MOVEMENT OF A CAULIMOVIRUS AND TOBAMOVIRUSES

By

YUELIN ZHANG

Bachelor of Science

Fudan University

Shanghai, P.R.China

1989

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirement of the
the degree of
DOCTOR OF PHILOSOPHY
July, 1995

OKLAHOMA STATE UNIV

MOVEMENT OF A CAULIMOVIRUS AND

TOBAMOVIRUSES

Thesis Approved:

Ulrich Melcher
Thesis Adviser

David W. Hinds

Franklin R. Leach

Richard C Essenberg

John L. Sherwood

Thomas C. Collins
Dean of the Graduate College

ACKNOWLEDGMENTS

I wish to express sincere appreciation to my advisor Dr. U.K. Melcher, for his support, encouragement, and advice throughout my graduate program. I would like to thank my graduate committee members, Dr. J.L. Sherwood, Dr. D.W. Meinke, Dr. R.C. Essenberg and Dr. F.R. Leach, for their suggestions, interests and support. I would also like to thank Janet Rodgers in the DNA/Protein Core Facility, Oklahoma State University, for synthesizing the oligonucleotide primers and sequencing some of the plasmids used in this study. I extend many thanks to Dr. Robert Lartey and Mrs. Ann Williams, who provided suggestions and assistance for this study.

I am gratefully indebted to my parents, Mr. Guoqi Zhang and Mrs. Chunlan Xiong, whose foresight was fundamental in framing my education during childhood.

Finally, special gratitude is expressed to my wife, Xin Li, for her love, understanding, encouragement and many sacrifices.

TABLE OF CONTENTS

Chapter Page

I. PREFACE 1

II. EXCHANGE OF MOVEMENT PROTEIN GENES BETWEEN TOBACCO MOSAIC VIRUS (TMV) AND CAULIFLOWER MOSAIC VIRUS (CaMV) 3

1. Introduction 3
Plant Virus Movement Protein 3
TMV and TMV Movement Protein 4
CaMV and CaMV Movement Protein 5
Movement Protein and Host Specificity 6
2. Materials and Methods 11
Construction of Plasmids 11
Construction of pCaT30 11
Generating TMV 30K Gene Fragment 11
Restriction Digestion 13
Ligation, Transformation, and Screening 13
Construction of pTMVCaNM38 14
Construction of pTMVCaMC38NA 16
Construction of pTMVCaM38 18
Construction of pTCaNM38, pTCaM38, pTCaMC38 and pTCa38 19
Construction of pCaTNC30, pCaTN30 and pCaTC30 . 19
Construction of pCaBB1:P30 20
Construction of pTB2:ORF1-Sp6 and pTVCV:ORF1 . . 20
Plants and Inoculation 23
Assay for CaMV DNA 24
3. Results 25
Inoculation of CaMV Chimeras on Turnip, Tobacco and Arabidopsis Plants 25
Inoculation of TMV Chimeras on Tobacco Plants 28
Inoculation of pCaBB1:P30 on Turnip Plants 33
Inoculation of pTB2:ORF1-Sp6 on Tobacco Plants 34
Inoculation of TVCV:ORF1 on Tobacco and Turnip Plants . . . 36

Chapter Page

4. Discussion 37
Functions of CaMV and TMV Movement Protein are not Exchangeable 37
Possible Recombination between TMV Chimeras and Movement Protein Transgene 39
Interference between the Functions of TMV and CaMV Movement Proteins 41

III. GENETIC DETERMINANTS OF TURNIP HOST SPECIFICITY IN TOBAMOVIRUSES 45

1. Introduction 45
2. Materials and Methods 48
Construction of pTVCV5'SP6, pTVCV50, pTMV445TVCV and pTVCV445TMV 48
Construction of pTMV4904TVCV 51
Construction of pTVCV4279TMV and pTVCV4967TMV 51
Construction of pTVCV4904TMV 53
Construction of pTZ200 55
Plants and Inoculation 58
3. Results 59
Construction of an Infectious TVCV cDNA Clone 59
Test of TMV and TVCV Chimeras on Plants 60
4. Discussion 63

BIBLIOGRAPHY 67

LIST OF TABLES

Table Page

1. CaMV P38 and TMV P30 Alignment 10

2. PCR Oligonucleotide Primers 12

3. Primers Used in the Study 17

4. Results of Turnip Plants Inoculated with CaMV MP Chimeras 27

5. Results of Samsun Plants Inoculated with CaMV MP Chimeras 27

6. Results of Tobacco Plants Inoculated with TMV Chimeras 29

7. Sequences of CaBB1:P30 and Viruses Recovered from Turnip Plants Infected with CaBB1:P30 35

8. Results of Testing TMV-TVCV Chimeras on Plants 62

LIST OF FIGURES

Figure Page

1. CaMV and TMV Movement Protein Chimeras Constructed for Use in the Study 9

2. Construction of pTMVCaNM38 15

3. Construction of pTVCV:ORF1 and pTZ195 22

4. TMV and TVCV Chimeras 47

5. Construction of pTVCV445TMV and pTVCV50 49

6. Construction of pTMV445TVCV and pTMV4904TVCV 50

7. Construction of pTZ157 and pTZ158 52

8. Construction of pTVCV4279TMV and pTVCV4967TMV 54

9. Construction of pTVCV4904TMV 56

10. Construction of pTZ200 57

LIST OF ABBREVIATION

bp	Base pair
CaMV	Cauliflower mosaic virus
cDNA	Complementary DNA
CM4-184	Cauliflower mosaic virus, isolate 4-184
kb	Kilobases
K	Kilodalton
MP	Movement protein
ORF	Open reading frame
ORSV	Odontoglossum ringspot virus
^{32}P	Radioisotope of phosphorus
P30	TMV 30K protein
P38	CaMV 38K protein
PCR	Polymerase chain reaction
TMV	Tobacco mosaic virus
TVCV	Turnip vein clearing virus
DTT	Dithiothreitol

CHAPTER I

PREFACE

Virus coded movement proteins (MP) are required for cell-to-cell movement of viruses in plants. In some plant-virus interactions, infection is limited to inoculated cells, implying that MP are important determinants of the host specificity of viral infection. The focus of this study was the movement of cauliflower mosaic virus (CaMV), tobacco mosaic virus (TMV) and turnip vein clearing virus (TVCV) on three plant species (*Arabidopsis thaliana*, tobacco and turnip). Arabidopsis is a host for all three viruses. Tobacco only supports TMV and TVCV infection. Turnip is resistant to TMV, but not CaMV and TVCV.

To test whether the functions of TMV and CaMV MP are exchangeable and what their roles in host specificity are, eight TMV and CaMV chimeras were constructed by exchanging their MP genes or parts of their MP genes. The infectivity of the eight chimeras was evaluated on the three plant species. None of the chimeras was able to infect any wild type plants tested, but all four TMV chimeras infected MP transgenic tobacco plants. TMV with its MP gene replaced by the CaMV MP gene moved 5-6 times slower than wild-type TMV and other TMV chimeras on TMV MP transgenic plants. The results indicate that functions of CaMV and TMV MP are not

exchangeable and CaMV MP interferes with TMV MP function. Viruses able to infect wild-type tobacco plants were also recovered after passage of two TMV chimeras through the MP transgenic plants, possibly indicating recombination between TMV chimeras and the MP transgene.

To identify TMV genes responsible for limiting TMV movement on turnip, a full length TVCV cDNA plasmid was constructed from partial clones. In vitro transcripts of the plasmid infected tobacco and turnip plants. This plasmid and an analogous TMV cDNA plasmid served as starting materials for construction of chimeric genomes. A TVCV chimera with TVCV replicase gene replaced by that of TMV and a TVCV chimera with TVCV movement protein (MP) gene replaced by that of TMV moved like TVCV and TMV on tobacco plants, but their movement was limited on turnip plants. The results indicate that both TMV replicase and MP genes are genetic factors limiting TMV spread on turnip plants.

CHAPTER II

EXCHANGE OF MOVEMENT PROTEIN GENES BETWEEN TOBACCO MOSAIC VIRUS AND CAULIFLOWER MOSAIC VIRUS

1. Introduction

Plant Virus Movement Protein

For most plant viruses, virus particles are initially introduced into host cells by mechanical damage or a vector. For a systemic infection, the introduced plant virus replicates in the inoculated cells, moves from the inoculated cells to the adjacent cells (cell-to-cell movement) and then moves from the inoculated leaves to other leaves. This study focused on cell-to-cell movement of CaMV and TMV. Generally, cell-to-cell movement of a plant virus requires a virus-coded movement protein, that is actively involved in virus cell-to-cell movement (Hull, 1991; Atabekov & Taliansky, 1990; Deom *et al.*, 1992).

The putative movement proteins of many viruses have been identified (Melcher, 1990; Koonin *et al.*, 1991; for reviews, see Hull, 1991; Maule, 1991). Some

properties plant virus movement proteins have in common are location in cell walls in association with plasmodesmata; alteration of the size exclusion limit of plasmodesmata; sequence nonspecific binding of RNA; and formation of tubules associated with plasma membranes and cell walls (For review, see Deom *et al.*, 1992). Recently, it was found that microinjected, fluorescently tagged movement proteins rapidly moved to adjacent cells while fluorescently tagged control proteins remained in the originally microinjected cell (Pascal *et al.*, 1994; Fujiwara *et al.*, 1993; Noueiry *et al.*, 1994; Ding *et al.*, 1995). Fluorescently labeled RNA also moved rapidly to adjacent cells after coinjection with virus movement proteins.

TMV and TMV Movement Protein

TMV is the type member of the tobamoviruses. At least four polypeptides are encoded by the single-stranded positive sense RNA genome of TMV. The 126K and 183K polypeptides are components of the replicase which is involved in virus replication (Young *et al.*, 1986; Meshi *et al.*, 1987). The 17K polypeptide is the coat protein and is involved in virus long distance movement (Siegel *et al.*, 1962; Dawson *et al.*, 1988). The 30K polypeptide (P30) is the movement protein required for cell-to-cell movement (Meshi *et al.*, 1987; Deom *et al.*, 1987). The P30 of TMV is located in the plasmodesmata of virus-infected plants (Tomenius *et al.*,1987) or transgenic plants producing movement protein (Atkins *et al.*, 1991). In such plants the molecular size exclusion limit of the plasmodesmata is increased (Wolf *et al.*,

1989; Deom *et al.*, 1990). The discovery that the TMV movement protein binds cooperatively to single-stranded nucleic acids (Citovsky *et al.*, 1990) led to the suggestion that it has two functions, increasing the permeability of plasmodesmata and forming an unfolded RNA-movement protein complex that is able to move through the modified plasmodesmata. The region of TMV P30 responsible for increasing plasmodesmal size exclusion limits was mapped to the C-terminal part of the 268-amino acid residue protein, between amino acid residues 126 and 224 (Waigmann *et al.*, 1994).

CaMV and CaMV Movement Protein

CaMV is the type member of the caulimovirus group. The virus contains a double-stranded circular DNA molecule, with neither strand covalently closed. The positive-stranded DNA of CaMV contains six major open reading frames (ORFs). Functions have been assigned to some of the putative gene products: aphid transmission factor (ORF 2) (Armour *et al.*, 1983; Woolston *et al.*, 1983), coat protein precursor (ORF 4) (Daubert *et al.*, 1982), reverse transcriptase (ORF 5) (Toh *et al.*, 1983; Takatsuji *et al.*, 1986), main inclusion body matrix protein and transactivator of translation (ORF 6) (Covey and Hull, 1981; Gowda *et al.*, 1989).

CaMV ORF 1 encodes a 38K protein (P38). A significant sequence similarity exists between the putative P38 of CaMV and P30 of TMV. The strongest similarity between CaMV P38 and TMV P30 occurs in a central region of the polypeptide.

CaMV P38 was suggested as a good candidate for the CaMV movement protein (Hull and Covey, 1985; Hull *et al.*, 1986; Martinez-Izquierdo *et al.*, 1987; Melcher, 1990). The probable participation of the ORF 1 product in the control of cell-to-cell transport of CaMV infection is supported by its location around modified plasmodesmata between mesophyll cells and the ends of phloem parenchymal cells in small vascular elements. Sections of CaMV-infected turnip tissues showed modification of the plasmodesmata between mesophyll cells, including an increased diameter of the intersymplastic channel and the appearance of a fine granular tubular structure within a cell wall extension (Linstead *et al.*, 1988; Perbal *et al.*, 1993). The protein encoded by CaMV ORF 1 is an RNA binding protein (Citovsky *et al.*, 1991). Recently two groups have shown that CaMV mutants with different ORF 1 deletions replicated in single cells or protoplasts, but did not move from cell to cell (Thomas *et al.*, 1993; Tsuge *et al.*, 1994; Thomas & Maule, 1995), which strongly indicates that CaMV P38 is the movement protein of CaMV.

Movement Protein and Host Specificity

As TMV P30 is associated with plasmodesmata and binds to single-stranded RNA sequence nonspecifically, a viral RNA and movement protein complex may be formed which moves through the plasmodesmata by interacting with some putative host factors (Deom *et al.*, 1992; Citovsky, 1991). The implication that TMV moves from cell-to-cell through the interaction of P30 with a host component(s) suggests that the

P30 protein may be a determinant of host specificity of viral infection. This suggestion is supported by several experiments. *Tm2* is a recessive mutation of tomato that prevents tomato mosaic virus (ToMV) from moving from cell to cell (Motoyoshi *et al.*, 1975). Nucleic acid sequence analysis indicated that overcoming of the resistance of *Tm2* plants is due to mutation in the P30 gene (Meshi *et al.*, 1989). A hybrid TMV with its P30 gene replaced by the P30 gene of odontoglossum ringspot tobamovirus (ORSV) can infect vanilla orchids, which is a host of ORSV but not of TMV (Holt *et al.*, 1991).

Virus movement proteins are also host specificity determinants for other viruses. A large number of possible complementations of systemic spread between related and unrelated plant viruses were reported (Atabekov *et al.*, 1990). Bromovirus movement protein genes play a crucial role in host specificity (Mise *et al.*, 1993).

To study the genetic determinants for CaMV and TMV, three kinds of plants (turnip, tobacco and Arabidopsis) were used. Turnip is a host of CaMV, but not a host of TMV (Oshima et al., 1962). *Nicotiana tabacum* cv Samsun is a host of TMV, but not a host of CaMV. *Arabidopsis thaliana* is a host of both CaMV and TMV. In this study, a nonhost for a virus means no accumulation of the virus on systemic and inoculated leaves or a very low level accumulation of the virus only on the inoculated leaves. Two major questions were addressed in this study: (1) are the functions of TMV P30 and CaMV P38 exchangeable; and (2) what roles do TMV P30 and CaMV P38 play in host specificity? To answer these two questions, I made four CaMV plasmids (Figure 1) with their P38 gene partially or completely replaced

by the analogous TMV movement protein part and four chimeric TMV cDNA clones (Figure 1) with the TMV movement protein gene replaced by the CaMV P38 gene or with parts of the TMV P30 gene replaced by the analogous CaMV P38 parts. The exchanges were based on the sequence alignment (Melcher, 1990) and structure model proposed by Melcher (1993). CaMV P38 and TMV P30 sequences in the alignment were shown to be significantly related to each other. The alignment revealed three domains of the movement proteins: an N-terminal domain, a central domain and a C-terminal domain. The central domain sequences are the most highly conserved and also have significant similarity to lentiviral proteinase. Based on the similarity between the central domains of movement proteins and lentiviral proteinase, a prediction of the relative three-dimensional orientation of sequence domains of the movement proteins was made. In the structural model, the movement proteins are dimers, like the lentiviral proteinases. The core of the structure consists of a dimer of proteinase-similar domain (the central domain). The N-terminal domain of one protein interacts with the C-terminal domain of another protein in the dimer. The sequence alignment of CaMV P38 and TMV P30 and the junctions of exchanges are shown in Table 1. The junctions of the exchanges were designed to keep the three putative domains intact. A CaMV plasmid with an additional TMV P30 gene and a TMV cDNA clone with an additional CaMV P38 gene were also constructed. The plasmids were tested on turnip, tobacco and Arabidopsis plants.

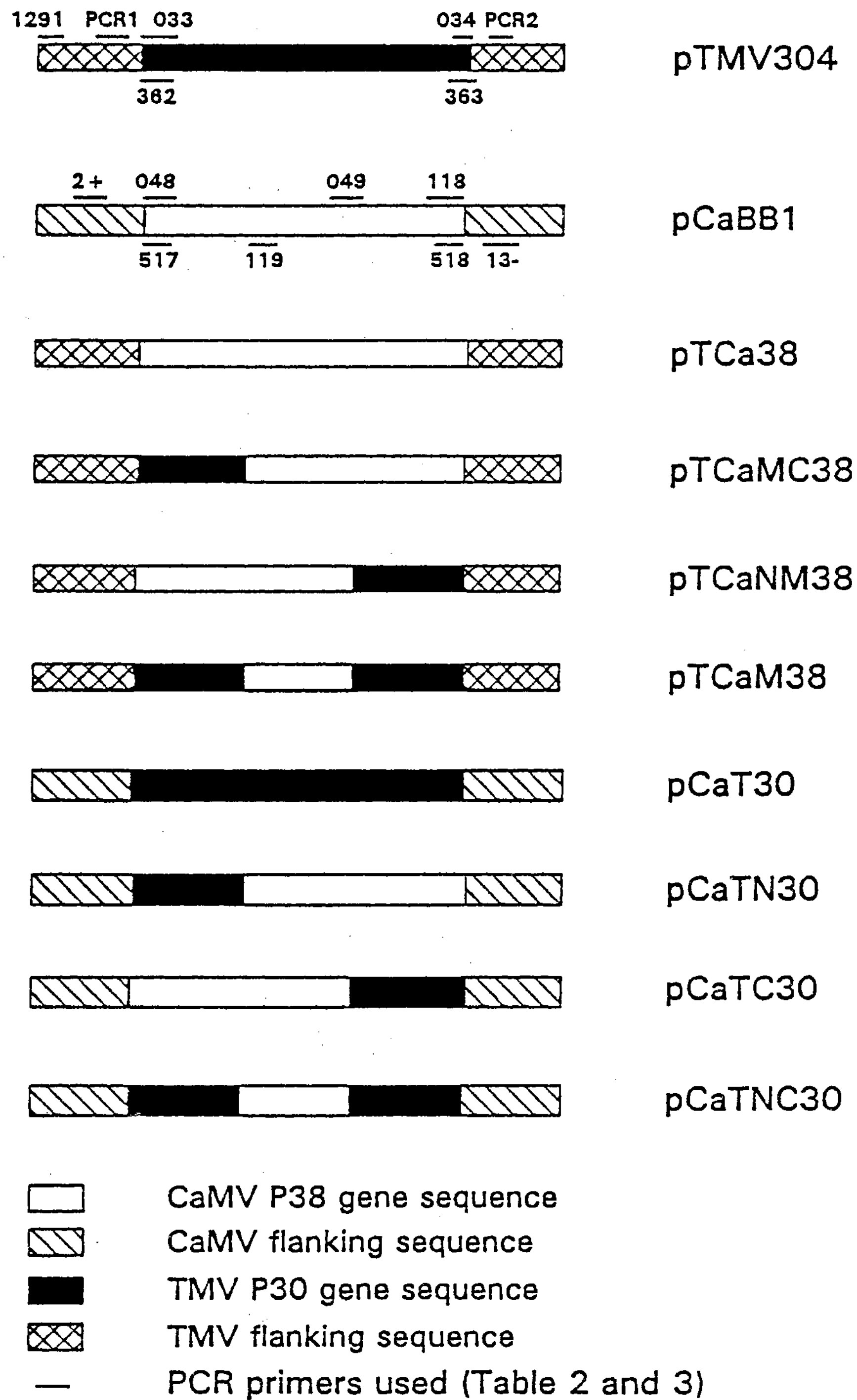

Figure 1. CaMV and TMV Movement Protein Chimeras constructed for use in the study.

TABLE 1

CaMV P38 AND TMV P30 ALIGNMENT

Protein	Amino Acid Sequences
CaMV P38	MDLYPEENTQSEQSQNSENNMQIFKSENSDGFSSDLMISNDQLKN
TMV P30	??MAL
	15I 30I 45I
CaMV P38	ISKTQLTLEKEKIFKMPNVLSQVMKKAFSRKNEILYCVSTKELSV
TMV P30	VVKGKVNINE--FIDLTK-MEKILPSMFTPVKSV------MCSKV
	60I 75I 90I
CaMV P38	DIHDATGKVYLPLITKEEINKRLSSL↓KPEVRKTMSMVHLGAVKIL
TMV P30	DKIMVHENESLSEV---------NLL↓KGVKLIDSGYVCLAGLVVT
	105I 120I 135I
CaMV P38	LKAQFRNGIDTPIKIALIDDRINSRRDCLLGAAKGNLAYGKFMFT
TMV P30	GEWNLPDNCRGGVSVCLVDKRMERADEATLGSYYTAAAKKRFQFK
	150I 165I 180I
CaMV P38	VYPKFGISLNTQRLNQTLSLIHDFENKNNMNKGDKVMTITYVVGY
TMV P30	VVPNYAITTQDAMKNVWQVLVN--IRNVKMSAGFCPLSLEFVSVC
	195I 210I 225I
CaMV P38	ALTNSHHSIDYQSN↓--------ATIELEDVFQEIGNVQQSEFCTI
TMV P30	IVYRNNIKLGLREK↓ITNVRDGGPMELTEEVVDEFMEDVPMSIRLA
	240I 255I 270I
CaMV P38	QNDEC-NWAIDIAQNKALLGAKTKTQIGNNLQIGNSASSSNTE--
TMV P30	KFRSRTGKKSDVRKGKNSSNDRSVPNKN---------YRNVKDFG
	285I 300I 315I
CaMV P38	NELARVSQN-IDLLKNKLKEICGE?
TMV P30	GMSFKKNNLIDDD-SEATVAESDSF
	330I

Note: 1. ↓ indicates the exchange sites between CaMV and TMV MPs and corresponds to the junctions of the black and white bars in Figure 1.
2. The numbers indicate the alignment positions. "I" indicates the position of the amino acid with the number. Among the 122 amino acids in the internal region, 14 amino acids are identical.

(This table is modified from Melcher, 1990)

2. Materials and Methods

Construction of Plasmids

Construction of pCaT30

Generating TMV 30K Gene Fragment Two PCR primers (primer 033 and 034, Table 2) were designed from the known pCaBB1 (Brisson *et al.*, 1984) and TMV vulgare sequences (Goelet *et al.*, 1982). DNA was amplified from pTMV304 (Dawson, W. O., personal communication) in a 100 μl reaction mix containing 10 μl 10x PCR Mg^{2+} free buffer, 6 μl 25 mM Mg^{2+} buffer, 16 μl 1.25 mM dNTP mix, 500 ng each oligonucleotide primer and 2.5 units Taq polymerase (buffers and enzyme were from Promega) (Mullis *et al.*, 1987). After overlaying with 100 μl paraffin oil, PCR program 15 (25 rounds of cycling at 94°C for 1 min, 30°C for 2 min, 72°C for 3 min) was performed. The DNA fragments were electrophoresed through a 1% agarose gel. To recover fragments from the gel, the gel slice was cut from the gel, placed in a sterile eppendorf and broken into smaller pieces with a pipetman tip. After adding an equal volume of phenol and vortexing, the tube was placed at -70°C for 15 min, then thawed and microfuged. The aqueous phase was removed to a new tube and 2.5 volumes 95% ethanol were added. The tube was placed at -20°C for 2 hours. DNA was recovered by centrifuging, 70% ethanol washing, and drying. The amount of DNA was determined using a DNA fluorometer.

TABLE 2

PCR OLIGONUCLEOTIDE PRIMERS

Primer	Sequence
033	ccg¦**CTCGAG**¦gttaaaacgaatccgattcggc¦ *Xho*I TMV P30 seq.
034	gcgcg¦**GGTgACC**¦tgagatggctctagttgtt¦ *Bst*EII TMV P30 seq.
048	¦**ATGGCTCTAGTTGTTAAA**¦gaaaatacccaaagcg¦ **TMV P30 seq.** CaMV P38 seq.
049	¦**TTCTTCTGTAAGTTCCATGGG**¦attcgattgataatctatgc¦ **TMV P30 seq.** CaMV P38 seq.
118	gcgcgc¦**CTTAAG**¦cata¦ttattctccacagat¦ *Afl*II CaMV seq.
119	gccc¦**AAGCTT**¦aaacctgaagaagtcagaaa¦ *Hin*dIII CaMV seq.
362	cgccg¦**CTCGAG**¦atggctctagttgttaaag¦ *Xho*I TMV P30 seq.
363	cacgc¦**GTCGAC**¦tccgattcggcgacagtagcct¦ *SalI* TMV P30 seq.
517	acgc¦**GTCGAC**¦atggatttgtatccag¦ *Sal*I CaMV seq.
518	acgc¦**GTCGAC**¦ttattctccacagatctc¦ *Sal*I CaMV P38 seq.

Note: Primer locations are shown in Figure 1.

Restriction Digestion 2 μg pCaBB1 (Brisson, *et al.*, 1984) DNA were digested with *Xho*I and *Bst*EII, and subjected to gel electrophoresis. The large band (about 11 kb) was purified as described above. 140 ng purified PCR fragment was first digested with 6 units *Xho*I for 5 hours at 37°C, then with another 6 units *Xho*I for 20 hours at 20°C. The DNA was precipitated by adding 1/10 volume of 3 M sodium acetate and 2.5 volumes of 95% ethanol and storing at -20°C for 2 hours. The DNA was pelleted by centrifugation, washed with cold 70% ethanol, dried in a vacuum dryer and dissolved in water. All the DNA was subjected to 6 units *Bst*EII digestion overnight at 37°C, 7 hours at 20°C, and 1 hour at 60°C. The DNA was purified by phenol extraction and dissolved in 28 μl water.

Ligation, Transformation, and Screening 28 μl pCaBB1 (3 ng/μl) vector, 18 μl digested PCR fragment, 12 μl 5x ligation buffer (BRL) and 2 μl water containing 0.2 unit T4 DNA ligase were mixed together. The mixture was incubated at 15°C overnight and then diluted to 240 μl by adding 144 μl water and 36 μl 5x ligation buffer. The mixture was further incubated at 15°C for 6 hours. After heat treatment at 70°C for 5 min, the DNA was precipitated by adding 24 μl 3 M sodium acetate and 750 μl 95% ethanol and storing at -20°C for 2 hours. The DNA was pelleted, dried and dissolved in 10 μl water. 20 μl DH5α competent cells (BRL) were transformed with 1 μl of DNA as recommended by BRL. Plasmid DNA was isolated from cultures of the resulting colonies by the alkaline SDS lysis as described (Sambrook *et al.*, 1989) and digested with *Xho*I and *Bst*EII to test the size of insert. The plasmid

with the right insert was called pCaT30.

Construction of pTMVCaNM38

pTMVCaNM38 (Figure 2) was made by the methods described by Clackson *et al.* (1989). PCR primers 048 and 049 (Table 2) were designed from TMV vulgare and pCaBB1 sequences respectively. Primer 048 was first phosphorylated in a 30 μl reaction mix containing T4 polynucleotide kinase buffer, 200 ng oligonucleotide primer, 10 mM ATP, 4 units of T4 polynucleotide kinase at 37°C for 30 minutes. DNA was then amplified from pCaBB1 plasmid DNA in a 100 μl reaction mix containing 10 μl PCR Mg^{2+} free buffer, 6 μl Mg^{2+} buffer, 2.5 units Taq polymerase (Promega), 100 ng phosphorylated primer 048, 100 ng primer 049, 16 μl 1.25 mM dNTP mix and 10 ng template. After overlaying with paraffin oil, PCR program 15 was performed as above. The DNA fragments were electrophoresed through a 1% agarose gel, purified by freeze-phenol extraction method as described above and taken up in 10μl water. Single-stranded pTMV DNA template (500 ng) and the PCR product (5 μl) were annealed in a final volume of 10 μl containing PCR Mg^{2+}-free buffer, Mg^{2+} buffer, 500 μM dNTPs and 2.5 units Taq polymerase, overlaid with paraffin oil. The annealing reaction was incubated at 92°C for 3 min, 67°C for 2 min and 37°C for 2 min in a DNA thermal cycler with 1 min intervals between each incubation. 10μl of buffer and enzyme mixture [10 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 500 μM ATP, 10 mM DTT, 2 units T4 DNA ligase (BRL) and 3.25 units

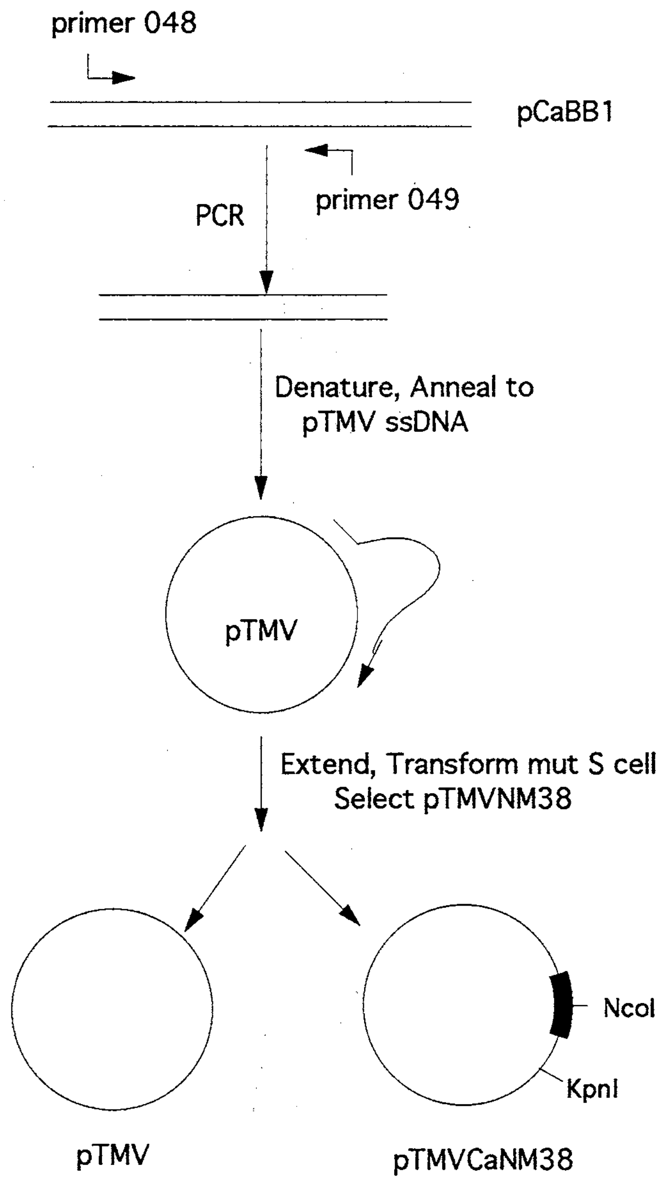

Figure 2. Construction of pTMVCaNM38

Sequenase version 2.0 (USB)] was then added to the annealing reaction and the incubation continued at 37°C for 1 hour. The synthesized DNA was then precipitated by ethanol, and dissolved in 16 μl TEN buffer (10 mM Tris-HCl, 1 mM EDTA and 10 mM NaCl, pH 8.0). Aliquots of 2 μl were used to transform BMH 71-18 mut S competent cells. One-tenth of the transformation reaction was plated to check the number of transformants. 4 ml LB medium (Sambrook *et al.*, 1989) containing 75 μg/ml ampicillin was then added to the remaining reaction and the reaction was shaken at 37°C at a moderate speed. Plasmid DNA was isolated from the overnight bacterial culture. 200 ng of the DNA was digested in a 30 μl reaction containing Promega restriction enzyme digestion buffer B and 6 units *Hin*dIII at 37°C for 3 hours. 0.5 μl of the reaction was then used to transform DH5*α* competent cells. Plasmid DNA was isolated from cultures of the colonies and digested by *Hin*dIII to check the identity of the plasmids. Plasmids which had no *Hin*dIII site were further checked by *Xba*I digestion and one of those plasmids was partially sequenced by dideoxy sequencing with TMV PCR1 primer and PCR2 primer (Table 3) which are complementary to sequences just outside TMV P30 gene. The resulting mutant plasmid was called pTMVCaNM38.

Construction of pTMVCaMC38NA

Primers 118 and 119 (Table 2) were designed from pCS101 (equivalent to pCa37, Lebeurier *et al.*, 1980) and pTMV3'NA sequences (Lapidot, M., personal

TABLE 3

PRIMERS USED IN THE STUDY

Primer	Sequence
TMV PCR1	GCCCCTCCAGGTTCGTTTGTTATAAA
TMV PCR2	GAACACGAACTGAGATGGAGTAGTGA
1291	GGAGTACCCGGCTTTGCAGA
CaMV 2+	AAGCCTTCCTCAGGAAGTACCTT
CaMV 13-	TAAACATGCGGTTGTCCCGTAATGCT
038	GCTCTCCTTGACCGAGGGAA
915	GGCTCTAGATTTAGGTGACACTATAGTTTAGTTTTATTGCAAC
037	AGAATCTCGACTCTTTTGAC
1048	GCCGCTCGAGTTATCAAACAAAGAACAAAT

Note: Locations of TMV PCR1, TMV PCR2, 1291, CaMV 2+ and CaMV 13- are shown in Figure 1. Locations of TVCV primers 038, 915, 037 and 1048 are shown in Figure 4 (Chapter III).

communication). DNA was amplified from pCS101 DNA in a 100 μl reaction mix containing 10 μl PCR Mg^{2+} free buffer, 6 μl Mg^{2+} buffer, 2.5 units Taq polymerase, 16 μl 1.25 mM dNTP mix, 500 ng primer 118, 500 ng primer 119 and 10 ng template with PCR program 15 as described above. After gel electrophoresis, the DNA was purified by the freeze-phenol extraction method as described above and digested by *Hin*dIII and *Afl*II. pTMVCaMC38NA was obtained by ligating the digested PCR fragments with the vector part of pTMV3'NA digested with *Hin*dIII and *Afl*II. Identity of plasmids resulting from transformation of DH5α cells was checked by digestion with *Xba*I.

Construction of pTMVCaM38

The CaMV P38 one-third center fragment was amplified from pCaBB1 DNA in a 100 μl reaction mix containing 10 μl PCR Mg^{2+} free buffer, 6 μl Mg^{2+} buffer, 2.5 units Taq polymerase, 16 μl 1.25 mM dNTPs mix, 500 ng primer 119, 500 ng primer 049 and 10 ng template with PCR program 15 as described above. The DNA was purified as described above, completely digested by *Hin*dIII and then partially digested by *Sty*I in a 30 μl reaction containing 1 unit *Sty*I (Promega) at 37°C for 15 min. The digested DNA was purified and ligated to the vector part of pTMV (Lapidot, M., personal communication) digested by *Hin*dIII and *Sty*I to obtain pTMVCaM38. Identity of plasmids resulting from transformation of DH5α cells was checked by digestion with *Xba*I and *Hin*dIII.

Construction of pTCaNM38, pTCaM38, pTCaMC38 and pTCa38

pTMVCaNM38, pTMVCaM38 and pTMVCaMC38NA were digested by *Bam*HI and *Kpn*I. The fragments with viral sequence were purified and ligated to the vector containing fragment of pTMV304 digested with *Bam*HI and *Kpn*I. The resulting plasmids were named pTCaNM38, pTCaM38 and pTCaMC38, respectively. Identity of plasmids resulting from transformation of DH5α cells was checked by digestion with *Hin*dIII and *Bgl*II. For construction of pTCa38, both pTMVCaMC38 and pTCaNM38 were digested by *Nco*I and *Kpn*I. The large fragment (about 8.4 kb) of pTCaNM38 was ligated to the small fragment (about 1 kb) of pTMVCaMC38 to obtain pTCa38. To purify the DNA fragments, the DNAs were electrophoresed through a 1% agarose gel and the gel was stained in 0.5 μg/ml ethidium bromide solution. The DNA band was excised, placed in a sterile eppendorf and incubated in 70°C for 15 min. The tube was then put in -70°C freezer for 5 min, thawed and spun in a microcentrifuge for 5 min. The supernatant was transferred to another eppendorf and was used directly for ligation. Identity of plasmids resulting from the transformation of DH5α cells was checked with *Bgl*II digestion.

Construction of pCaTNC30, pCaTN30 and pCaTC30

pCaTNC30 was constructed the same way as pCaT30 except that the template for the PCR reaction was pTCaM38. Identity of pCaTNC30 was checked by digestion

with *Eco*RI and *Xba*I. For construction of pCaTN30 and pCaTC30, both pCaBB1 and pCaTNC30 were digested by *Afl*II and *Mlu*I. The small fragment (about 2.6 kb) of pCaBB1 was ligated to the large fragment (about 7 kb) of pCaTNC30 to obtain pCaTC30. The small fragment (about 2.2 kb) of pCaTNC30 was ligated to the large fragment (about 7 kb) of pCaBB1 to obtain pCaTN30. Identity of plasmids resulting from the transformation of DH5α cells was checked with *Sty*I digestion.

Construction of pCaBB1:P30

The TMV P30 gene was amplified from pTMV304 by PCR using primer 362 and primer 363 (Table 2). The PCR product was digested with *Sal*I and *Xho*I and was cloned into *Xho*I-digested pCaBB1. The direction of the insert was checked by digesting the plasmids resulting from transformation with *Sal*I and *Xho*I together. The plasmid with the P30 gene inserted in the same direction as the CaMV genes was called pCaBB1:P30.

Construction of pTB2:ORF1-Sp6 and pTVCV:ORF1

PCR primers 517 and 518 (Table 2) were designed from the pCaBB1 sequence and used to amplify the ORF 1 fragment from a pCaBB1 template by PCR. The PCR DNA fragment was digested with *Sal*I and ligated to *Sal*I-digested pBluescript SK(+) to obtain pB:ORF1 (Figure 3). pB:ORF1 was then digested with *Sal*I. The 1.0 kb

ORF1 *Sal*I was purified by gel electrophoresis and ligated to *Xho*I-digested pTB2 (Donson *et al.*, 1991). To identify the plasmid with the insertion among plasmids resulting from transformation, the transformants were cultured in 4 ml LB medium with ampicillin after transformation. Plasmid DNA was isolated from the overnight culture and digested with *Xho*I. The digested DNA was used to transform DH5α competent cells. Plasmids from the resulting transformants were screened for size and for the pattern of *Eco*RI digestion products. The plasmid with the CaMV ORF 1 inserted at the *Xho*I site of pTB2 in the right direction was called pTB2:ORF1. For construction of pTB2:ORF1-Sp6, both pTMV304 and pTB2:ORF1 were digested with *Bam*HI and *Kpn*I. The 6.3 kb pTMV304 fragment was ligated to the 4.1 kb pTB2:ORF1 fragment to obtain pTB2:ORF1-Sp6. Identity of plasmids resulting from transformation of DH5α cells was checked by digestion with *Hin*dIII.

Both pB:ORF1 and pTZ171 (described in Chapter 3) were first digested with *Xho*I and *Hin*dIII. The 1.0 kb pB:ORF1 fragment was ligated to the 5.4 kb pTZ171 fragment to obtain pTZ185 after transformation (Figure 3). Both pTZ185 and pTVCV50 were then digested with *Pst*I and *Kpn*I. The 3.5 kb pTZ185 fragment was ligated to the 6.6 kb pTVCV50 fragment to obtain pTVCV:ORF1 (Figure 3). Identity of plasmids resulting from transformation of DH5α cells was checked by digestion with *Hin*dIII and *Xho*I.

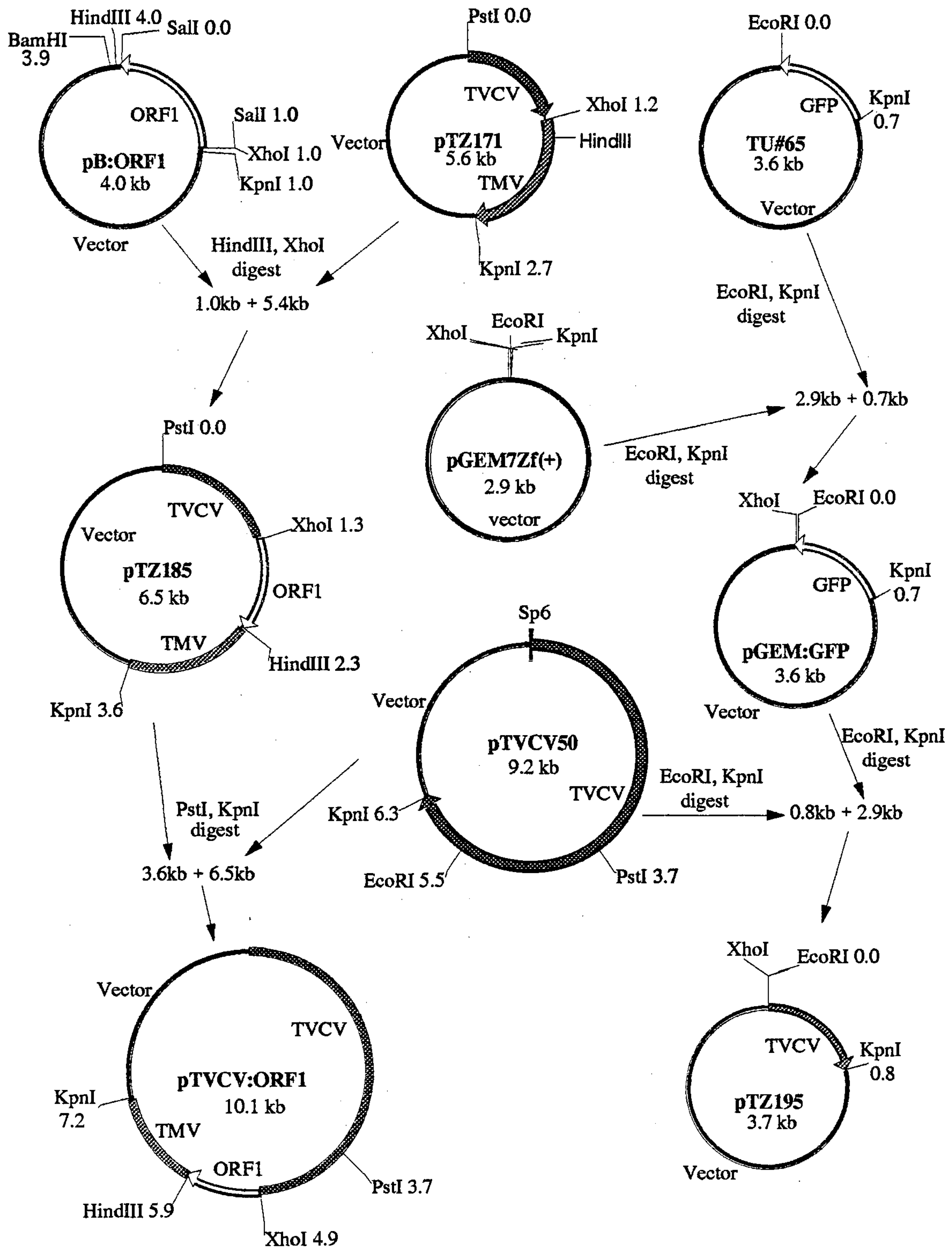

Figure 3. Construction of pTVCV:ORF1 and pTZ195

Plants and Inoculation

N. tabacum cv. Xanthi-nc, *N. tabacum* cv. Samsun, 30K TMV movement protein transgenic Xanthi-NN (plant line 2005, Deom *et al.*, 1990) and Xanthi-nn (plant line 277, Deom *et al.*, 1990) were used. They will be referred to as Xanthi-nc, Samsun-nn, MP2005 plants and MP277 plants, respectively. *Arabidopsis thaliana* ecotype Columbia and *Brassica rapa* cv. Just Right were also used. They will be referred to as Arabidopsis and turnip, respectively.

All CaMV plasmids used in the study have CaMV viral DNA inserted in the SalI site of a plasmid vector. CaMV inocula were prepared by excising viral sequence DNA from the plasmid vector by *Sal*I. The inocula contained 20 μg/ml digested DNA in 20x SSC and 3 mg/ml Celite. All TMV cDNAs were inserted in a plasmid vector. In vitro transcripts were made from *Kpn*I-linearized plasmids by Ambion (Austin, Texas) mMessage mMachine™ in vitro transcription kit (catalog #1340). In vitro transcripts derived from viral cDNAs were diluted 1 to 4 in RNAse-free water before inoculation on plants. Homogenates from infected leaves were also used as sources of inoculum. To grind tobacco leaf tissue, about 0.5 g of virus infected leaf tissue and 2.5 ml sterile water were added to a disposable 17x100 mm polypropylene round-bottom tube (FALCON 2059 from Becton Dickinson Labware, Lincoln Park, New Jersey) and ground with a 2 ml sterile disposable plastic pipet.

For each experiment, at least two tobacco plants or two turnip plants were inoculated with each inoculum. For each tobacco plant, the third to the sixth leaves

were inoculated when the plant was at the seven-leaf stage. For each turnip plant, the third to the fifth leaves were inoculated when the plant was at the six-leaf stage. For each Arabidopsis plant, the third to the sixth leaves were inoculated when the plant was four weeks old. For each chimera, plasmids from four independent colonies were tested. The inoculation was done by gently rubbing a 20 μl (5 μl for Arabidopsis) drop of inoculum over the leaf surface with a gloved finger. Plants inoculated with tobamovirus inoculum were dusted with carborundum before inoculation. The inoculated plants were kept in a growth chamber (12-hr light, 23°C/12-hr dark, 21°C dark) and assayed for symptoms over a two-week period for tobacco plants and a four-week period for Arabidopsis and turnip plants.

Assay for CaMV DNA

A "virion DNA" fraction was isolated from systemic or upper uninoculated tobacco leaves by the method of Gardner and Shepherd (1980) and subjected to gel electrophoresis. Total DNA was isolated from inoculated or systemic tobacco leaves by the method of Rogers and Bendich (1988).

DNA prepared from upper uninoculated leaves by both methods was used as template to attempt to amplify TMV 30K gene fragments by primer 033 and primer 034. After adding PCR buffer, dNTP mixture and Taq polymerase (Promega), the reactions were overlaid with mineral oil and subjected to 40 cycles of 1 min at 94°C, 2 min at 60°C and 3 min at 72°C. DNA from plants inoculated with buffer was used

as negative control. Plasmid DNA was used as positive control.

*Eco*RI digested and undigested "virion fraction" DNA were Southern blotted and hybridized with a ^{32}P-dCTP labeled probe. The probe was prepared by nick translation of pCaT30 DNA (Melcher *et al.*, 1981). *Eco*RI digested and undigested pCaT30 DNA were used as positive controls. Prehybridization for 2 to 4 hours at 42°C and hybridization for 18 hours at 42°C were in 50% formamide, 5x SSPE (1x SSPE is 0.18 M-NaCl, 0.01 M-sodium phosphate, 1 mM-EDTA, pH 7.4), 5x Denhardt's solution (Denhardt, 1966), 0.5% SDS, 0.2 mg/ml sheared, denatured salmon sperm DNA. After hybridization, the membrane was washed 4 times with 2x SSC (1x SSC is 0.15M-NaCl, 0.015 M-sodium citrate), 0.1% SDS at 25°C for 15 min, followed by a final wash under the same conditions for 30 min. The washed membrane was autoradiographed. For nonradioactive hybridization, a biotin-labeled probe was prepared from pCaBB1 DNA by random priming using BRL (Gaitherburg, MD) BIOPRIMER DNA Labeling System (Cat. No 18094-011). The PhotoGene™ Nucleic Acid Detection System (BRL, Cat. No. 18192-057) was used for the detection of hybridized nucleic acids.

3. Results

Inoculation of CaMV Chimeras on Turnip, Tobacco and Arabidopsis Plants

As shown in Table 4, a total of 14 turnip plants were inoculated with pCaT30 in

three different tests. A total of 8 turnip plants were inoculated with each of the other three CaMV chimeras. None of the turnip plants inoculated showed any symptoms when compared with buffer-inoculated control plants. A "virion DNA" fraction was extracted from both inoculated and upper uninoculated leaves of turnip plants in test 3 two weeks post-inoculation. Southern blotting and hybridization using a pCaBB1 biotin-labeled probe was able to detect pCaT30 DNA control, but failed to detect any CaMV DNA in the "virion DNA" fraction.

As shown in Table 5, a total of 22 Samsun plants in 4 tests were inoculated with pCaT30. In test 1, systemic chlorotic spots were found on 4 out of 7 tobacco plants 7 days post-inoculation with pCaT30. None of the three buffer-inoculated plants had similar symptoms. A "virion DNA" fraction and plant total DNA were extracted from the symptomatic tobacco leaves. Analysis of the "virion" DNA fraction by ethidium bromide staining of an agarose gel after electrophoretic separation failed to reveal a DNA band. Southern blotting and hybridization also failed to detect CaMV DNA at a sensitivity level of about 10 pg. PCR amplification (using CaMV primers 2+ and 13-, Table 3) from the total DNA of symptomatic leaves gave no bands of the same size as DNA amplified from the plasmid DNA. Inocula made from homogenates of the symptomatic leaves were back-inoculated on Samsun plants. None of the inoculated plants developed any symptoms. In tests 2, 3 and 4, none of the tobacco plants inoculated with pCaT30 developed any symptoms. None of the tobacco plants inoculated with pCaTN30, pCaTC30 and pCaTNC30 developed any symptoms. A "virion DNA" fraction was extracted from the inoculated turnip leaves

TABLE 4

RESULTS OF TURNIP PLANTS INOCULATED WITH CaMV MP CHIMERAS

Plasmids	Test 1	Test 2	Test 3
pCaT30	0/6	0/4	0/4
pCaTN30	N.D.	0/4	0/4
pCaTC30	N.D.	0/4	0/4
pCaTNC30	N.D.	0/4	0/4

Note: The number A/B means that A number of plants showed symptoms in a total of B number plants inoculated. "N.D." means not determined. Plants inoculated with pCaBB1 inoculum (positive control) always showed disease symptoms.

TABLE 5

RESULTS OF SAMSUN PLANTS INOCULATED WITH CaMV MP CHIMERAS

Plasmids	Test 1	Test 2	Test 3	Test 4
pCaT30	0/7	0/7	0/4	0/4
pCaTN30	N.D.	N.D.	0/4	0/4
pCaTC30	N.D.	N.D.	0/4	0/4
pCaTNC30	N.D.	N.D.	0/4	0/4

Note: The number A/B means that A number of plants showed symptoms in a total of B number of plants inoculated. "N.D." means not determined.

in test 4. Southern blotting and hybridization with a nonradioactive pCaBB1 probe failed to detect any CaMV DNA at a sensitivity level of about 10 pg.

At least 30 Arabidopsis plants were inoculated with pCaBB1 inoculum in three experiments. No plant inoculated developed any symptoms, while plants inoculated with CaBB1 virions developed systemic chlorosis symptom. Thus I could not infect Arabidopsis plants with CaMV plasmid DNA because the efficiency of plasmid DNA inoculum is very low compared with virion inoculum.

The results of inoculating CaMV chimeras on turnip and tobacco plants indicate that CaMV chimeras are not infectious on turnip and tobacco plants.

Inoculation of TMV Chimeras on Tobacco Plants

In vitro transcripts were made from pTCa38, pTCaNM38, pTCaM38 and pTCaMC38. The transcripts were used to inoculate Xanthi-nc plants and MP2005 plants. As shown in Table 6, a total of 6 Xanthi-nc plants were inoculated with in vitro transcripts from two different transcription reactions for each chimera. None of the plants developed any local lesions on the inoculated leaves. The same in vitro transcripts used in test 2 were used in test 3 to inoculate MP2005 plants. All the inoculated leaves developed local lesions. There were more than 100 lesions on each leaf. The lesions on leaves inoculated with transcripts from pTCaNM38, pTCaM38 and pTCaMC38 developed two days post-inoculation and the sizes of those lesions were the same as those on control plants inoculated with TMV (about 5 mm in

TABLE 6

RESULTS OF TOBACCO PLANTS INOCULATED WITH TMV CHIMERAS

Plasmids	Test 1: Xanthi-nc	Test 2: Xanthi-nc	Test 3: MP2005
pTCaNM38	0/4	0/2	2/2
pTCaM38	0/4	0/2	2/2
pTCaMC38	0/4	0/2	2/2
pTCa38	0/4	0/2	2/2

Plasmids	Test 4: Xanthi-nc	Test 5: MP2005	Test 6: Xanthi-nc
pTCaNM38	0/2	2/2	0/2
pTCaM38	0/2	2/2	0/2
pTCaMC38	4/2+2	2/2	N.D.
pTCa38	4/2+2	2/2	N.D.

Note:

1. The number A/B means that A number of plants showed symptoms in a total of B number plants inoculated. "2+2" means that two experiments were done and two plants were inoculated in each experiment.

2. Inocula in test 1, test 2 and test 3 were in vitro transcripts. Inocula in test 4 and test 5 were homogenates of infected MP2005 leaves in test 3. Inoculum in test 6 was homogenate of infected MP2005 leaves in test 5.

3. Xanthi-nc plants inoculated with TMV as positive control always developed local lesion on the inoculated leaves.

diameter 7 days post-inoculation). Lesions developed on leaves inoculated with pTCa38 four days post-inoculation. The shape of the lesions was irregular and sizes of the lesions ranged from less than 1 mm to about 3 mm 7 days post-inoculation, smaller than lesions produced by TMV.

MP2005 plant leaves infected with the chimeras were ground and the homogenates were inoculated on Xanthi-nc (test 4) and MP2005 plants (test 5). As shown in Table 6, all inoculated MP2005 plants were infected by the chimeras. Local lesions developed on all MP2005 plants two days post-inoculation. The sizes of the lesions were the same as lesions produced by TMV. The shapes of lesions produced by inoculum derived from pTCa38 were round and similar to those of lesions produced by TMV and the other chimeras. Systemic necrosis developed on all the inoculated MP2005 plants 4-5 days post-inoculation. In test 4 of Table 6, Xanthi-nc plants inoculated with inoculum derived from pTCaNM38 and pTCaM38 did not develop any lesions on the inoculated leaves, but more than 100 lesions developed on each leaf inoculated with inoculum derived from pTCaMC38 and pTCa38 in two sets of experiments. The lesions developed two days post-inoculation and sizes of the lesion were the same as lesions produced by TMV. In test 6 of Table 6, inocula prepared from homogenates of systemically infected MP2005 plant leaf tissue in test 5 were inoculated on Xanthi-nc. None of the plants inoculated with inoculum derived from pTCaM38 and pTCaNM38 developed any local lesions.

MP277 plants were inoculated with pTCaMC38 and pTCaM38 inoculum prepared from the infected MP2005 plants in test 3. Systemic mosaic symptoms were visible 5

days after inoculation. The symptoms on plants inoculated with pTCaMC38-derived inoculum were similar to symptoms on TMV-infected plants. The mosaic symptom on plants inoculated with pTCaM38-derived inoculum was milder than the mosaic symptom on TMV-infected plants. Total RNAs were extracted from the symptomatic leaves. Reverse transcription PCRs were done using the total RNA as template and TMV PCR1 and PCR2 (Table 3) as primers. Both primers bind to regions outside the TMV P30 gene. A fragment of about 900 bp was amplified from total RNA of plants inoculated with pTCaMC38 or pTCaM38 derived inoculum. The size of the fragments is the same as expected. There is a unique *Xba*I site in the middle of CaMV P38 gene and no *Xba*I site in TMV P30 gene. The PCR fragments were precipitated, dissolved and digested with *Xba*I. Analysis of the digestion products by gel electrophoresis revealed that there was no *Xba*I site in the PCR fragment amplified from plants inoculated with pTCaMC38-derived inoculum and a single *Xba*I site in that from plants inoculated with pTCaM38-derived inoculum.

Reverse transcription PCRs were also done using the total RNA as template and primers 1291 and TMV PCR2 (Table 3) as primers. Primer 1291 binds to a region in the TMV 183K gene. A fragment of about 1.8 kb was amplified from total RNA of plants inoculated with TMV or pTCaMC38-derived inoculum. The size of the fragment was the same as expected. A unique *Nde*I site is present at the start of the movement protein gene in pTCaMC38 but not present in wild type TMV. The PCR fragments were precipitated, dissolved and digested with *Nde*I. Analysis of the digestion products by gel electrophoresis revealed that there was no *Nde*I site in the

PCR fragment amplified from plants inoculated with TMV or pTCaMC38-derived inoculum.

To repeat the tests 2, 3 and 4 (Table 6) of pTCa38 and pTCaMC38, the following experiments were done. Transcripts were made from the plasmids and inoculated on MP2005 plants. After 5 days, inocula were made from the infected plant leaves and used to inoculate Xanthi-nc plants and MP2005 plants. After local lesions developed on the MP2005 plants, inocula were then made from the infected MP2005 plants and used to inoculate Xanthi-nc plants again.

In the first experiment, no Xanthi-nc plants developed any local lesions in the two rounds of inoculation. Local lesions that developed on MP2005 plants inoculated with pTCaMC38 transcripts always were of the same size as lesions produced by TMV. Local lesions that developed on MP2005 plants inoculated with pTCa38 transcripts were always smaller and had irregular shapes. But when the plants were inoculated with pTCa38 inoculum derived from MP2005 plants, the size and shape of lesions were the same as those of lesions produced by TMV.

In a repeat of the experiment, one local lesion developed on one Xanthi-nc plant leaf inoculated with pTCaMC38 inoculum from transcript-inoculated MP2005 plants. No local lesions developed on Xanthi-nc plants inoculated with pTCa38-derived inoculum.

Arabidopsis plants were inoculated with pTCaNM38 and pTCaM38 inoculum prepared from the infected MP2005 plants in test 3. TMV was used as positive control. After four weeks, the inoculated leaves and upper uninoculated leaves were

collected and homogenized. The homogenates were used to inoculate MP2005 plants. No lesions developed on MP2005 plants inoculated with homogenates from Arabidopsis plants previously inoculated with the two TMV chimeras. Local lesions developed on MP2005 plants inoculated with homogenates from Arabidopsis previously inoculated with TMV.

The results of inoculating TMV chimeras on plants indicate that TMV chimeras can not move from cell to cell on Xanthi-nc plants, but they can move from cell to cell on MP2005 plants. Viruses able to infect Xanthi-nc were recovered from MP2005 plants previously inoculated with transcripts of two chimeras.

Inoculation of pCaBB1:P30 on Turnip Plants

A total of 12 turnip plants in two tests were inoculated with *Sal*I-digested pCaBB1:P30 DNA. While systemic chlorotic spots developed on turnip plants inoculated with pCaBB1 DNA 20 days after inoculation, symptoms were first visible on plants inoculated with pCaBB1:P30 30 days after inoculation.

Virion DNA was extracted from the symptomatic leaves of turnip plants inoculated with pCaBB1:P30. The viral DNA and pCaBB1:P30 plasmid DNA were digested with *Sal*I and electrophoresed to compare the sizes. The size of the recovered viral DNA was smaller than the size of the virus sequence fragment from the plasmid. *Xho*I digestion of the viral DNA showed that there was no *Xho*I site in the viral DNA although there is an *Xho*I site in pCaBB1:P30. The absence of the

*Xho*I site suggests that the TMV P30 gene was probably deleted in the virus. To further confirm that, the *Sal*I-digested viral DNA was then cloned into the *Sal*I site of pBluescript to create plasmid pB:CaBB1:P30. To sequence the region of the TMV P30 gene insert, the 2.7 kb *Bam*HI fragment of pB:CaBB1-p30 was deleted to make pZ135. Primer SK was then used to sequence the region between CaMV ORF 1 and ORF 3. The sequence analysis (Table 7) showed that the P30 gene fragment and five nucleotides originally present between ORF 1 and ORF 3 in pCaBB1 were deleted. I can not assign the deletion end point precisely because of a two nucleotides (GA) ambiguity.

The results of testing pCaBB1:P30 on turnip plants indicate that CaBB1:P30 was not stable on turnip plants and the P30 gene insert was deleted.

Inoculation of pTB2:ORF1-Sp6 on Tobacco Plants

In vitro transcripts were made from pTB2:ORF1-Sp6 and inoculated on Xanthi-nc plants and MP2005 plants. Local lesions developed on both MP2005 plants and Xanthi-nc plants. The sizes of lesions developed on MP2005 plants were the same as those of lesions produced by TMV on control plants. The sizes of lesions developed on Xanthi-nc plants were about 1 mm in diameter seven days post-inoculation, smaller than the lesions produced by TMV at the same time stage (5 mm in diameter).

Inoculum was made from the infected MP2005 plants and inoculated on Xanthi-nc plants again. Local lesions developed on the inoculated leaves. The sizes of the

TABLE 7

SEQUENCES OF CaBB1:P30 AND VIRUSES RECOVERED FROM TURNIP PLANTS INFECTED WITH CaBB1:P30

Plasmid	Sequences
pCaBB1:P30	---AGAATAACCTCGAG(...)GTGAAATG---
pZ135	---AGAATAACCTCGA..........AATG---

Note: 1. (...) indicates the P30 gene insert sequence.

2. pZ135 is the plasmid used for sequencing the P30 gene insert region of the recovered virus.

3. The sequencing was done by the Core Facility at Oklahoma State University.

lesions were about 1 mm in diameter 7 days post-inoculation, as were those produced by inoculation with transcripts.

The results of testing pTB2:ORF1-Sp6 on tobacco plants indicate that TB2:ORF1 moves slower on Xanthi-nc plants, but moves at about the same rate as TMV on MP2005 plants.

Inoculation of TVCV:ORF1 on Tobacco and Turnip Plants

In vitro transcripts were made from pTVCV:ORF1 and inoculated on Xanthi-nc plants and MP2005 plants. Local lesions developed on MP2005 plants but no local lesions developed on Xanthi-nc plants. The sizes of lesions produced on MP2005 plants were about 3 mm in diameter seven days post-inoculation, smaller than the lesions with size of 5 mm in diameter produced by TMV at the same time stage. At 32°C, TVCV:ORF1 did not move systemically on MP2005 plants.

Inoculum was made from the infected MP2005 plants and inoculated on Xanthi-nc and turnip plants. No local lesions developed on the inoculated Xanthi-nc leaves. Inocula were made from the inoculated turnip leaves 12 days post-inoculation and inoculated on Xanthi-nc plants. No local lesions developed on the inoculated Xanthi-nc leaves. The results of inoculating TVCV:ORF1 on turnip and tobacco plants indicate that TVCV:ORF1 can not infect the Xanthi-nc and turnip plants.

4. Discussion

Functions of CaMV and TMV Movement Protein are not Exchangeable

Although CaMV P38 and TMV P30 have significant sequence similarity (Melcher, 1990) and a similar function of facilitating viral cell-to-cell movement, the results obtained indicate that they are not exchangeable. In pCaT30, the CaMV P38 gene was replaced with the TMV P30 gene. As CaMV DNA was not detected from the inoculated and upper uninoculated turnip leaves by Southern blotting and hybridization, the CaMV construct is probably not infectious on turnip plants. I can not rule out the possibility of an extremely low level of infection below the detection limit by hybridization. Considering that TMV can move at a slow rate on turnip plants (Chapter 3), the inability of pCaT30 to infect turnip suggests that TMV P30 is unable to facilitate CaMV cell-to-cell movement or replacement of CaMV ORF1 with TMV P30 gene sequence destroys CaMV replication.

In pTCa38 and pTVCV:ORF1, the tobamovirus P30 genes were replaced with the CaMV P38 gene. As both viruses were able to produce local lesions on MP2005 plants and unable to produce local lesions on Xanthi-nc plants, both viruses were able to replicate in tobacco cells, but not able to move from cell-to-cell without assistance from the transgene. The fact that no viruses were recovered from turnip plants inoculated with TVCV:ORF1 demonstrated that TVCV:ORF1 had no cell-to-cell movement on turnip plants, considering that TVCV:ORF1 has a TVCV replicase and

should be able to replicate on turnip. The inability of TCa38 and TVCV:ORF1 to move from cell to cell suggests that CaMV P38 is unable to facilitate TMV or TVCV cell-to-cell movement. However, a recombinant virus consisting of a movement-defective mutant of TMV and putative peanut chlorotic streak caulimovirus (PClSV) movement protein gene was capable of cell-to-cell movement (Richins *et al.*, 1993). My results are not consistent with the PClSV results, perhaps due to the difference between movement proteins of CaMV and PClSV. It is interesting to see whether functions of CaMV and PClSV movement proteins are exchangeable.

One possible explanation for the inability to exchange functions of CaMV and TMV movement protein is that CaMV and TMV use different mechanisms to move from cell-to-cell. The TMV virion is not essential for the virus to move from cell-to-cell as a TMV coat protein minus mutant moves from cell-to-cell at the same rate as wild type TMV (Dawson *et al.*, 1988). TMV may move from cell-to-cell as free RNA or some kind of RNA-protein complex which does not involve coat protein. During CaMV infection, fine granular tubular structures within cell wall extensions were found (Linstead *et al.*, 1988). CaMV virions were also found in the tubular structures (Maule, 1991). Thus CaMV probably moves from cell-to-cell in the virion form.

In pTCaNM38, pTCaM38, pTCaMC38, pCaTN30, pCaTNC30 and pCaTC30, the movement proteins are chimeric. As none of them were able to infect turnip or tobacco plants, the chimeric movement proteins are probably not functional. Although the similarity between TMV P30 and CaMV P38 is significant, the overall

similarity is very low and sequences in N-terminal and C-terminal regions are highly diverged (Melcher 1990). The amino acid sequences from CaMV P38 and TMV P30 in the chimeric movement proteins are probably incompatible and the chimeric proteins probably fold improperly or are unable to fold at all.

As the functions of CaMV P38 and TMV P30 are not exchangeable, my results with TMV and CaMV movement protein chimeras can not answer the questions of whether CaMV P38 is a host specificity determinant on tobacco and whether TMV P30 is a host specificity determinant on turnip. Approaches like the one described in Chapter 3 should answer these questions.

Possible Recombination between TMV Chimeras and Movement Protein Transgene

Although pTCaMC38 and pTCa38 transcripts are not able to move from cell-to-cell on Xanthi-nc plants, inocula prepared from MP2005 plants inoculated with the transcripts were able to infect Xanthi-nc plants. This indicated that viruses able to move from cell-to-cell were recovered. In pTCaMC38 and pTCa38, the promoter for synthesis of the coat protein subgenomic RNA was removed during the replacement of the TMV P30 gene with CaMV P38 gene. The viruses from the two plasmids should not express the coat protein. The fact that further passage of the inocula on MP2005 plants produced systemic necrosis indicated that the recovered viruses were able to move long distances. As coat protein is required for efficient virus long

distance movement, the recovered viruses should have regained expression of their coat proteins. A unique *Xba*I site is in the one-third middle part of CaMV P38 gene and is present in the original pTCaMC38 constructs. PCR amplification of the movement protein gene region and *Xba*I digestion of the PCR fragments indicated that there was no *Xba*I site in the fragments. A unique *Nde*I site is present at the start of the movement protein gene in pTCaMC38. PCR amplification of the movement protein gene region and *Nde*I digestion of the PCR fragments indicated that there was no *Nde*I site in the fragment. As the viruses recovered from the MP2005 plants inoculated with pTCaMC38 and pTCa38 inoculum can infect Samsun plants and produce the same symptoms as the symptoms produced by wild type TMV, the difference between the recovered viruses and TMV are probably very small. As the recovered viruses have no *Xba*I site in the movement protein gene and can move from cell-to-cell on Xanthi-nc and long distances on MP2005 plants and produced systemic mosaic symptoms on Samsun plants, the recovered viruses were not the same as the original constructs and were similar to TMV. In the side-by-side experiments I did, no viruses able to move from cell-to-cell on Xanthi-nc were recovered from MP2005 plants inoculated with inoculum from pTCaM38 and pTCaNM38.

There are at least two explanations for the recovery of viruses able to infect Xanthi-nc plants. One is that the recovered viruses are contaminants. As Xanthi-nc plants inoculated with the same inocula of pTCaMC38 and pTCa38 did not develop any local lesions, the original inocula were not contaminated. Also considering that no viruses able to infect Xanthi-nc were recovered from MP2005 plants inoculated

with pTCaNM38 and pTCaM38 inoculum in the side-by-side experiment and several other experiments, contamination is very unlikely. The other explanation is that recombination between the original viruses and the TMV P30 transgene occurred.

As the coat protein gene and its promoter was preserved in TCaNM38 and TCaM38 on replacement of the TMV P30 gene parts with CaMV P38 parts, the TMV coat protein should have been produced. The ability of TCaNM38 and TCaM38 to move systemically on MP2005 plants was consistent with coat protein expression and virion formation. In TCaMC38 and TCa38, the coat protein gene promoter was absent. Thus no virion should have formed during infection with transcripts of these plasmids. During infection by TCaM38 and TCaNM38, some viral RNA should be packaged into the virions after replication. Viral RNA of TCaMC38 and TCa38 could not be packaged into virions as no coat protein was expressed.

The differences in packaging behavior support the view that recombination rather than contamination occurred with TCa38 and TCaMC38. The packaging of TCaNM38 and TCaM38 viral RNA into virions should lower their free RNA (unpackaged RNA) concentration. Thus their free RNA concentrations must have been substantially lower than those of TCa38 and TCaMC38 on the MP2005 plants. The low free RNA concentrations could make recombination impossible.

Interference between the Functions of TMV and CaMV Movement Proteins

As TMV was restricted to those regions of local lesions (Holmes, 1931; Sela,

1981), the size of local lesions should reflect the rate of virus spread. My results showed that sizes of the lesions were the same on MP2005 plants inoculated with TMV, TCaNM38, TCaMC38 and TCaM38, but lesions were smaller and appeared later on MP2005 plants inoculated with TCa38. These results indicated that TCa38 moved much slower on MP2005 plants than TMV while pTCaNM38, TCaMC38 and TCaM38 moved at the same rate as TMV did. This suggests that P38 produced by TCa38 interfered with the function of the TMV movement protein produced from the transgene while the chimeric movement proteins did not interfere. An interference between CaMV P38 and TMV P30 was supported by the observation that TVCV:ORF1 moved slower locally on MP2005 plants compared to TMV. The interference is also supported by the observation that TVCV:ORF1 was not able to move systemically at a temperature >30°C, while TMV is able to move systemically on MP2005 plants at the same temperature.

In comparison, Lapidot *et al.* (1993) showed that a defective TMV movement protein in transgenic plants interferes with the cell-to-cell movement of TMV and two other tobamoviruses. Further studies showed that transgenic plants with the defective TMV movement protein gene are also resistant to several viruses from other virus groups (Cooper *et al.*, 1995) including peanut chlorotic streak virus, which is a caulimovirus. The defective movement protein only interfered with the long distance movement and had little or no effect on cell-to-cell movement of those viruses from other virus groups. My results indicate that a caulimovirus movement protein can interfere with the cell-to-cell movement of a tobamovirus movement protein. This

suggests that although their overall functions are not exchangeable, CaMV P38 and TMV P30 have some common functions and CaMV P38 can interfere with at least one or more of these functions.

The stronger interference by the movement protein of TCa38 than by that of TVCV:ORF1 is probably because the N-terminal 6 amino acids in the P38 from TCa38 are from TMV. These extra residues may make the protein even less functional than CaMV P38. As TB2:ORF1 moves at the same rate as TMV on MP2005 plants, P38 from TB2:ORF1 does not interfere with the TMV P30 transgene product. In TB2:ORF1, the insertion of the P38 gene places the TMV P30 gene 1 kb further from the 3' end and the position effect should lower the expression of TMV P30 significantly (Culver *et al.*, 1993). This is supported by the observation that lesions produced by TB2:ORF1 were smaller than those produced by TMV on Xanthi-nc plants. As P30 expressed from the transgene on MP2005 plants is able to complement the movement of TMV movement protein mutants to wild type levels, the expression level of P30 from the transgene should be much higher than that from TB2:ORF1. TMV P30 concentration in MP2005 plants infected with TB2:ORF1 should not be different significantly from that in uninfected MP2005 plants. Thus the P30 concentration difference on MP2005 plants during the infection of TB2:ORF1 and TCa38 does not explain the different behavior of TB2:ORF1 and TCa38. The P38 in TB2:ORF1 is expressed under a coat protein subgenomic promoter, which is later in expression than the movement protein subgenomic promoter (Ogawa & Sakai, 1984). No interference between P38 from TB2:ORF1 and P30 from the transgene

could be due to the way P38 was expressed in TB2:ORF1. This suggests that the timing for the expression of P38 could be important for the interference.

As a partially functional movement protein can interfere with TMV movement protein functions, the fact that the three chimeric movement proteins do not interfere with TMV movement protein functions suggests that the chimeric movement proteins may not fold sufficiently properly to be at least partially functional and interfere with the TMV movement protein function.

Passage of virus from the small lesions of MP2005 plants inoculated with pTCa38 transcripts produced normal lesions. The results suggest that TCa38 was not stable on MP2005 plants and probably accumulated mutations in the P38 gene. My results also showed that CaBB1:P30 is not stable on turnip plants and the P30 gene inserted was deleted on the plants.

As CaBB1 is a relatively stable viral expression vector (Brisson *et al.*, 1984) , the instability of CaBB1:P30 suggests that P30 may interfere with normal function of CaMV movement protein and that leads to the selection of viruses with the inserted P30 gene deleted. A similar deletion was found in a PClSV construct (Mushegian *et al*, 1995).

In conclusion, functions of CaMV and TMV MP are not exchangeable and CaMV MP interferes with TMV MP function. Possible recombination between TMV chimeras and the MP transgene may occur under specific conditions.

CHAPTER III

GENETIC DETERMINANTS OF TURNIP HOST SPECIFICITY IN TOBAMOVIRUSES

1. Introduction

Tobamoviruses, a group of rod-shaped plant viruses with a single-component positive-sense RNA genome of about 6400 nucleotides, have a diverse host range. Although turnip is reported not to be a host for tobacco mosaic virus (TMV) *vulgare*, the type member of tobamoviruses, there are at least 4 reported crucifer-pathogenic tobamoviruses which can infect turnip plants (Oshima *et al.*, 1962; Dorokhov *et al.*,1994; Lartey *et al.* 1993; Yamanaka *et al.*, 1994). Turnip vein clearing virus (TVCV) is a tobamovirus isolated from *Brassica rapa* (turnip) (Lartey *et al.*, 1993). It causes vein clearing in turnip, systemic mosaic in *N. tabacum* cv. Samsun and local lesions in *N. tabacum* cv. Xanthi-nc. Both the systemic mosaic and local lesion symptoms are similar to those caused by TMV *vulgare* in the respective plants (Lartey *et al.*, 1993). The complete genomic sequence of TVCV showed that it has a genome of about 6300 nucleotides and a genomic organization similar to TMV except that

there is a 25 codon overlap between TVCV movement protein and coat protein genes (Lartey *et al.*, 1993; Lartey *et al.*, 1995).

At least four polypeptides are encoded by tobamoviruses. The 126K and 183K polypeptides are components of a replicase which is involved in virus replication (Young *et al.*, 1986; Meshi *et al.*, 1987). The 30K protein is the movement protein required for cell-to-cell movement (Meshi *et al.*, 1987; Deom *et al.*, 1987). The 17K protein is the coat protein and is also involved in virus long distance movement (Siegel *et al.*, 1962; Dawson *et al.*, 1988). There is evidence that each of the polypeptides can be host specificity determinants. The 126K and/or 183K polypeptides were suggested to be involved in the interaction with the *Tm-1* resistance gene of tomato and the N gene of tobacco (Meshi *et al.*, 1988; Padgett *et al.*, 1993). The 30K protein was found to be involved in *Tm-2* resistance in tomato (Meshi *et al.*, 1989). The coat protein is responsible for the induction of the hypersensitive reaction (HR) in *Nicotiana sylvestris* (for a review see Culver et al., 1991).

Turnip protoplasts support TMV vulgare replication (Lartey *et al.*, 1992), although turnip was a host for TMV vulgare (Oshima *et al.*, 1962). Leaf skeleton hybridization experiments showed that local movement of TMV on turnip plants was restricted (Lartey *et al.*, 1992). The movement protein of TMV was probably the genetic factor that limits TMV cell-to-cell movement on turnip. With the complete nucleotide sequence of TVCV known, an infectious TVCV cDNA clone was constructed and used to create a series of TMV and TVCV chimeric viral genomes (Figure 4). The in vitro transcripts from the plasmids or chimeric viruses derived

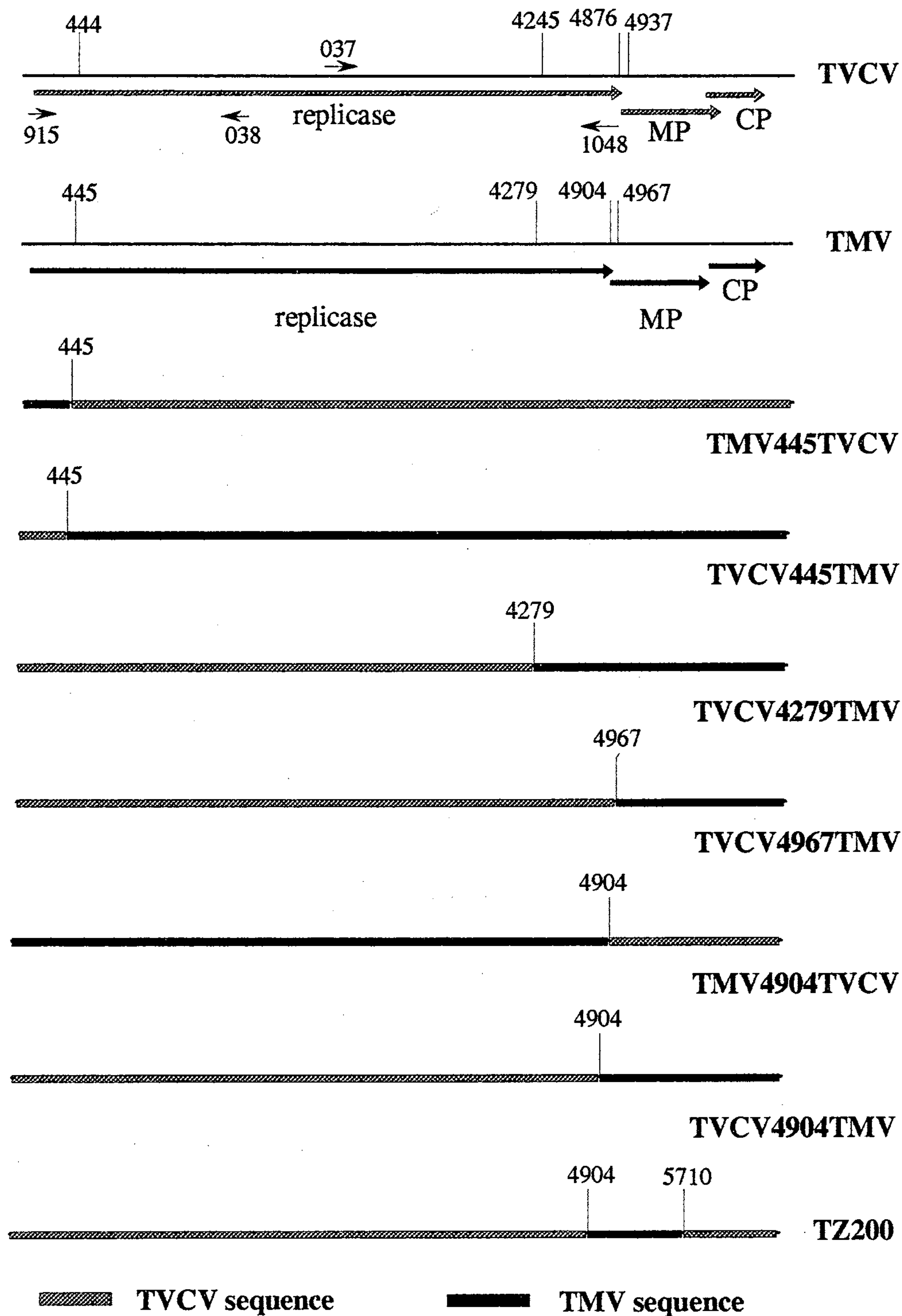

Figure 4. TMV and TVCV Chimeras

from the plasmids were inoculated on tobacco and turnip plants to test whether TMV movement protein gene is the genetic determinant for the limited spread of TMV on turnip or whether other TMV genes are involved in the process.

2. Materials and Methods

Construction of pTVCV5'SP6, pTVCV50, pTMV445TVCV and pTVCV445TMV

Primer 915 (Table 3) was designed with the Sp6 promoter sequence linked to the 5' end sequence of TVCV so that transcription by Sp6 RNA polymerase should start exactly at the first nucleotide of TVCV RNA. A 1.5 kb TVCV cDNA fragment was amplified by reverse transcription-PCR using primers 915 and 038 (Table 3) and TVCV RNA as template. The DNA fragments were electrophoresed through a 1% agarose gel, purified by the freeze-phenol extraction method as described in Chapter 2. The purified DNA was then digested with *Xba*I. The digested DNA was precipitated, redissolved and ligated to *Xba*I digested pBluescipt SK(+) DNA. The sizes of the plasmids of transformants were screened for the presence of a 0.8 kb insert and by *Eco*RV digestion for the orientation of the insert. The plasmid with the Sp6 promotor close to the *Sac*I site on the vector was called pTVCV5'Sp6 (Figure 5).

To construct pTVCV50, pTVCV445TMV (Figure 5) and pTMV445TVCV (Figure 6), pTVCV5'Sp6, pTMV304 and pTVCV42 (R. Lartey, T. Voss & U. Melcher, unpublished) were digested with *Sph*I and *Kpn*I. The large fragment (3.3 kb) of

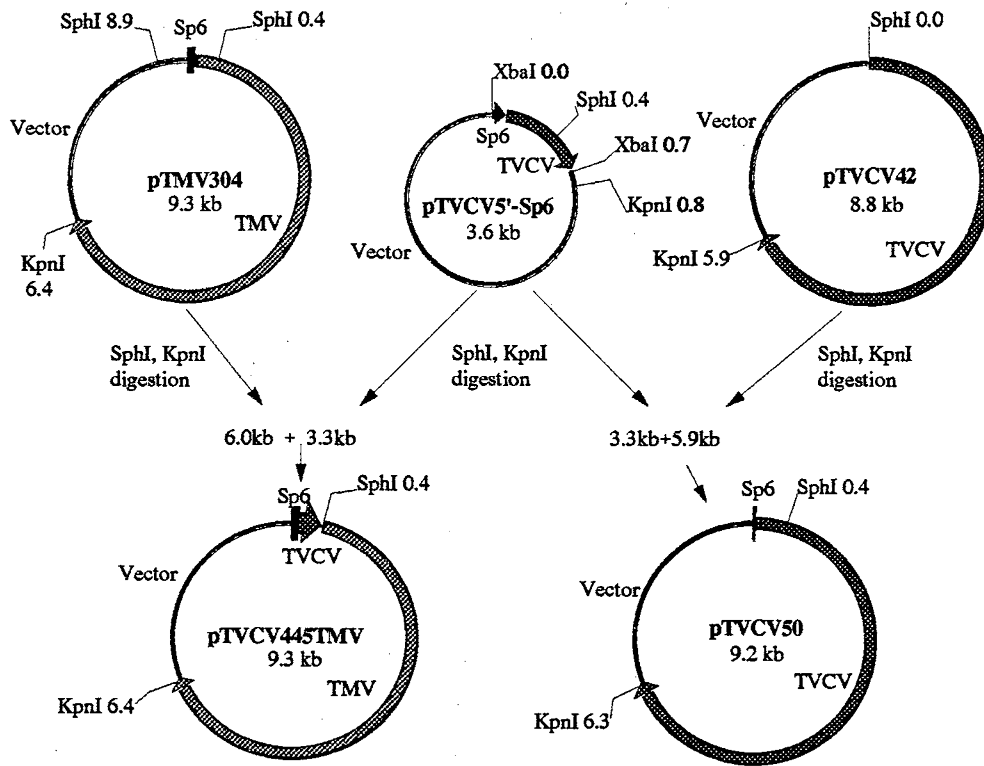

Figure 5. Construction of pTVCV445TMV and pTVCV50

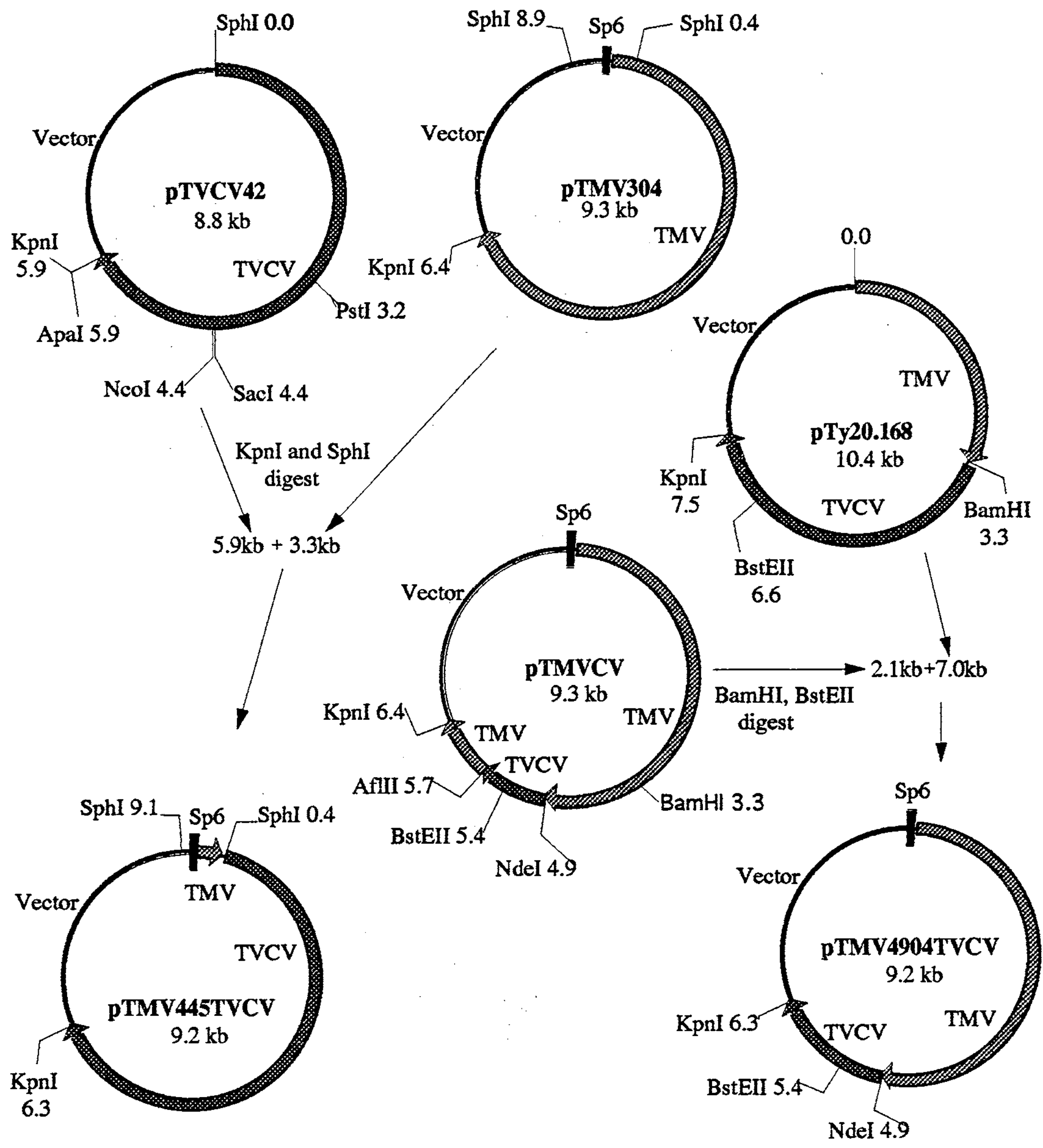

Figure 6. Construction of pTMV445TVCV and pTMV4904TVCV

pTVCV5'Sp6 was ligated to the large fragment (5.9 kb) of pTVCV42 to create pTVCV50. The large fragment (3.3 kb) of pTVCV5'Sp6 was ligated the large fragment (6.0 kb) of pTMV304 to create pTVCV445TMV. The small fragment (about 3.4 kb) of pTMV304 was ligated to the large fragment (about 5.9 kb) of pTVCV42 to create pTMV445TVCV.

Construction of pTMV4904TVCV

pTMVCV is a plasmid constructed by R. Lartey from pTMV304 by replacing the TMV movement protein gene with the TVCV movement protein gene. pTVCV20.168 is a plasmid constructed by T. Voss with a 4.4 kb TVCV cDNA cloned in pTMV304. Both pTMVCV and pTVCV20.168 were digested with *Bam*HI and *Bst*EII. The 7.2 kb pTVCV20.168 fragment was ligated to the 2.1 kb pTMVCV fragment to produce pTMV4904TVCV (Figure 6).

Construction of pTVCV4279TMV and pTVCV4967TMV

For construction of a TVCV 3' half subclone, pTVCV42 was digested with *Pst*I and *Apa*I. The 2.7 kb fragment was purified and cloned into *Pst*I and *Apa*I digested pBluescript SK(+). The resulting plasmid was called pTVCV3'H (Figure 7). A plasmid called pTZ155 (Figure 7) was obtained by deleting the 0.8 kb *Sac*I fragment of pTVCV3'H. There is a unique *Bgl*II site in pTZ155. Both pTZ155 and

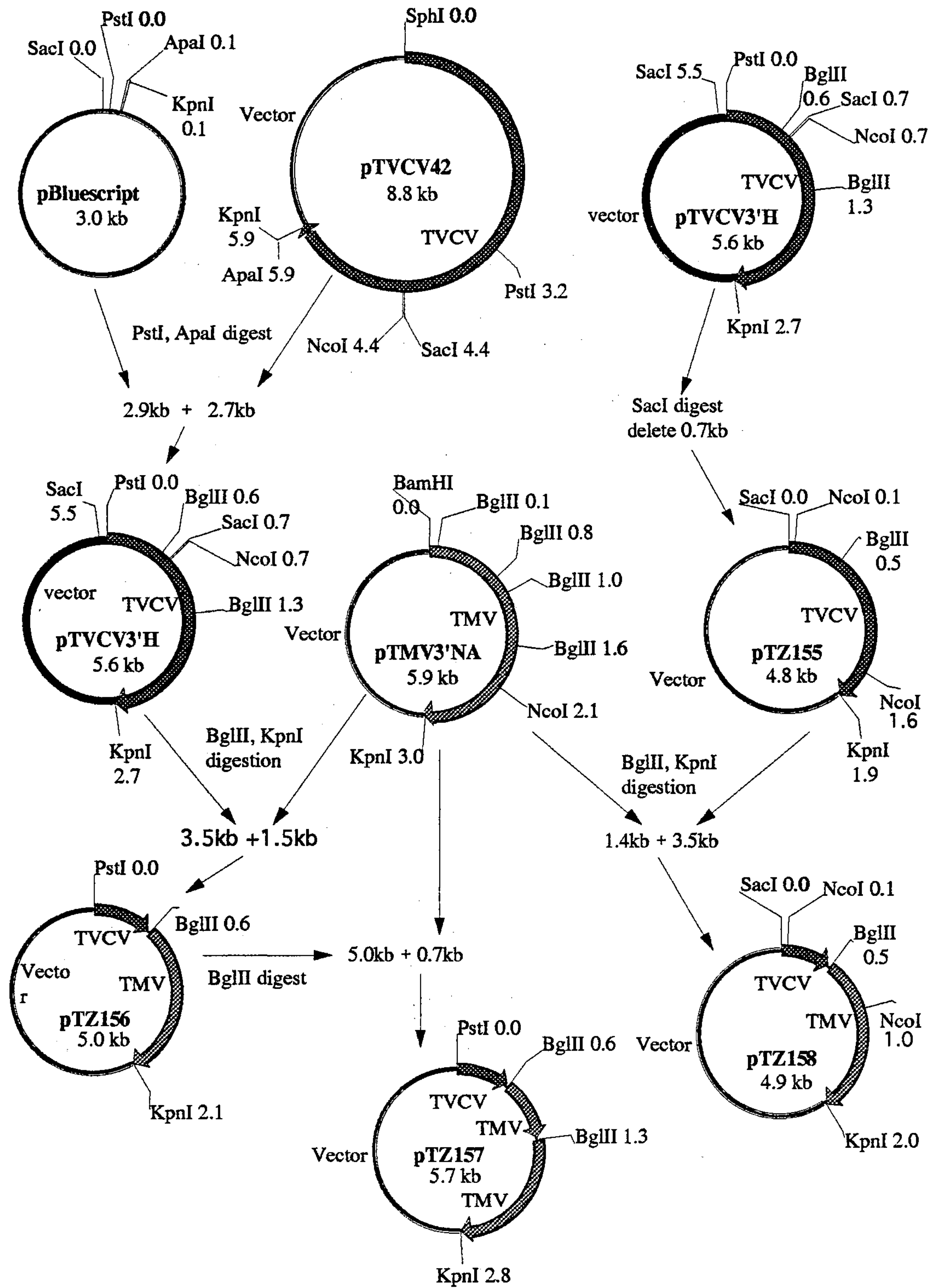

Figure 7. Construction of pTZ157 and pTZ158

pTMV3'NA were digested with *Bgl*II and *Kpn*I. The 1.5 kb pTMV3'NA fragment was ligated to the 3.4 kb pTZ155 fragment to create pTZ158 (Figure 7). To make pTVCV4967TMV, pTZ158 was digested with *Kpn*I completely and *Nco*I partially. The 1.9 kb *Nco*I-*Kpn*I pTZ158 fragment was ligated to the 7.4 kb *Nco*I-*Kpn*I pTVCV50 fragment to create pTVCV4967TMV (Figure 8).

For construction of a TVCV and TMV chimera joined at the common *Bgl*II site at TMV 4279 (Figure 8), pTVCV3'H and pTMV3'NA were digested with *Bgl*II and *Kpn*I. The 1.5 kb pTMV3'NA fragment was ligated to the 3.6 kb pTVCV3'H fragment. The resulting plasmid was called pTZ156 (Figure 7). The *Bgl*II TMV fragment from 4279-4967 was obtained by digesting pTMV3'NA with *Bgl*II. It was then ligated to *Bgl*II-digested and phosphatase HK™-treated 4.9 kb pTZ156 fragment. Plasmids with insertions of the expected size were obtained. Orientation of insertion was checked with *Nde*I and *Bsp*MI digestion. The plasmid with the insertion in frame was called pTZ157 (Figure 7). Both pTZ157 and pTVCV50 were then digested with *Pst*I and *Kpn*I. The 2.7 kb pTZ157 fragment was ligated to the 6.6 kb pTVCV50 fragment to create pTVCV4967TMV (Figure 8).

Construction of pTVCV4904TMV

The 560 bp *Bgl*II-*NcoI* fragment of pTZ156 was removed and replaced with the 2.1 kb *Bam*HI-*Nco*I pKK1 (Dawson *et al.* 1988) fragment to create pTZ168 (Figure 9). Primer 1048 is complementary to the 5' end of the TVCV replicase gene and has

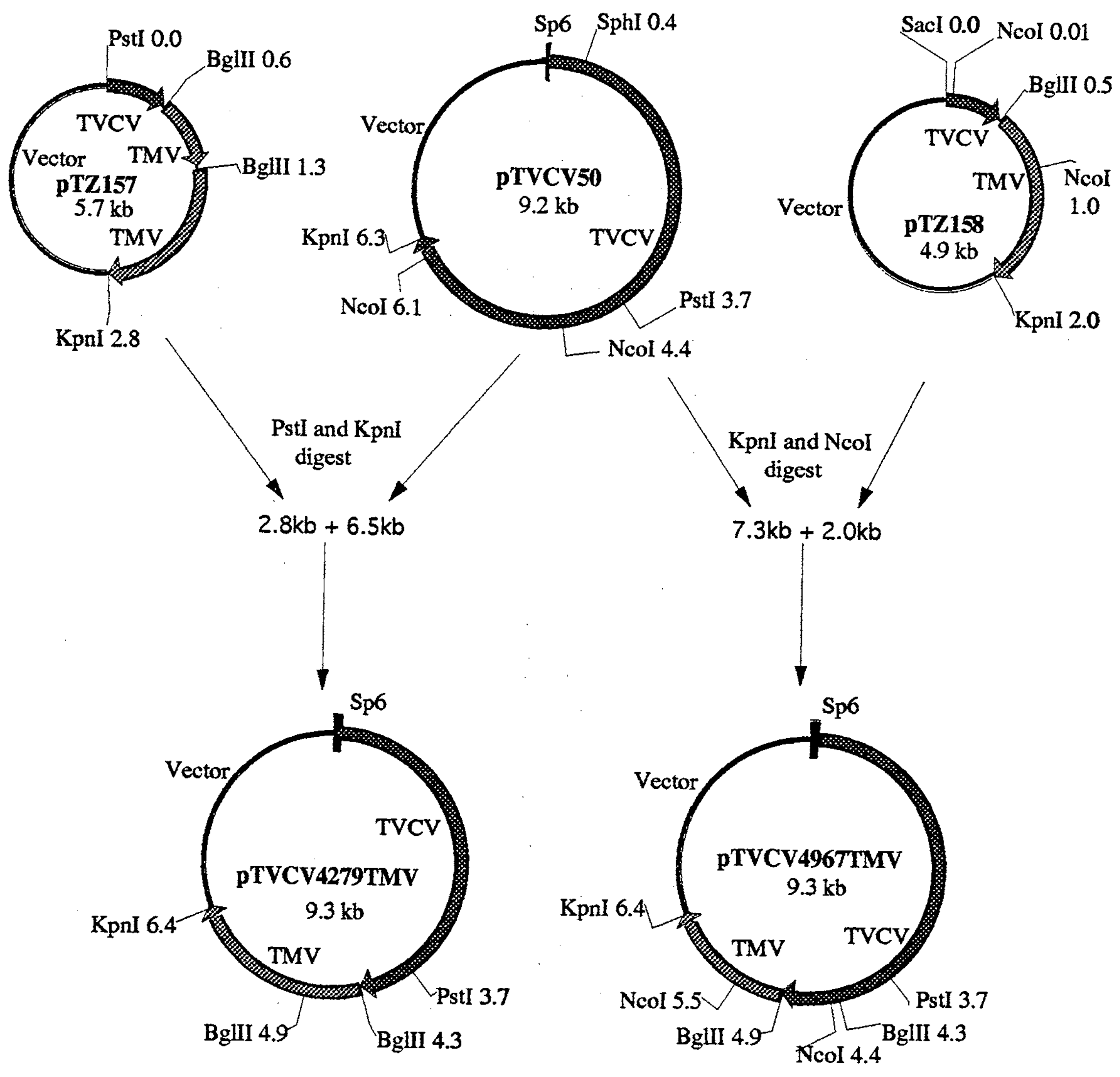

Figure 8. Construction of pTVCV4279TMV and pTVCV4967TMV

an *Xho*I site at the 5' end. A DNA fragment was amplified from pTVCV50 by using primer 1048 and primer 037. The DNA fragment was digested with *Pst*I and *Xho*I. The digested DNA fragment was ligated to the 4.4 kb *PstI-Xho*I fragment of pTZ168 to create pTZ171 (Figure 9). The 2.7 kb *Pst*I-*Kpn*I fragment of pTZ171 was used to replace the 2.6 kb *Pst*I-*Kpn*I fragment of pTVCV50. The resulting plasmid was called pTVCV4904TMV (Figure 9).

Construction of pTZ200

Both pTVCV50 and pGEM:GFP (Zhang & Melcher, unpublished) were digested with *Kpn*I and *EcoR*I. The 0.8 kb pTVCV50 fragment was then ligated to the 3.0 kb pGEM:GFP fragment to obtain pTZ195 (Figure 3). The 3.2 kb pTB2 *Bam*HI-*Kpn*I fragment was ligated to the 2.9 kb *Bam*HI- and *Kpn*I-digested pBluescript fragment to create pTB3'H. Both pTZ195 and pTB3'H were then digested with *Xho*I and *Kpn*I. The 0.8 kb pTZ195 fragment was ligated to the 5.3 kb pTB3'H fragment to obtain pTZ196 (Figure 10). Both pTZ196 and pTZ171 were then digested with *Hin*dIII and *Kpn*I. The 1.9 kb pTZ196 fragment was ligated to 3.8 kb pTZ171 fragment to obtain pTZ197 (Figure 10). Both pTVCV50 and pTZ197 were digested with *Pst*I and *Kpn*I. The 2.8 kb pTZ197 fragment was ligated to the 6.6 kb pTVCV50 fragment to construct pTZ200 (Figure 10).

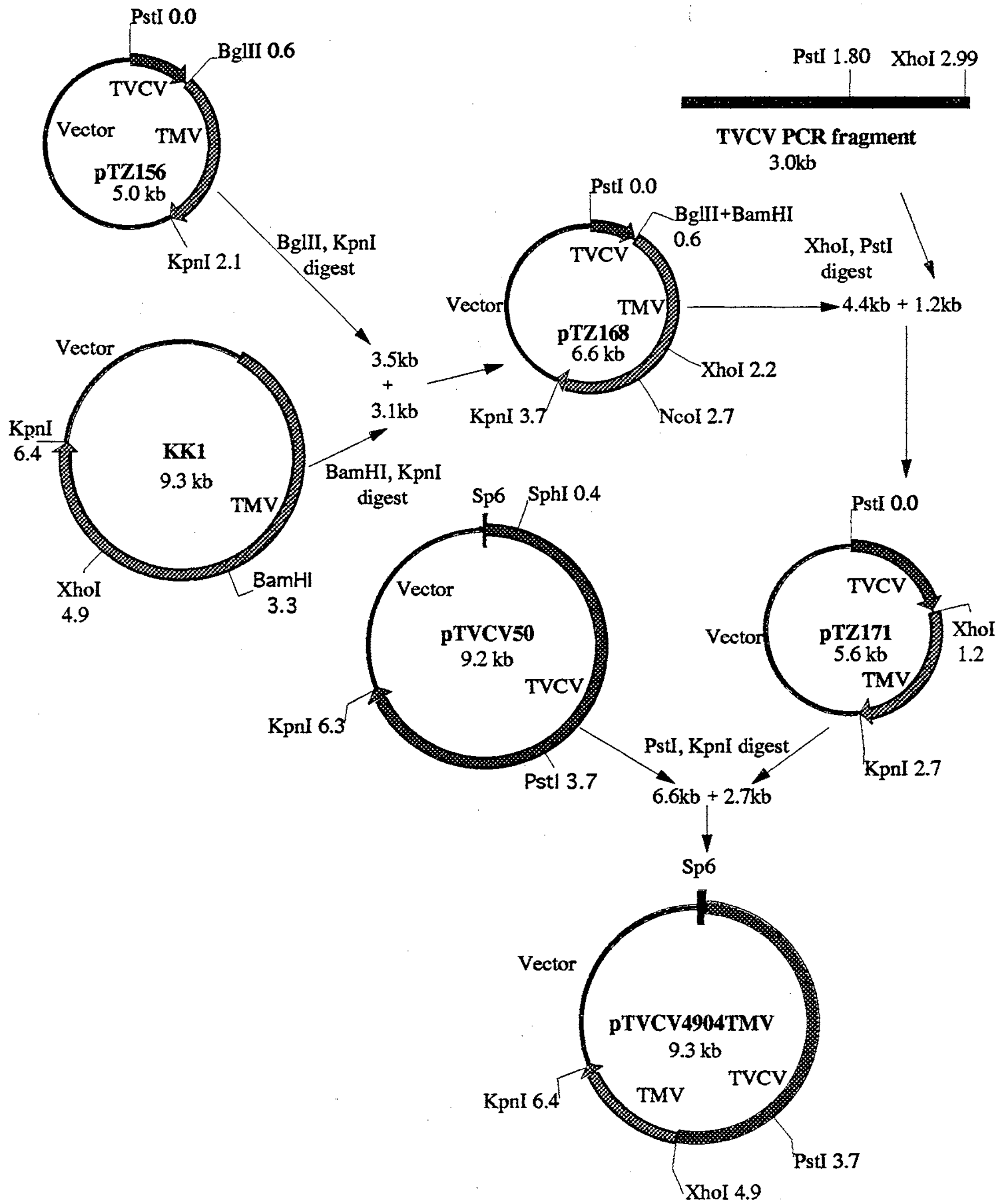

Figure 9. Construction of pTVCV4904TMV

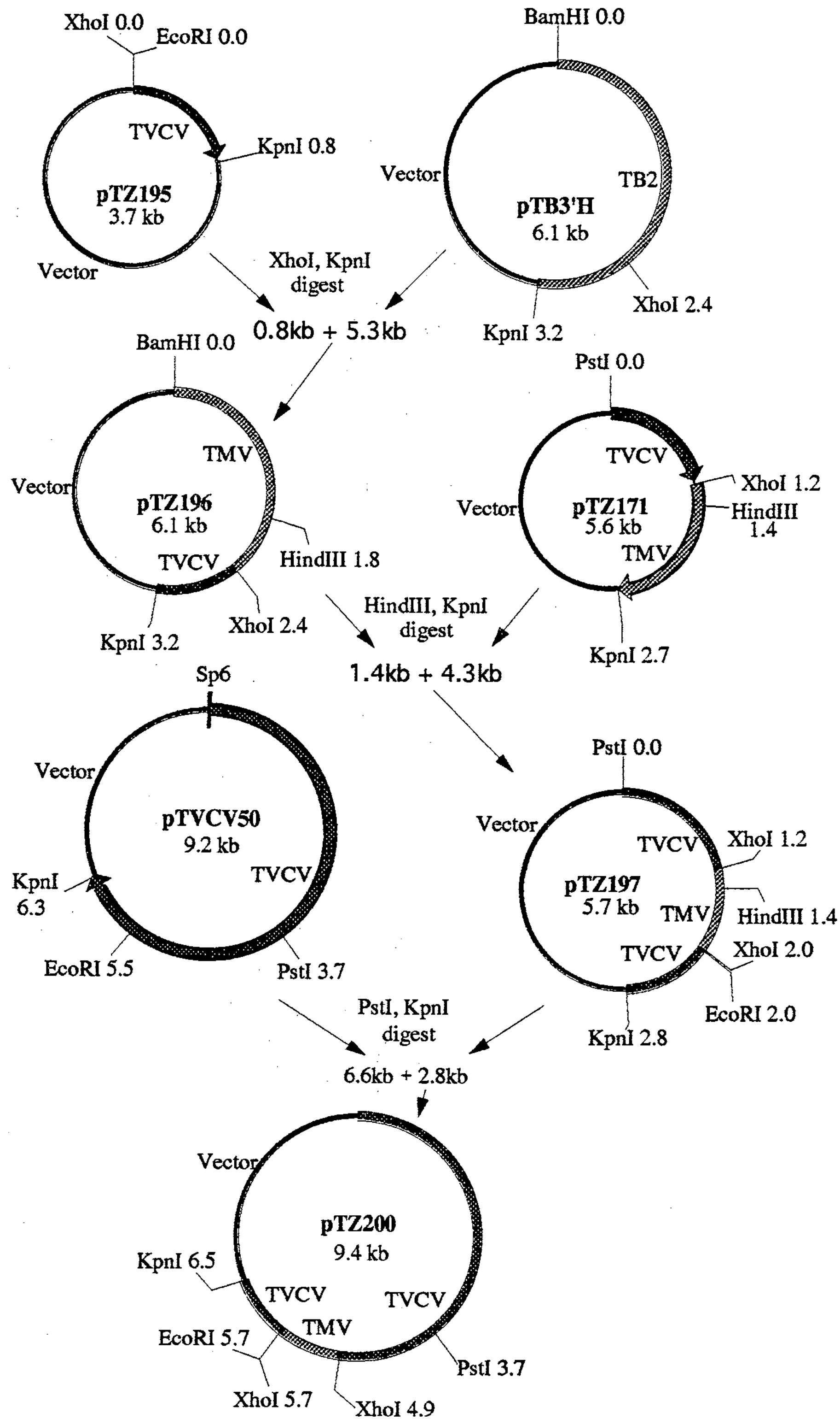

Figure 10. Construction of pTZ200

Plants and Inoculation

N. tabacum cv. Xanthi-nc, *N. tabacum* cv. Samsun, TMV movement protein transgenic Xanthi-NN (plant line 2005, Deom *et al.*, 1990) and Xanthi-nn (plant line 277, Deom *et al.*, 1990) were used. They will be referred to as Xanthi-nc, Samsun-nn, MP2005 line and MP277 line, respectively. *Arabidopsis thaliana* cv. Columbia and *Brassica rapa* cv. Just right were also used. They will be referred to as Arabidopsis and turnip, respectively. In vitro transcripts were made from the *Kpn*I-linearized plasmids with viral cDNAs by Ambion (Austin, Texas) mMessage mMachine™ kit (catalog #1340). In vitro transcripts derived from viral cDNAs or homogenates from infected leaves were used as sources of inoculum. For each experiment, at least two tobacco plants or two turnip plants were inoculated with each inoculum. For each tobacco plant, the third to sixth leaf were inoculated when the plant was at the seven-leaves stage. For each turnip plant, the third to fifth leaf were inoculated when the plant was at the six-leaves stage. The inoculation was done by gently rubbing a 20 μl drop of inoculum over the leaf surface dusted with carborundum. Plants were maintained in a growth chamber (12-hr light, 23°C/12-hr dark, 21°C dark period). For each experiment, at least two tests were done.

3. Results

Construction of an Infectious TVCV cDNA Clone

A full length cDNA clone of TVCV (pTVCV50) and two TVCV-TMV chimeric cDNA clones (pTMV445TVCV, 5'-end 445 nucleotides from TMV and other parts from TVCV; pTVCV445TMV, 5'-end 444 nucleotides from TVCV and other parts from TMV) were constructed by assembling several cDNA clones. In vitro-synthesized transcripts were made from *Kpn*I-linearized pTVCV50, pTMV445TVCV and pTVCV445TMV. The transcripts from pTMV445TVCV and pTVCV445TMV were used to inoculate Xanthi-nc plants. The transcripts from pTVCV50 were used to inoculate Xanthi-nc and turnip plants. No local lesions developed on Xanthi-nc leaves inoculated with transcripts from pTMV445TVCV or pTVCV445TMV. Local lesions developed on Xanthi-nc 2 days after inoculation with transcripts from pTVCV50. Both the lesion size and the timing of lesion development were the same as in control plants inoculated with TVCV. The upper uninoculated leaves of turnip plants inoculated with pTVCV50 transcripts were homogenized to make inoculum and tested on Xanthi-nc plants. Local lesions developed on inoculated Xanthi-nc leaves 2 days after inoculation. The results indicate that pTVCV50 transcripts are infectious to turnip as well as tobacco plants.

Test of TMV and TVCV Chimeras on Plants

In vitro-synthesized transcripts were made from *Kpn*I-linearized pTVCV4279TMV (a chimera with TMV from its nucleotide 4279 to the 3'-end and other parts from TVCV), pTVCV4967TMV (a chimera with TMV from its nucleotide 4967 to the 3'-end and other parts from TVCV), pTZ200 (a chimera with TVCV P30 gene replaced by TMV P30 gene), pTVCV4904TMV (a chimera with TMV from its nucleotide 4904 to the 3'-end and other parts from TVCV) and pTMV4904TVCV (a chimera with TMV from its 5'-end to nucleotide 4904 and other parts from TVCV). The transcripts were inoculated on Xanthi-nc plants. No local lesions developed on Xanthi-nc leaves inoculated with transcripts from pTVCV4279TMV or pTVCV4967TMV. Local lesions developed on Xanthi-nc leaves inoculated with transcripts from pTZ200, pTVCV4904TMV and pTMV4904TVCV 2 days after inoculation. The lesion sizes were the same as those on TMV-inoculated control leaves. Since the chimeric gene in pTVCV4967TMV is the movement protein gene, the transcripts from pTVCV4967TMV were inoculated on MP2005 plants to test whether the movement protein transgene is able to complement TVCV4967TMV movement. Local lesions developed on the inoculated leaves 3 days after inoculation with the chimera while lesions developed on the control MP2005 plants two days after inoculation with TMV. The lesions produced by TVCV4967TMV were smaller than those produced by TMV. Inoculum was prepared from the homogenates of infected MP2005 plant leaves.

Samsun plants were inoculated with homogenates of Xanthi-nc plants infected by TMV, TZ200, TVCV4904TMV or TMV4904TVCV. All inoculated Samsun plants developed systemic mosaic symptoms 7 days post-inoculation. Symptoms on plants inoculated with TMV or TZ200 were more severe than those on plants inoculated with TMV4904TVCV or TVCV4904TMV. Inocula prepared from the systemically infected Samsun plant leaves were inoculated on Xanthi-nc plants to test the titer of the inocula. Virus concentration in the systemic leaves infected by TVCV4904TMV or TMV4904TVCV was about ten times less than that in the systemic leaves infected with TMV or TZ200. The inocula concentrations were then adjusted by dilution to obtain inocula which produced about 500 local lesions on each Xanthi-nc leaf. Inoculum concentration of pTVCV4967TMV was also adjusted by dilution to obtain inoculum which produced about the same number of local lesions as TVCV4904TMV on each MP2005 plant leaf. Turnip plants were then inoculated with the adjusted inocula. After 4 days and 12 days, the inoculated turnip leaves were homogenized. After 2 weeks and 4 weeks post-inoculation, the upper uninoculated turnip leaves were also homogenized. The homogenates were then inoculated on Xanthi-nc plants. The results are shown in Table 8. An average of 0.3, 0, 2.0 or 15.3 local lesions per leaf developed on Xanthi-nc leaves inoculated with homogenates of turnip leaves inoculated 4 days previously with TMV, TMV4904TVCV, TVCV4904TMV and TZ200 respectively. An average of 4.3, 0.4, 11.3 or 65.6 local lesions per leaf developed on Xanthi-nc leaves inoculated with homogenates of turnip leaves inoculated 12 days previously with TMV, TMV4904TVCV, TVCV4904TMV and

TABLE 8

RESULTS OF TESTING TMV-TVCV CHIMERAS ON PLANTS

Chimeras	4 days inocula	12 days inocula
TMV*	2/8****	52/12
TMV4904TVCV*	0/8	5/12
TVCV4904TMV*	16/8	135/12
TZ200*	122/8	787/12
TVCV4967TMV**	0/8	0/12
TVCV4904TMV**	N.D.***	142/12

* Inoculum from turnip was inoculated on Xanthi-nc plants.

** Inoculum from turnip was inoculated on MP2005 plants.

*** N.D.: Not determined.

**** X/Y means a total of X lesions developed on a total of Y leaves.

TZ200, respectively. No lesions developed on Xanthi-nc leaves inoculated with homogenates from any of the upper uninoculated leaves. Xanthi-nc leaves inoculated with inoculum from TVCV-inoculated turnip leaves or systemic leaves always developed more than 500 local lesions. No lesions developed on MP2005 plant leaves inoculated with homogenates of turnip leaves inoculated 12 days previously with TVCV4967TMV, but an average of about 12 lesions developed on MP2005 plant leaves inoculated with homogenates of turnip leaves inoculated 12 days previously with TVCV4904TMV. The results indicate that TMV4904TVCV, TVCV4904TMV, TZ200 and TMV are able to infect inoculated turnip leaves limitedly, but are not able to infect turnip systemically.

4. Discussion

I have reported here the construction of an infectious cDNA clone of TVCV. Transcripts from the cDNA clone were infectious to tobacco and turnip. The symptoms of transcript-inoculated plants were the same as the symptoms on TVCV-inoculated plants, suggesting that there is no difference between the virus derived from the infectious clone and TVCV. As reported here, I used the infectious TVCV cDNA clone to determine the genetic factors limiting TMV spread on turnip by making chimeras between TMV and TVCV.

As there is a 50-60% identity between TMV and TVCV (Lartey, *et al.*, 1994), my results demonstrate that chimeras constructed between genes whose amino acid

sequences are only 50-60% identical are non-functional. In pTVCV445TMV, pTMV445TVCV and pTVCV4279TMV, the replicase gene is chimeric. In pTVCV4967TMV, the movement protein gene is chimeric. None of these four chimeras infected wild type tobacco plants which are the common host of TMV and TVCV. A complementation test showed that TVCV4967TMV can replicate and move from cell-to-cell in movement protein transgenic plants. This indicates that the only gene wrong with TVCV4967TMV was the movement protein gene. My results with these chimeras suggest that there is a sequence compatibility requirement for the chimeric replicase or movement proteins to be functional. For chimeric viral proteins to function, amino acid sequences of the parental proteins probably need to be more than 60% identical. Chimeric genes between TMV and tomato mosaic virus, whose sequences are 80% identical, are functional (Saito *et al.*, 1987).

The smaller lesions produced by TVCV4967TMV on MP2005 plants suggest that the dysfunctional movement protein from the virus interfered with the wild type TMV movement protein from the transgene. Such interference is consistent with the observation that a defective movement protein of TMV in transgenic plants interfered with the movement of TMV and two other tobamoviruses (Lapidot *et al.*, 1993).

As TVCV4967TMV has an intact TVCV replicase gene and a dysfunctional movement protein gene, TVCV4967TMV should be able to replicate but not to move from cell-to-cell on turnip plants. My results showed that no local lesions developed on MP2005 plants inoculated with homogenates of turnip leaves previously inoculated with TVCV4967TMV, while local lesions developed on Xanthi-nc plants inoculated

with homogenates from turnip leaves previously inoculated with TMV. These results indicate that TMV is able to replicate and move from cell-to-cell at a limited rate on turnip plants. The results support the conclusion from protoplast and leaf skeleton hybridization experiments (Lartey *et al.*, 1992) that TMV is able to replicate in turnip plants but its cell-to-cell movement is limited.

TMV4904TVCV and TVCV4904TMV accumulated to ten-fold lower levels than TMV and TZ200 on Samsun plants. The explanation for this result is that TMV replicase recognizes the sequence (or structure) of 3' end noncoding region of TVCV less efficiently than that of TMV and vicé versa, considering that the nucleotide sequence of 3' end noncoding regions of TVCV and TMV have only 40-50% identity. Chimeric TMVs which carry heterologous combinations of replicase genes and 3' noncoding regions replicate less efficiently than the wild type virus (Ishikawa *et al.*, 1988). TMV replicase was also found to recognize brome mosaic virus 3'-end less efficiently than the wild type TMV 3'-end (Ishikawa *et al.*, 1991).

As TMV4904TVCV has a TMV replicase and a TVCV movement protein and produces a very limited infection on turnip plants, TMV replicase is likely a limiting factor for its infection on turnip plants. The fact TMV4904TVCV replicates less efficiently than TMV due to the imperfect interaction between TMV replicase and the TVCV 3' end may explain the results that fewer viruses were recovered from the turnip leaves inoculated with TMV4904TVCV than from the leaves inoculated with TMV. As both TVCV4904TMV and TZ200 have an intact TVCV replicase gene and a TMV movement protein gene and both viruses produce a limited infection on turnip

plants, TMV movement protein is another factor that limits the infection of TMV on turnip plants. That both viruses accumulated to higher levels than TMV on turnip plants further confirmed that TMV replicase is one of the limiting factors for the infection of TMV on turnip.

The mechanisms for TMV replicase and movement protein to limit the infection of TMV on turnip plants are unknown. It could be that TMV replicase or TMV movement protein or both of them initiate a very efficient host response which leads to the restriction of TMV spread on turnip plants. It could also be that TMV replicase or TMV movement protein or both of them do not interact with the host factors required for virus replication and virus movement very well and that leads to a limited spread of TMV on turnip.

BIBLIOGRAPHY

Armour, S. L., Melcher, U., Pirone, T. P., Lyttle, D. J. & Essenberg, R. C. (1983). Helper component for aphid transmission encoded by region II of cauliflower mosaic virus DNA. *Virology* **129**, 25-30.

Atabekov J. G. & Talianski M. E. (1990). Expression of a plant virus-encoded transport function by different viral genomes. *Adv. Virus Res.* **38**, 201-248.

Atkins, D., Hull, R., Robert, K., Moore, P. & Beachy, R. N. (1991). The tobacco mosaic virus 30K movement protein in transgenic tobacco plants is localized to plasmodesmata. *J. Gen. Virol.* **72**, 209-211.

Brisson, N., Paszkowski, J., Penswick, J. R., Gronenborn, B., Potrykus I. & Hohn, T. (1984). Expression of a bacterial gene in plants by using a viral vector. *Nature* **310**, 511-513.

Citovsky, V., Knorr, D., Schuster, G. & Zambryski, P. (1990). The P30 movement protein of tobacco mosaic virus is a single-stranded nucleic acid binding protein. *Cell* **60,** 637-647.

Citovsky, V., Knorr, D. & Zambryski, P. (1991). Gene I, a potential cell-to-cell movement locus of cauliflower mosaic virus, encodes an RNA-binding protein. *Proc. Natl. Acad. Sci. USA* **88**, 2476-2480.

Citovsky, V. & Zambryski, P. (1991). How do plant virus nucleic acids move through intercellular connections? *BioEssays* **13**, 373-379.

Citovsky, V., Wong, M. L., Shaw, A. L., Prasad, B. V. V. & Zambryski, P. (1992). Visualization and characterization of tobacco mosaic virus movement protein binding to single-stranded nucleic acids. *Plant Cell* **4**, 397-411.

Clackson, T. & Winter, G. (1989). 'Sticky feet'-directed mutagenesis and its application to swapping antibody domains. *Nucleic Acids Res.* **17**, 10163-10170.

Covey, S. N. & Hull, R. (1981). Transcription of cauliflower mosaic virus DNA. Detection of transcripts, properties and location of the gene encoding the virus inclusion body protein. *Virology* **111**, 463-474.

Culver, J. N., Lindbeck, A. & Dawson, W. O. (1991). Virus-host interactions: Induction of chlorotic and necrotic responses in plants by tobamoviruses. *Annu. Rev. Phytopathol.* **29**, 193-217.

Culver, J. N., Lehto, K., Close, S. M., Hilf, M. E. & Dawson, W. O. (1993). Genomic position affects the expression of tobacco mosaic-virus movement and coat protein genes. *Proc. Natl. Acad. Sci. USA* **90**, 2055-2059.

Daubert, S., Richins, R., Shepherd, R. J. & Gardner, R. C. (1982). Mapping of the coat protein gene of cauliflower mosaic virus by its expression in a prokaryotic system. *Virology* **122**, 444-448.

Dawson, W. O., Bubrick, P. & Grantham, G. L. (1988). Modification of the tobacco mosaic virus coat protein gene affecting replication, movement, and symptomatology. *Phytopathology* **78**, 783-789.

Denhardt, D. T. (1966). A membrane-filter technique for the detection of complementary DNA. *Biochem. Biophy. Res. Commun.* **23**, 641-646.

Deom, C. M., Schubert, K. R., Wolf, S., Holt, C. A., Lucas, W. J. & Beachy, R. N. (1990). Molecular characterization and biological function of the movement protein of tobacco mosaic virus in transgenic plants. *Proc. Natl. Acad. Sci. USA* **87**, 3284-3288.

Deom, C. M., Lapidot, M. & Beachy, R. N. (1992). Plant virus movement proteins. *Cell* **69**, 221-224.

Deom, C. M., Oliver, M. J. & Beachy, R. N. (1987). The 30-kilodalton gene product of tobacco mosaic virus potentiates virus movement. *Science* **237**, 389-394.

Ding, B., Li, Q., Nguyen, L., Palukaitis, P. & Lucas, W. J. (1995). Cucumber mosaic virus 3a protein potentiates cell-to-cell trafficking of CMV RNA in tobacco plants. *Virology* **207**, 345-353.

Donson, J., Kearney, C. M., Hilf, M. E. & Dawson, W. O. (1991). Systemic expression of a bacterial gene by a tobacco mosaic virus-based vector. *Proc. Natl. Acad. Sci. USA* **88**, 7204-7208.

Dorokhov, Y. L., Ivanov, P. A., Novikov, V. K., Agranovsky, A. A., Morozov, S. Y., Efimov, V. A., Casper, R. & Atabekov, J. G. (1994). Complete nucleotide sequence and genome organization of a tobamovirus infecting cruciferae plants. *FEBS Lett.* **350**, 5-8.

Fujiwara, T., Giesman-Cookmeyer, D., Ding, B., Lommel, S. A. & Lucas, W. J. (1993). Cell-to-cell trafficking of macromolecules through plasmodesmata potentiated by the red clover necrotic mosaic virus movement protein. *Plant Cell* **5**, 1783-1794.

Gardner, R. C. & Shepherd, R. J. (1980). A procedure for rapid isolation and analysis of cauliflower mosaic virus DNA. *Virology* **106**, 159-161.

Goelet, P., Lomonossoff, G. P., Butler, P. J. G., Akam, M. E., Gait, M. J. & Karn, J. (1982). Nucleotide sequence of tobacco mosaic virus RNA. *Proc. Natl. Acad. Sci. USA* **79**, 5818-5822.

Gowda, S., Wu, F. C., Scholthof, H. B. & Shepherd, R. J. (1989). Gene VI of figwort mosaic virus (caulimovirus group) functions in posttranscriptional expression of genes on the full-length RNA transcript. *Proc. Natl. Acad. Sci. USA* **86**, 9203-9207.

Greene, A. E. & Allison, R. F. (1994). Recombination between viral RNA and transgenic plant transcripts. *Science* **263**, 1423-1425.

Holmes, F. O. (1931). Local lesions of mosaic in *Nicotiana tabacum* L. *Contr. Boyce Thompson* **3**, 163-172.

Holt, C. A., Vaewhong, A. A. & Beachy, R. N. (1991). Analysis of host-range limitations of tobacco mosaic virus using an infectious cDNA clone. International Society of Plant Molecular Biology, Third International Congress, Abstract 1220.

Hull, R. & Covey, S. N. (1985). Cauliflower mosaic virus: pathways of infection. *BioEssays* **3**, 160-163.

Hull, R., Sadler, J. & Longstaff, M. (1986). The sequence of carnation etched ring virus DNA: comparison with cauliflower mosaic virus and retroviruses. *EMBO J.* **5**, 3083-3090.

Hull, R. (1991). The movement of viruses within plants. *Sem. Virol.* **2**, 89-95.

Ishikawa, M., Meshi, T., Watanabe, Y. & Okada, Y. (1988) Replication of chimeric tobacco mosaic viruses which carry heterologous combinations of replicase genes and 3' noncoding regions. *Virology* **164**, 290-293.

Ishikawa, M., Kroner, P., Ahlquist, P. & Meshi, T. (1991). Biological activities of hybrid RNAs generated by 3'-end exchanges between tobacco mosaic and brome mosaic viruses. *J. Virol.* **65**, 3451-3459.

Joseph G. A. & Mikhail E. T. (1990). Expression of a plant virus coded transport function by different viral genomes. *Adv. Virus Res.* **38**, 201-243.

Koonin, E. V., Mushegian, A. R., Ryabov, E. V. & Dolja, V. V. (1991). Diverse groups of plant RNA and DNA viruses share related movement proteins that may possess chaperone-like activity. *J. Gen. Virol.* **72**, 2895-2903.

Lapidot, M., Gafny, R., Ding, B., Wolf, S., Lucas, W. J. & Beachy, R. N. (1993). A dysfunctional movement protein of tobacco mosaic-virus that partially modifies the plasmodesmata and limits virus spread in transgenic plants. *Plant J.* **4**, 959-970.

Lartey, R. T., Hartson, S. D. & Melcher, U. (1992). Deficiency in intercellular movement limits tobacco mosaic virus infection of turnip. *Phytopathology* **82**, 1077.

Lartey, R. T., Hartson, S. D., Pennington, R. E., Sherwood, J. L. & Melcher, U. (1993). Occurrence of a vein-clearing tobamovirus in turnip. *Plant Dis.* **77**, 21-24.

Lartey, R. T., Melcher, U. & Voss, T. C. (1993). The complete nucleotide sequence of turnip vein-clearing virus. *GenBank* **U03387**.

Lartey, R. T., Lane, L. C. & Melcher, U. (1994). Electron microscopic and molecular characterization of turnip vein-clearing virus. *Arch. Virol.* **138**, 287-298.

Lartey, R.T., Voss, T.C. & Melcher, U. (1995). Overlap gene organization of a crucifer-pathogenic subgroup of tobamoviruses. *J. Gen. Virol.*, submitted.

Lebeurier, G., Hirth, L., Hohn, T. & Hohn, B. (1980). Infectivities of native and cloned DNA of CaMV. *Gene* **12**, 139-146.

Lehto, K. & Dawson, W. O. (1990). Changing the start codon context of the 30K gene of tobacco mosaic virus from "weak" to "strong" does not increase expression. *Virology* **174**, 169-176.

Linstead, P. J., Hills, G. J., Plaskitt, K. A., Wilson, I. G., Harker, C. L. & Maule, A. J. (1988). The subcellular location of the gene I product of cauliflower mosaic virus is consistent with a function associated with virus spread. *J. Gen. Virol.* **69**, 1809-1818.

Martinez-Izquierdo, J. A., Futterer, J. & Hohn, T. (1987). Protein encoded by ORF I of cauliflower mosaic virus is part of the viral inclusion body. *Virology* **160**,

527-530.

Maule, A. J. (1991). Virus movement in infected plants. *Crit. Rev. Plant Sci.* **9**, 457-473.

Melcher, U., Gardner, C. O., JR. & Essenberg, R. C. (1981). Clones of cauliflower mosaic virus identified by molecular hybridization in turnip leaves. *Plant Mol. Biol.* **1**, 63-74.

Melcher, U., Choe, I. S., Lebeurier, G., Richards, K. & Essenberg, R. C. (1986). Selective allele loss and interference between cauliflower mosaic virus DNAs. *Mol. Gen. Genet.* **203**, 230-236.

Melcher, U. (1990) Similarities between putative transport proteins of plant viruses. *J. Gen. Virol.* **71**, 1009-1018.

Melcher, U. (1993). HIV-1 proteinases as structural model of intercellular transport proteins of plant viruses. *J. Theor. Biol.* **162**, 61-74.

Meshi, T., Watanabe, Y., Saito, T., Sugimoto, A., Maeda, T. & Okada, Y. (1987). Function of the 30 Kd protein of tobacco mosaic virus: involvement in cell-to-cell movement and dispensability for replication. *EMBO J.* **6**, 2557-2563.

Meshi, T., Motoyoshi, F., Adachi, A., Watanabe, Y., Takamatsu, N. & Okada, Y. (1988). Two concomitant base substitutions in the putative replicase genes of tobacco mosaic virus confer the ability to overcome the effects of a tomato resistance gene, Tm-1. *EMBO J.* **7**, 1575-1580.

Meshi, T., Motoyoshi, F., Maeda, T., Yoshiwoka, S., Watanabe, H. & Okada, Y. (1989). Mutations in the tobacco mosaic virus 30-Kd protein gene overcome Tm-2 resistance in tomato. *Plant Cell* **1**, 515-522.

Mise, K., Allison, R. F., Janda, M. & Ahlquist, P. (1993). Bromovirus movement protein genes play a crucial role in host specificity. *J. Virol.* **67**, 2815-2823.

Motoyoshi, F. & Oshima, N. (1975). Infection with tobacco mosaic virus of leaf mesophyll protoplasts from susceptible and resistant lines of tomato. *J. Gen. Virol.* **29**, 81-91.

Mullis, K. B. & Faloona, F. A. (1987). Specific synthesis of DNA in vitro via polymerase-catalyzed chain reaction. *Meth. Enzymol.* **155**, 335-350.

Mushegian, A. R., Wolff, J. A., Richins, R. D. & Shepherd, R. J. (1995).

Molecular analysis of the essential and nonessential genetic elements in the genome of peanut chlorotic streak caulimovirus. *Virology* **206**, 823-834.

Noueiry, A. O., Lucas, W. J. & Gilbertson, R. L. (1994). Two proteins of a plant DNA virus coordinate nuclear and plasmodesmal transport. *Cell* **76**, 925-932.

Ogawa, M. & Sakai, F. (1984). A message RNA for tobacco mosaic virus coat protein in infected tobacco mesophyll protoplasts. *Phytopathol. Z.* **109**, 193-203.

Oshima, N., Goto, T. & Sato, R. (1962). A strain (TMV-C) of tobacco mosaic virus pathogenic to crucifer plants. *Hokkaido Natl. Agri. Exp. Stat. Res. Bull.* **78**, 74-80.

Padgett, H. S. & Beachy, R. N. (1993). Analysis of a tobacco mosaic virus strain capable of overcoming N gene-mediated resistance. *Plant Cell* **5**, 577-586.

Pascal, E., Sanderfoot, A. A., Ward, B. M., Medville, R., Turgeon, R. & Lazarowitz, S. G. (1994). The geminivirus BR1 movement protein binds single-stranded DNA and localizes to the cell nucleus. *Plant Cell* **6**, 995-1006.

Perbal, M. C., Thomas, C. L. & Maule, A. J. (1993). Cauliflower mosaic virus gene I product (P1) forms tubular structures which extend from the surface of infected protoplasts. *Virology* **195**, 281-285.

Richins, R. D., Donson, J., Lewandowski, D. J., Ducasse, D. A., Dawson, W. O. & Shepherd, R. J. (1993). Temperature-dependent complementation of movement-deficient mutants of tobacco mosaic virus by gene I of peanut chlorotic streak caulimovirus. *Phytopathology* **83**, 1349.

Rogers, S. O. & Bendich, A. J. (1988). Extraction of DNA from plant tissues. Plant Molecular Biology Manual A6, 1-10. Kluwer Academic Publishers, Dordrecht, Belgium.

Saito, T., Meshi, T., Takamatsu, N. & Okada, Y. (1987). Coat protein gene sequence of tobacco mosaic virus encodes a host response determinant. *Proc. Natl. Acad. Sci. USA* **84**, 6074-6077.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). Molecular Cloning. Second edition, Cold Spring Harbor Laboratory Press, New York.

Sela, I. (1981). Plant-virus interactions related to resistance and localization of viral infections. *Adv. Virus Res.* **26**, 201-237.

Siegel, A., Zaitlin, M. & Sehgal, O. P. (1962). The isolation of defective tobacco

mosaic virus strains. *Proc. Natl. Acad. Sci. USA* **48**, 1845-1851.

Takatsuji, H., Hirochika, H., Fukushi, T. & Ikeda, J. E. (1986). Expression of cauliflower mosaic virus reverse transcriptase in yeast. *Nature* (London) **319**, 240-243.

Taliansky, M. E., Malyshenko, S. I., Kaplan, I. B., Kondakova, O. A. & Atabekov J. G. (1992). Production of the tobacco mosaic virus (TMV) transport protein in transgenic plants is essential but insufficient for complementing foreign virus transport: a need for the full-length TMV genome or some other TMV-encoded product. *J. Gen. Virol.* **73**, 471-474.

Thomas, C. L., Perbal, C. & Maule, A. J. (1993). A mutation of cauliflower mosaic virus gene I interferes with virus movement but not virus replication. *Virology* **192**, 415-421.

Thomas, C. L. & Maule, A. J. (1995). Identification of structural domains within the cauliflower mosaic virus movement protein by scanning deletion mutagenesis and epitope tagging. *Plant Cell* **7**, 561-572.

Toh, H., Hayashida, H. & Miyata, T. (1983). Sequence homology between retroviral reverse transcriptase and putative polymerases of hepatitis B virus and cauliflower mosaic virus. *Nature* **305**, 827-829.

Tomenius, K., Clapham, D. & Meshi, T. (1987). Localization by immunogold cytochemistry of the virus-coded 30K protein in plasmodesmata of leaves infected with tobacco mosaic virus. *Virology* **160**, 363-371.

Tsuge, S., Kobayashi, K., Nakayashiki, H., Okuno, T. & Furusawa, I. (1994). Replication of cauliflower mosaic virus ORF 1 mutants in turnip protoplasts. *Annals of the Phytopathological Society of Japan* **60**, 27-35.

Waigmann, E., Lucas, W. J., Citovsky, V. & Zambryski, P. (1994). Direct functional assay for tobacco mosaic virus cell-to-cell movement protein and identification of a domain involved in increasing plasmodesmal permeability. *Proc. Natl. Acad. Sci. USA* **91**, 1433-1437.

Wolf, S., Deom, C. M., Beachy, R. N. & Lucas, W. J. (1989). Movement protein of tobacco mosaic virus modifies plasmodesmatal size exclusion limit. *Science* **246**, 377-379.

Woolston, C. J., Covey, S. N., Penswick, J. R. & Davies, J. W. (1983). Aphid transmission and a polypeptide are specified by a defined region of the cauliflower mosaic virus genome. *Gene* **23**, 15-23.

Yamanaka, T., Komatani, H., Ishikawa, M., Naito, S. & Ohno, T. (1994). Complete nucleotide sequence of a crucifer strain of tobacco mosaic virus, TMV-Cg. *DDBJ* **D3844.**

Young, N. D. & Zaitlin. M. (1986). An analysis of tobacco mosaic virus replicative structures synthesized in vitro. *Plant Mol. Biol.* **6**, 455-465.

Ziegler-Graff, V., Guilford, P. J. & Baulcombe, D. C. (1991). Tobacco rattle virus RNA-1 29K gene product potentiates viral movement and also affects symptom induction in tobacco. *Virology* **182**, 145-155.

VITA

Yuelin Zhang

Candidate for the Degree of

Doctor of Philosophy

Thesis: MOVEMENT OF A CAULIMOVIRUS AND TOBAMOVIRUSES

Major Field: Biochemistry and Molecular Biology

Biographical:

Personal Data: Born in Anhui, P.R. China, April 7, 1968, the son of Guoqi Zhang and Chunlan Xiong, married to Xin Li.

Education: Graduated from Tongchen High School, Anhui, P.R. China, in July, 1985; received Bachelor of Science Degree in Genetics and Genetic Engineering, Fudan University, Shanghai, P.R. China, in July, 1989; attended Graduate School, Institute of Biochemistry, Academia Sinica, Shanghai, P.R. China from September, 1989 to September, 1991; completed requirements for the Doctor of Philosophy Degree at Oklahoma State University in July, 1995.