateful appreciation is extended to Dr. Ong Ching Ang a member of the supervisory committee, for providing the CMV isolates, constructive suggestions and comments at various stages of this study.

I owe my sincere gratitude and appreciation to Assoc. Prof. Dr. Khatijah Mohd. Yusoff and Dr. Suhaimi Napis, members of the supervisory committee for their constructive comments in the preparation of the final manuscript.



I am very much grateful to all the staff of Department of Biochemistry and Microbiology for their support, cooperation and friendship.

The financial support from the University of Omar Al-Mukhtar El-Bida Libya and the National Council for Research and Development, Malaysia (IRPA) is gratefully acknowledged.

The companionship of friends is highly appreciated for their motivation throughout the study.



## TABLE OF CONTENTS

		Page
ACKNOWLEDGEMENTS.....		ii
LIST OF TABLES .....		ix
LIST OF FIGURES.....		xi
LIST OF PLATES.....		xii
LIST OF ABBREVIATIONS .....		xiv
ABSTRACT.....		xvi
ABSTRAK.....		xix
<b>CHAPTER</b>		
<b>I</b>	INTRODUCTION.....	1
<b>II</b>	LITERATURE REVIEW .....	4
	Structure and Composition of CMV .....	4
	Particle Structure .....	4
	Genome .....	5
	Antigenic Properties.....	6
	Immunogenicity.....	6
	Serological Identification .....	6
	Strain Classification .....	7
	Biological Properties .....	8
	Host Range and Symptomatology .....	8
	Aphid Transmission.....	10
	Polymerase Chain Reaction (PCR) .....	11
	The Relationship with Other Cucumovirus Species.....	15
	Genetic Basis of Host Specificity and Symptom Induction .	16
	RNA 1 .....	16
	RNA 2 .....	17
	RNA 3 .....	19
	Satellite RNAs of CMV .....	20



<b>III</b>	<b>GENERAL MATERIALS AND METHODS.....</b>	<b>23</b>
	Virus Isolates.....	23
	Antisera.....	23
	Chemicals .....	23
	Inoculation and Propagation of Virus .....	27
	Aphid Transmission .....	27
	Virus Purification .....	28
	Serological Techniques .....	29
	Fixation of CMV with Glutaraldehyde .....	29
	Production of Antisera to CMV .....	30
	Agar-gel Immunodiffusion Tests .....	30
	Purification of $\gamma$ - Globulin .....	31
	Conjugation of Enzyme with $\gamma$ -Globulins.....	31
	Enzyme-Linked Immunosorbent Assays.....	32
	Isolation of Viral RNA .....	34
	Precaution Against Ribonuclease .....	34
	Extraction of Viral RNA .....	34
	Electrophoresis of Viral RNA .....	35
	Infectivity and Purity of Fractionated RNA Components.....	36
	Spectrophotometry.....	37
<b>IV</b>	<b>HOST RESPONSE, SYMPTOMATOLOGY AND APHID TRANSMISSION OF CMV ISOLATES.....</b>	<b>38</b>
	Introduction .....	38
	Experimental .....	39
	Host Response and Symptomatology .....	39
	Aphid Transmission .....	48
	Conclusions.....	49
<b>V</b>	<b>VIRUS PURIFICATION AND RNA ISOLATION .....</b>	<b>50</b>
	Introduction.....	50
	Experimental.....	51
	Virus Purification.....	51
	RNA Extraction.....	54
	Conclusions.....	56
<b>VI</b>	<b>SEROLOGICAL PROPERTIES OF CMV ISOLATES.....</b>	<b>57</b>
	Introduction .....	57
	Experimental .....	58
	Properties of Antisera Used .....	58
	Agar gel Immunodiffusion Test .....	58
	Enzyme-linked Immunosorbent Assays .....	61

	Determination of the Optimum Concentration of Coating and Enzyme Labelled $\gamma$ -Globulin.....	61
	Determination of Serological Relationships Between CMV Isolates by ELISA.....	64
	Serological Relationships of CMV Isolates with D and Q Strain as Revealed by ELISA.....	64
	Conclusions.....	73
VII	POLYMERASE CHAIN REACTION.....	74
	Introduction.....	74
	Experimental.....	75
	Primers .....	75
	Synthesis of cDNA .....	75
	PCR Amplification .....	76
	PCR Product .....	76
	Restriction Digests of PCR products .....	77
	Enzymatic Digestion Pattern of PCR Products.....	77
	Conclusions.....	80
VIII	SATELLITE-RNA (sat-RNA).....	81
	Introduction.....	81
	Experimental.....	83
	Isolation of Sat-RNA.....	83
	Inoculation with Sat-RNA .....	83
	Elimination of Sat-RNA from Genomic RNA.....	84
	Response of The CMV Isolates to Sat-RNA.....	84
	Conclusions.....	90
IX	GENETIC ANALYSIS OF CMV ISOLATES GENOME BY PSEUDORECOMBINANT FORMATION.....	91
	Introduction.....	91
	Experimental.....	92
	Infectivity and Purity of Isolated RNA Components .....	92
	<i>In vitro</i> Construction of Pseudorecombinants .....	92
	Examination of Pseudorecombinants.....	94
	Symptomatology and Host Range of Pseudorecombinants.....	95
	Conclusions.....	109
X	GENERAL DISCUSSION.....	110
	Characterisation of CMV Isolates .....	110
	Satellite RNA .....	115
	Genetic Studies of CMV Isolates .....	119



XI SUMMARY AND CONCLUSION .....	123
REFERENCES .....	127
VITAE.....	144





## LIST OF TABLES

Table		Page
1	Synonyms of CMV Subgroups.....	9
2	PCR Primers for the Amplification of Coat Protein Gene of CMV.....	13
3	Sources of CMV Isolates.....	24
4	Chemicals Used .....	25
5	The Host Response and Symptoms of CMV Isolates.....	41
6	The Transmission Efficiency of the CMV Isolates by the Two Aphid Species.....	48
7	Influence of Phosphate and Citrate Buffer on Extraction of the CMV Isolates .....	52
8	Antisera Titre at Different Bleeding Periods.....	59
9	Serological Relationship Among the CMV Isolates. ....	60
10	Characteristics of CMV Antisera Used for ELISA Tests.....	63
11	The Effect of Sat-RNA on Virus Concentration.....	85
12	The Symptoms Induced by CMV Isolates in the Presence of Sat-RNA6 on Three Different Hosts.....	87
13	The Symptoms Induced by CMV Isolates in the Presence of Sat-RNA7 on Three Different Hosts.....	88
14	Infection of CMV Isolates in the Presence of Sat-RNA6 and Sat-RNA7 in Chilli cv. MC4 and Tomato cv. Eggtomato.....	89



15	Test of Purity and Infectivity of RNA1, RNA2 and RNA3 of CMV-3, CMV-4 and CMV-7 Separated by Gel Electrophoresis.....	93
16	The Response of Different Hosts to the Pseudorecombinants Formed Between CMV-3 and CMV-4.....	96
17	The Response of Different Hosts to the Pseudorecombinants Formed Between CMV-4 and CMV-7.....	104



## LIST OF FIGURES

Figure		Page
1	Influence of Different Concentration of Coating $\gamma$ -Globulins of CMV-7 Antiserum on the Colour Production in ELISA.....	64
2	The Serological Relationship Between CMV-3 and the Other CMV Isolates as Revealed by DAS-ELISA Tests Using IgG and Enzyme Conjugated IgG to CMV-3.....	66
3	The Serological Relationship Between CMV-4 and the Other CMV Isolates as Revealed by DAS-ELISA Tests Using IgG and Enzyme Conjugated IgG to CMV-4.....	67
4	The Serological Relationship Between CMV-6 and the Other CMV Isolates as Revealed by DAS-ELISA Tests Using IgG and Enzyme Conjugated IgG to CMV-6.....	68
5	The Serological Relationship Between CMV-7 and the Other CMV Isolates as Revealed by DAS-ELISA Tests Using IgG and Enzyme Conjugated IgG to CMV-7.....	69
6	The Serological Relationship Between the CMV Isolates and D Strain as Revealed by DAS-ELISA Tests Using IgG and Enzyme Conjugated IgG to D Strain .....	70
7	The Serological Relationship Between the CMV Isolates and Q Strain as Revealed by Direct and Indirect-ELISA Tests Using Enzyme Conjugated to Goat-Antirabbit-IgG.....	71
8	The Serological Relationship Between the CMV Isolates and D and Q Strain as Revealed by Indirect-ELISA Tests Using Enzyme Conjugated to Goat-Antirabbit-IgG .....	72



## LIST OF PLATES

Plate		Page
1	Mosaic Symptoms Produced in <i>C. annuum</i> cv. MC4. by CMV-4 (A) and CMV-6 (B).....	43
2	Symptoms Induced by CMV Isolates in <i>C. sativa</i> cv. Oriental.....	44
3	Symptoms Produced by CMV Isolates in <i>L. esculentum</i> cv. Eggtomato.....	45
4	The Infection of <i>N. glutinosa</i> by CMV Isolates.....	46
5	The Symptoms Produced by CMV Isolates in <i>N. tabacum</i> cv White Burley.....	47
6	Electron Micrograph of Purified Virus Particles Stained with Uranyl Acetate.....	53
7	Electrophoretic Analysis of the RNA Components of the CMV Isolates in 2% Agarose Gel, Stained with Ethidium Bromide.....	55
8	Immunodiffusion Tests Between CMV Isolates.....	61
9	Analysis of RT-PCR-Amplified CMV Coat Protein cDNAs Using Electrophoresis on 1.5 Agarose Gel Stained with Ethidium Bromide.....	78
10	Restriction Enzyme Digestion of PCR Product of CMV Isolates by <i>EcoRI</i> and <i>MspI</i> Enzyme.....	79
11	The Response of <i>C. annuum</i> cv. MC4 to Pseudorecombinants Formed from CMV-3 and CMV-4.....	98
12	The Response of <i>L. esculentum</i> cv. Eggtomato to Pseudorecombinants Formed from CMV-3 and CMV-4.....	99
13	The Response of <i>N. glutinosa</i> to Pseudorecombinants Formed from CMV-3 and CMV-4.....	101



14	The Response of <i>N. tabacum</i> cv. White Burley to Pseudorecombinants Formed from CMV-3 and CMV-4.....	102
15	The Response of <i>L. esculentum</i> cv. Eggtomato to Pseudorecombinants Constructed from CMV-4 and CMV-7.....	105
16	The Response of <i>N. tabacum</i> cv. White Burley to Pseudorecombinants Constructed from CMV-4 and CMV-7.....	106
17	The Response of <i>N. glutinosa</i> to Pseudorecombinants Constructed from CMV-4 and CMV-7.....	108



## LIST OF ABBREVIATIONS

bp	=	base pair
CARNA5	=	CMV Associated Ribonucleic Acid 5
CMV	=	Cucumber Mosaic Virus
cv	=	Cultivar
DAS-ELISA	=	Double Antibody Sandwich Enzyme Linked Immunosorbent Assay
DEPC	=	Diethyl Pyrocarbonate
DNA	=	Deoxyribonucleic Acid
cDNA	=	Complementary deoxyribonucleic acid
ds	=	Double Stranded
EDTA	=	Ethylenediaminetetra Acetic Acid
g	=	Gravity
Kb	=	Kilo bases
dNTP	=	Deoxynucleotides Triphosphate
PBS	=	Phosphate Buffer Saline
PCR	=	Polymerase Chain Reaction
PSV	=	Peanut Stunt Virus
PVP	=	Polyvinylpyrrolidone
RNA	=	Ribonucleic Acid



RT-PCR	=	Reverse Transcription Polymerase Chain Reaction
SDI	=	Serological Differentiation Indices
SDS	=	Sodium Dodecyl Sulphate
ss	=	Single Stranded
TAV	=	Tomato Aspermy Virus
v/v	=	Volume/Volume
w/w	=	Weight/Weight



Abstract of dissertation submitted to the Senate of Universiti Putra Malaysia  
in fulfilment of the requirements for the degree of Doctor of Philosophy

**DIFFERENTIATION AND GENETIC STUDIES OF SEVERAL  
ISOLATES OF CUCUMBER MOSAIC VIRUS**

By

OMAR MUSSA EL-SANOUSI

June 1997

Chairman: Associate Prof. Dr. Norani Abdul Samad

Faculty : Science and Environmental Studies

Four cucumber mosaic virus (CMV) isolates from different host and localities were differentiated on the basis of biological and serological properties and polymerase chain reaction (PCR). The first two isolates (CMV-3 and CMV-7) were isolated from tobacco in Telong, Kelantan; the third (CMV-4) was from chilli in MARDI Jalan Kebun, Klang; and the fourth isolate (CMV-6) was from purple cleome in Sri Kembangan, Selangor. These isolates could be distinguished from each other by the symptoms produced in several plant species. CMV-3 and CMV-7 were more similar to each other than to the other isolates. Aphid transmission test revealed that all the isolates could be transmitted by *A. gossypii* with higher efficiency than *A. craccivora*.





Immunodiffusion tests revealed that all the isolates were closely related since all the heterologous titres were within the same two-fold dilutions of each other. In the DAS-ELISA tests, there were variations observed between the homologous and the heterologous antigens which revealed that the isolates CMV-3, -6, -7 had a closer relationship to each other than to CMV-4; and the isolate CMV-7 had a more distant relationship to isolate CMV-4 than to the others. All the isolates showed a closer relationship to D strain (DTL serogroup) but could not be detected by Q strain (ToRS serogroup) when the DAS-ELISA was used. By indirect-ELISA, all the isolates could be detected by D and Q strains.

A single band of about 487 bp were successfully amplified from the coat protein gene of the CMV isolates. The PCR product could be digested by *Msp* I to produce two bands of approximately 337 and 151 bp but could not be digested by *Eco*R I. Result of analysis of the biological and serological properties as well as PCR confirmed that the isolates belonged to subgroup I of CMV.

To determine the gene or genes location of symptoms determinants in the RNA segments, six pseudorecombinants were constructed *in vitro* between the RNAs of CMV-4 and CMV-3; and two pseudorecombinants by exchanging RNA3 between CMV-4 and CMV-7. The observations on chilli cv. MC4, tomato cv. Eggtomato, *N. glutinosa* and *N. tabacum* cv. White Burley infected with these



pseudorecombinants indicated that RNA 2 or 3 or both were involved in symptoms production.

Two different types of sat-RNA, named sat-RNA6 and sat-RNA7 were found to be naturally associated with CMV-6 and CMV-7, respectively. The association of these sat- RNAs with the CMV isolates reduced the virus concentration up to ten times. These sat-RNAs induced systemic necrosis in tomato and attenuated the symptoms produced by the genomic RNAs in MC4 chilli and White Burley tobacco. Sat-RNA6 showed a higher virulence by inducing systemic necrosis in tomato and produced local necrosis in inoculated White Burley tobacco leaves.



Abstrak disertasi yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi syarat keperluan untuk Ijazah Doktor Falsafah

**PEMENCILAN DAN KAJIAN GENETIK BEBERAPA PENCILAN  
CUCUMBER MOSAIC VIRUS**

Oleh

OMAR MUSSA EL-SANOUSI

June 1997

Pengerusi: Prof. Madya Dr. Norani Abdul Samad

Fakulti : Sains dan Pengajian Alam Sekitar

Empat pencilan cucumber mosaic virus (CMV) dari beberapa tumbuhan perumah dan keadaan persekitaran telah diperbeza berasaskan ciri-ciri biologi dan serologi serta kaedah tindakbalas berangkai polimerase (PCR). Dua pencilan pertama (CMV-3 dan CMV-7), telah diperolehi dari pokok tembakau di Telong, Kelantan; pencilan ketiga (CMV-4) telah diperolehi dari pokok cili di MARDI, Jalan Kebun, Klang; dan pencilan keempat (CMV-6) telah diperolehi dari pokok mami liar di Sri Kembangan, Selangor. Pencilan-pencilan ini boleh dibezakan di antara satu dengan yang lain melalui simptom yang dihasilkan di beberapa jenis tumbuhan. Pencilan-pencilan CMV-3 dan CMV-7 adalah hampir serupa berbanding dengan pencilan yang lain. Ujian transmisi afid menunjukkan



kesemua pencilan boleh ditransmisikan oleh *A. gossypii* dengan keupayaan yang lebih tinggi jika dibandingkan dengan *A. craccivora*.

Ujian-ujian imunoserapan dua hala menunjukkan bahawa ke semua pencilan mempunyai pertalian yang rapat antara satu sama lain berdasarkan kepada persamaan titer heterolog dalam lingkungan pencairan dua kali. Dalam ujian DAS-ELISA, terdapat beberapa perbezaan di antara antigen homolog dan antigen heterolog yang menunjukkan bahawa CMV-3, -6, -7 mempunyai pertalian yang lebih rapat di antara satu sama lain daripada pertalian dengan CMV-4; sementara pencilan CMV-7 pula mempunyai pertalian yang paling jauh dengan pencilan CMV-4 berbanding dengan yang lain. Dengan menggunakan DAS-ELISA, kesemua pencilan menunjukkan pertalian yang rapat dengan strain D (kumpulan serum DTL) tetapi tidak dapat dikesan oleh strain Q (kumpulan serum ToRS). Akan tetapi dengan melakukan ujian "indirect-ELISA", kesemua pencilan dapat dikesan oleh strain D dan Q.

Kesemua pencilan-pencilan CMV telah berjaya menghasilkan satu jalur hasil PCR bersaiz 487 bp dari cDNA yang mengkod protein virus dengan menggunakan primer-primer yang direka untuk gen protein kot. Hasil PCR boleh dihadamkan dengan enzim pembatas *Msp* I dengan menghasilkan dua jalur bersaiz



sekitar 337 bp dan 151 bp , tetapi tidak boleh dihadamkan oleh enzim *EcoR* I. Pencirian biologi dan serologi dan teknik PCR membuktikan bahawa pencilan-pencilan tersebut tergolong dalam CMV sub-kumpulan I.

Untuk menentukan gen atau lokasi gen bagi penentu simptom dalam RNA, enam 'pseudorecombinant' telah dibentuk *in vitro* di antara segmen-segmen RNA dari CMV-4 dan CMV-3; dan dua pseudorecombinant melalui pertukaran RNA3 di antara CMV-4 dan CMV-7. Pemerhatian-pemerhatian pada cili cv. MC4, tomato cv. Egg tomato, *N. glutinosa* dan *N. tabacum* cv White Burley yang disuntik dengan pseudorecombinant tersebut menunjukkan bahawa RNA2 atau 3, atau keduanya sekali terlibat di dalam penghasilan simptom.

Dua jenis sat-RNA yang berbeza, sat-RNA6 dan sat-RNA7 telah ditemui yang mempunyai hubungan semulajadi dengan CMV-6 dan CMV-7, masing-masing. Hubungan di antara sat-RNA tersebut dengan pencilan-pencilan CMV menurunkan kepekatan virus pada daun hingga sepuluh kali ganda. Sat-RNA ini mengaruhkan nekrosis sistemik pada tomato dan melemahkan kesan simptom genom RNA pada cili MC4 dan tembakau White Burley. Sat-RNA6 menunjukkan daya kevirulenan yang tinggi dengan mengaruhkan nekrosis sistemik pada tomato dan menghasilkan nekrosis setempat dalam daun tembakau White Burley.

# CHAPTER I

## INTRODUCTION

Cucumber mosaic virus (CMV) is the type species of the genus Cucumovirus of Bromoviridae Family (Murphy *et al.*, 1995). CMV was first reported as the causal agent of cucumber mosaic disease in U.S.A. (Doolittle, 1916; Jagger, 1916). Since then CMV has been found in most countries of the world. CMV is often the most prevalent virus in a number of surveys on plant virus infections in different parts of the world including Hungary (Gaborjanyi and Nagy, 1972), Iran (Rahimian and Izadpanah, 1978), Germany (Schimanski *et al.*, 1976), France (Quiot *et al.*, 1979a), Korea (Choi and Park, 1982), Spain (Garcia-Luque *et al.*, 1983; Luis-Arteaga *et al.*, 1988; Blas *et al.*, 1993), New Zealand (Burgman *et al.*, 1986), Morocco (Bouhid and Lockhart, 1990), U.S.A. (Provvidenti *et al.*, 1984; Rist and Lorbeer, 1989, 1991), Malaysia (Mohamad Roff and Ong, 1992), Indonesia, Malaysia, Sri Lanka, Taiwan and Thailand (Green, 1992). In the last two decades, CMV has been identified as the causal agent of several disease epidemics, for instance, necrosis of tomato in France (Quiot *et al.*, 1979b), Italy (Gallitelli *et al.*, 1990) and Spain (Jorda *et al.*, 1992); severe stunting and leaf epinasty of lupine in Australia (Alberts *et al.*, 1985), mosaic in sweet potato in



Israel (Cohen *et al.*, 1988) and in banana in Morocco (Bouhida and Lockhart, 1990). In China, severe losses caused by CMV were reported in vegetable crops (Tien and Wu, 1991).

The CMV has a very broad natural host range throughout the temperate, tropical and subtropical areas. It is pathogenic to cereals, forages, woody and herbaceous ornamental, vegetables and fruit crops (Kaper and Waterworth, 1981).

In Malaysia, CMV was reported as a major virus disease on cucurbits (Ong and Ting, 1977) and one of the main virus diseases on chilli (Mohamad Roff and Anang, 1989; Mohamad Roff and Ho, 1989; Fujisawa *et al.*, 1990; Mohamad Roff and Ong, 1992; Green 1992).

Based on symptomology, the CMV was first reported in Malaya by Milsun and Grist earlier in 1941. Later in 1968 Miller reported the infection of squash and pumpkin with CMV (Ong, 1972). Ong (1972) studied CMV isolated from infected cucumber in more detail by using physical properties, transmission, extensive host range, serology and cross protection. Since then several studies had been conducted to differentiate CMV isolates using selected hosts and electrophoretic patterns of double stranded RNA (dsRNA) (Ahmad, 1991 ; Abu-Baker, 1990; Mohd Nadzir 1992; Mustapha, 1992). Since information are still lacking on the existence of CMV

subgroup, and the association of satellite RNA, the main objectives of the study were to:

1. differentiate several CMV isolates from different hosts and locality and classify them into subgroup by using, i) biological properties; ii) serological properties and iii) molecular techniques.
2. study the transmission efficiency of these isolates by the two main aphid species in Malaysia.
3. study the presence of the satellite RNA with the CMV isolates and their interaction with the virus genome.
4. study the distribution of genetic determinants for symptom production and host range.



## CHAPTER II

### LITERATURE REVIEW

#### Structure and Composition of CMV

##### Particle Structure

CMV has isometric particles about 28-30 nm in diameter. The capsid is composed of 180 protein subunits in pentamer-hexamer cluster with T=3 surface lattice symmetry (Francki *et al.*, 1979). The stability of CMV particles depends largely on protein-RNA interaction which occur between the positively charged basic residues of capsid polypeptide and the negatively charged phosphate groups of RNA (Kaper, 1975 ; 1976). This stability can readily be disrupted in low concentration of ionic detergents such as sodium dodecyl sulphate or high concentration of neutral chloride salts (Francki *et al.*, 1966; Kaper and Geelen, 1971; Boatman and Kaper, 1976) and its infectivity is sensitive to ribonuclease (Francki, 1968; Kaper and Geelen, 1971; Habili and Francki, 1974a). The particles sediment as a single component with  $S = 98.6 - 1.04 C$  where  $C$  is the virus concentration in milligram/ millilitre. The extinction coefficient at 260 nm is 5 (Francki *et al.*, 1966).

