COMPETITION OF TOBAMOVIRUSES

IN TOBACCO PROTOPLASTS

By

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TABLE OF CONTENTS

Chapter		Page
I.	INTRODUCTION AND LITERATURE REVIEW	1
	Introduction to Tobamovirus	1
	Classification of Tobamovirus	2
	Potential Hazards of Recombined Virus	3
	Competition of Two Viruses	4
II.	METHODS AND MATERIALS	6
	Construction of pTVCVrMPCP	6
	Synthesis of Movement Protein Probes	12
	Chemiluminescent Detection of RNA	13
	Culture of BY-2 Tobacco Suspension Cells	14
	Preparation of BY-2 Tobacco Protoplasts for Infection	15
	Viral Infection of Tobacco BY-2 Protoplasts	15
	Sample Collection of Infected Tobacco BY-2 Protoplasts	16
	SDS-PAGE	17
	Western Blotting for Detection of Tobamovirus Coat Protein	17
III.	RESULTS	20
	Construction of pTVCVrMP	20
	Infection of Tobacco Protoplasts by Single Virus Species	24
	Co-infection of Tobacco Protoplasts by Two Viral Species	31
	Detection of TMV and TVCV RNA signals	34
	Standard Curve of Western Blotting	35
IV.	DISCUSSION	37
	Feasibility of using western blotting as a quantitative method	37
	CP amount used as an index of viral ability of replication	38
	TMV4904TVCV does not replicate well in single cells	40
	Competition between TMV4904TVCV and wild type viruses	40
	Virus Chimera TVCVrMP replicates well in single cells	41
	Competition has limited effects on wild type virus TMV and TVCV	41
	Competition decreased the replication of TVCVrMP	43
	Virus chimera like TVCVrMP may not be hazardous	44
BIBLIOGRAPHY		45

LIST OF FIGURES

Figure		Page
1	Construction of pTVCVrMPCP	9
2	Digestion results of pRQJ1	20
3	Digestion results of pRQJ2 by *Kpn*I and *Hin*dIII	22
4	Digestion results of pRQJ2 by *Xho*I	23
5	Digestion results of pTVCVrMP	23
6	Photograph of tobacco protoplasts under microscope	24
7	TMV single infection	25
8	Amount of coat protein accumulated in TMV single infection	26
9	TVCV single infection	26
10	Amount of coat protein accumulated in TVCV single infection	27
11	Single infection of TVCVrMP	28
12	Amount of protein accumulated in TVCVrMP single infection	28
13	Single infection of TMV4904TVCV	29
14	Single infection of TVCVMPB	30
15	TMV,TVCV co-infection	31
16	Amount of coat protein accumulated by TMV and TVCV	32
17	TMV, TVCVrMP co-infection 1	33
18	TMV, TVCVrMP co-infection 2	33
19	Amount of coat protein accumulated by TMV and TVCVrMP	34
20	Detection of TMV and TVCV RNA	35
21	TVCV western blotting detection standard curve	36
22	Amount of coat protein accumulated by single infections	39
23	Amount of coat protein accumulated by TMV	42
24	Amount of coat protein accumulated by TVCV	43
25	Amount of coat protein accumulated by TVCVrMP	44

CHAPTER I

Introduction and Literature Review

Tobacco mosaic virus (TMV) is the type-member of the tobamoviruses, which are characterized by their rod-shaped virions, single positive-sense RNA genomes and single species of capsid protein.[1] The 6.4 kilonucleotides (knt) RNA genome of TMV encodes at least four polypeptides. The 5-most gene produces two polypeptides --- the 126K/183K polypeptides[2], in which the longer polypeptide is produced by suppression of termination at a single UAG stop codon near the middle of the genome[1]. The 126K polypeptide contains at least two domains. The first one is recognized as responsible for the methyltransferase (MT) activity in RNA capping. The second domain is predicted as RNA helicase (Hel)[3,4,5]. The 183K polypeptide contains an additional domain identified as an RNA dependent RNA polymerase (RdRP)[6]. Two 3 co-terminal subgenomic RNAs are responsible for the other proteins. The longer subgenomic RNA serves as mRNA for a 30K polypeptide movement protein (MP) which is responsible for cell-to-cell movement[7,8]. The shorter subgenomic RNA is the mRNA for 17K coat protein (CP) polypeptide. There is evidence that the coat protein is required for virus long distance movement as well as encapsidation[9].

Turnip vein-clearing virus (TVCV) is a tobamovirus that causes disease in crucifers[10]. The TVCV causes vein clearing in *Brassica rapa* (turnip) and a mosaic in *Nicotiana tabacum* (tobacco). TVCV particles contain a single 6.3 knt RNA strand and a single species of 17kD coat protein[11]. The TVCV genome is about 60% identical in sequence to those of most other tobamoviruses whose sequence has been obtained[11]. Electron microscopy of TVCV viral particles revealed rigid rod-shaped[11] virions. The genomic organization of TVCV is similar to that of TMV. The 5'most polypeptide translated from its RNA genome is the 125/182 kD polypeptide. A reasonable suggestion is that the 182kD polypeptide could be obtained by a suppression of a UAG stop codon. The movement protein and the coat protein are obtained from 3'-half of the genome[12]. This is typical for tobamoviruses.

Tobamoviruses can be divided into three subgroups[13,14]. The divisions are based on the location of the virion assembly origin, the structure of the junction between MP and CP open reading frames (ORFs), and clustering in phylogenetic analysis. Subgroup 1 viruses originated predominantly from solanaceous hosts. Tobacco mosaic virus is within subgroup 1 viruses. Subgroup 2 viruses include sunn-hemp mosaic virus (SHMV) and cucumber green mottle mosaic virus (CGMMV). Subgroup 3 viruses are crucifer-infecting viruses including TVCV. One exception for subgroup 3 viruses was isolated from garlic[15]. Subgroup 1 and subgroup 3 were much less distant from one another than they were from subgroup 2. There is a hypothesis that subgroup 3 viruses evolved from a subgroup 1 ancestor adapted to crucifer infection[14].

Comparison of TMV with TVCV shows about 60% similarity in sequence yet with some major differences. One difference is at the junction of *MP* and *CP* genes. The TVCV genes overlapped 25 codons while the TMV genes have no overlap[12]. The sizes of MP protein for both viruses are similar, though coat protein for TVCV and TMV are distinguishable in SDS-PAGE. In SDS-PAGE, the TVCV coat protein migrates slightly faster than the TMV counterpart[11].

Plants can be genetically engineered to carry parts of viral genomes in the plants' genome. Such transgenic plants may be resistant to infection by the same virus whose genome was engineered as a part of the plant's genome[16,17]. This is a desirable trait, especially for crops. The engineered crop may be immune to certain kinds of viral diseases. Meanwhile, concerns about possible recombination of a second virus infecting the transgenic plants with the integrated viral genome were raised[18,19]. This viral recombination may result in generating new virus species, which could be more virulent and hazardous than either of the original viruses[20].

TMV and TVCV provide us a chance to test the risks of viral recombination. The risks of viral recombination are a combination of two aspects. The first one is the frequency of possible recombination events. The second one is the hazards associated with the recombined genome. The frequency of recombination between an infecting viral genome and a transgene is generally considered sufficiently high in natural infections. However, not much evidence has been provided to clarify the hazards associated with

recombined genomes. Chimeric viruses made from TMV and ToMV (tomato mosaic virus) successfully induced infection[21]. Both TMV and ToMV belong to the same subgroup (subgroup1) of tobamoviruses. They are more than 80% identical in nucleotide sequence. In contrast, TVCV belongs to tobamovirus subgroup 3 and has less than 60% identity in nucleotide sequence with TMV. A virus chimeric between TMV and TVCV named TVCVrMP, which has the TVCV *MP* gene replaced by TMV's, can infect tobacco plants successfully[22].

A virus' abilities to infect plants are not only related to its ability to replicate within a host cell, but to its ability to spread to neighboring cells, to spread throughout the plant, and to transfer from one plant to another as well. In other words, all abilities, including replication in single cells, local movement, and long distance movement are important for a successful systemic viral infection. Thus, an appearance of inability to infect one plant species can result from deficiency in replication, local movement, or long distance movement. In contrasts, an infection using plant protoplasts instead of the whole plant, on the contrary, involves the ability of replication only. Results of infection of protoplasts can demonstrate the viruses abilities of replication.

To measure the potential hazards of a virus chimera, it is important to know how well the virus chimera replicates when it competes with other viral genomes, especially with a wild type virus. An experiment of co-infection of the protoplasts with two different viruses was designed to test the potential hazards. One virus used in co-infection was a wild type virus, either TVCV or TMV. The second one was the chimeric virus of

interest. The potential hazard can be measured as relative fitness[23], which is the ratio of quantities of two genomes after one generation divided by the input ratio.

In my research work, TMV, TVCV, and viruses chimeric between TMV and TVCV were used to infect tobacco protoplasts to determine their ability of replication in single tobacco cells. Experiments based on single virus infection and co-infection by two different viruses have been performed. The amount of coat protein from the infected protoplasts sample has been measured. Results suggest that the hazard of recombined genomes is low.

CHAPTER II

Methods and Materials

Construction of Plasmid pTVCVrMPCP

TVCVrMPCP is a RNA virus chimeric between TMV and TVCV. The full cDNA clone of TVCVrMPCP is designated as pTVCVrMPCP and has a pBluesciptSK+ vector portion. Plasmid pSNC065 was obtained from Dr. Dawson's lab[25]. This plasmid is a full clone of TMV cDNA with an engineered *Xho*I site right after the CP gene. All transformations performed in construction of pTVCVrMPCP are with *E. coli* DH5α chemically competent cells prepared by Rb-Ca method (Fraij, B., unpublished). All DNA fragments obtained from restriction enzyme digestion were purified with QIAEX II gel extraction kit (Qiagen) after gel electrophoresis. The process is shown as fig. 1.

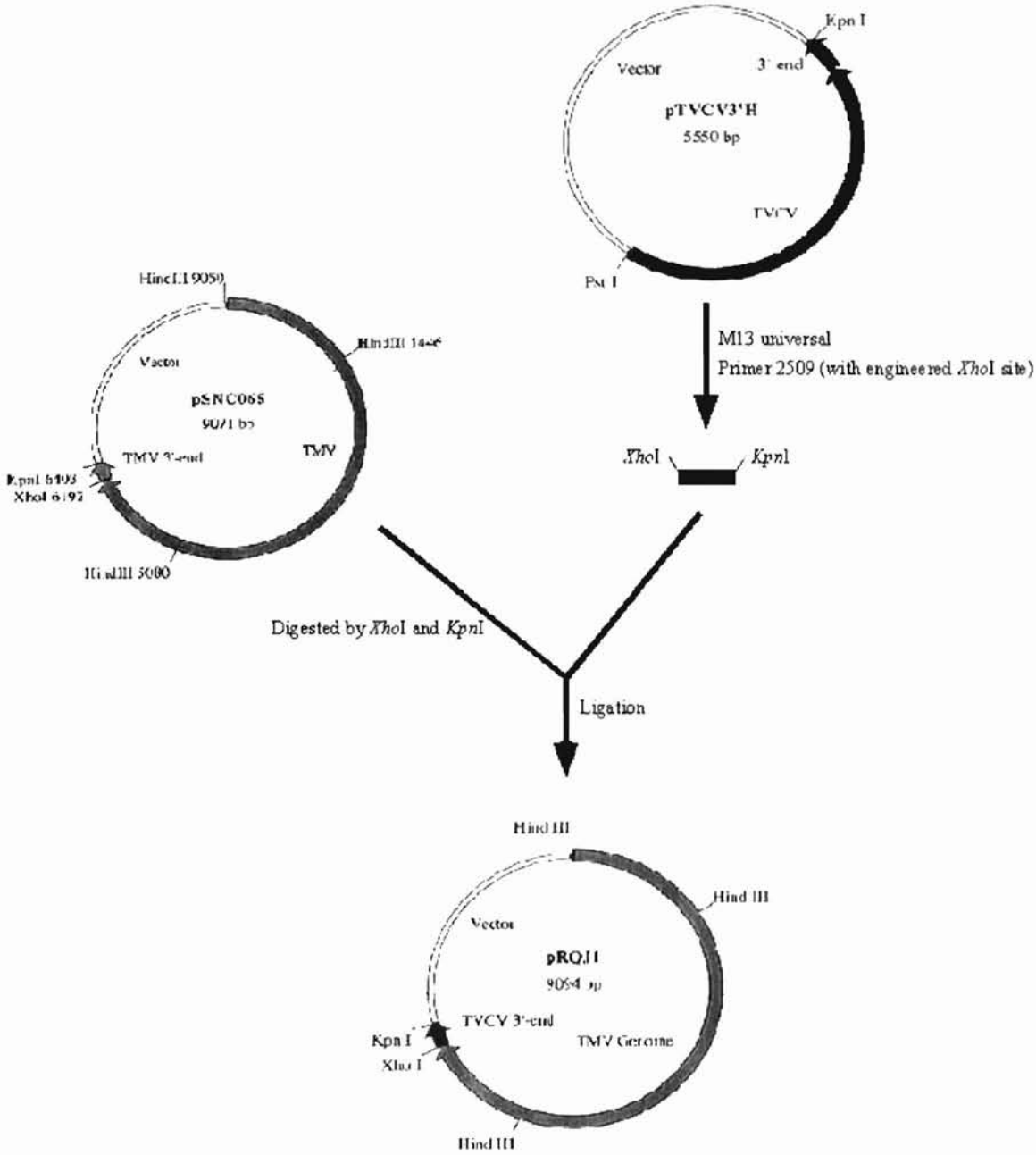
The 3'-end non-translated portion of TVCVrMPCP was obtained from a PCR reaction. Primer M13 forward (GTAAAACGACGGCCAGT) sequence is located in the vector pBluescriptSK+, 3' to the DNA fragment. Primer 2509 (CTTCTTAGCTCGAGTGCG) contained a mutation that created a *Xho*I site. PCR

reactions were performed in a 25 µl reaction with 20 ng primer M13 forward, 12.95 ng primer 2509, 1.5 mM $MgCl_2$, 200 nmol dNTPs, 260 ng pTVCV3'H[24] plasmid DNA and 5 units *Taq* polymerase (Promega). The PCR product was then digested by *Xho*I and *Kpn*I. Plasmid pSNC065 was also digested with *Xho*I and *Kpn*I. The PCR product was then ligated with the 9.0 kb fragment from pSNC065 digestion. This partial clone was screened and checked by enzyme digestion patterns (*Fsp*I and *Sma*I separately) and named as pRQJ1.

A 1.3 kb fragment was selected from four fragments by digestion of pRQJ1 with *Hin*dIII and *Kpn*I. A 4.3 kb fragment was obtained from pTZ171[24] digested by the same enzymes. Plasmid pTZ171 has a TVCV fragment from the *Pst*I site in the *RdRP* gene to an engineered *Xho*I site at the junction of *MP* and *RdRP* followed by a TMV fragment from the junction of *MP* and *RdRP* to the 3'-untranslated end. The 1.3 kb and 4.3 kb fragments were then gel purified and ligated together to yield plasmid pRQJ2. This second partial clone was checked by its restriction enzyme digestion pattern (*Kpn*I/*Hin*dIII and *Xho*I separately). Plasmid pRQJ2 has TMV's *MP/CP* genes and TVCV's non-translated 3'-end and part of its *RdRP* gene.

The final clone pTVCVrMPCP was obtained from an exchange of a *Pst*I-*Kpn*I fragment between pRQJ2 and pTVCV50, which is the full length clone of wild type TVCV. The plasmid pTVCVrMPCP was confirmed by the restriction digestion pattern (*Xho*I). The 3'-end fragment derived from the PCR reaction was then sequenced with primer M13 forward.

Fig 1. Construction of pTVCVrMPCP. White portion of the plasmids were vector parts. Shaded portions were from TMV genome. The solid black portions were from TVCV genome.


Kpn I
Vector
3' end
pTVCV3'H
5550 bp
TVCV
Pst I
M13 universal
Primer 2509 (with engineered XhoI site)
XhoI
KpnI
HincII 9050
Vector
pSNC065
9071 bp
TMV
TMV 3'-end
KpnI 6193
XhoI 6192
HindIII 5000
Digested by XhoI and KpnI
Ligation
Hind III
Vector
pRQJ1
9054 bp
TVCV 3'-end
TMV Genome
Kpn I
Xho I
Hind III

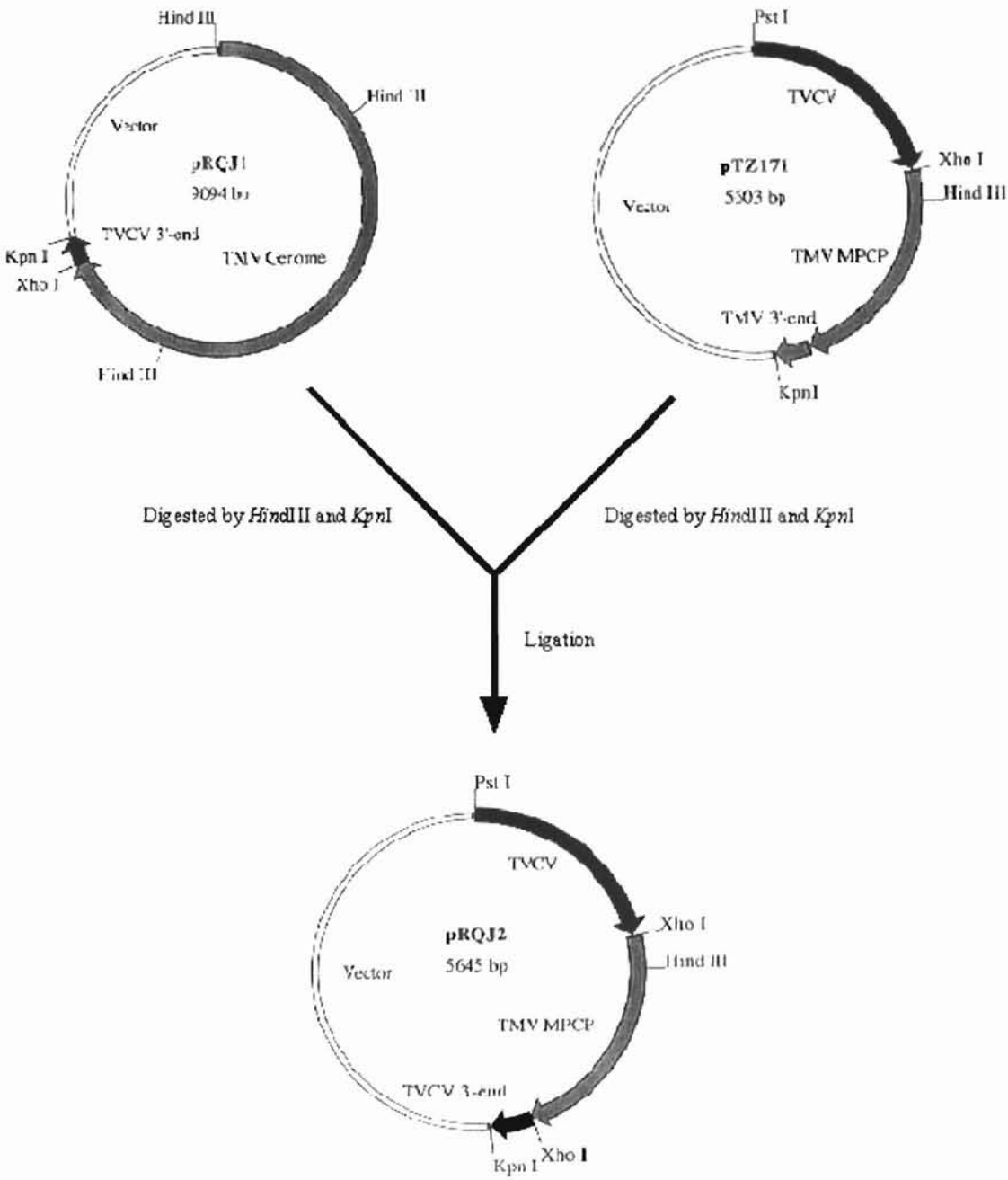


Fig 1. Construction of pTVCVrMPCP (continued)

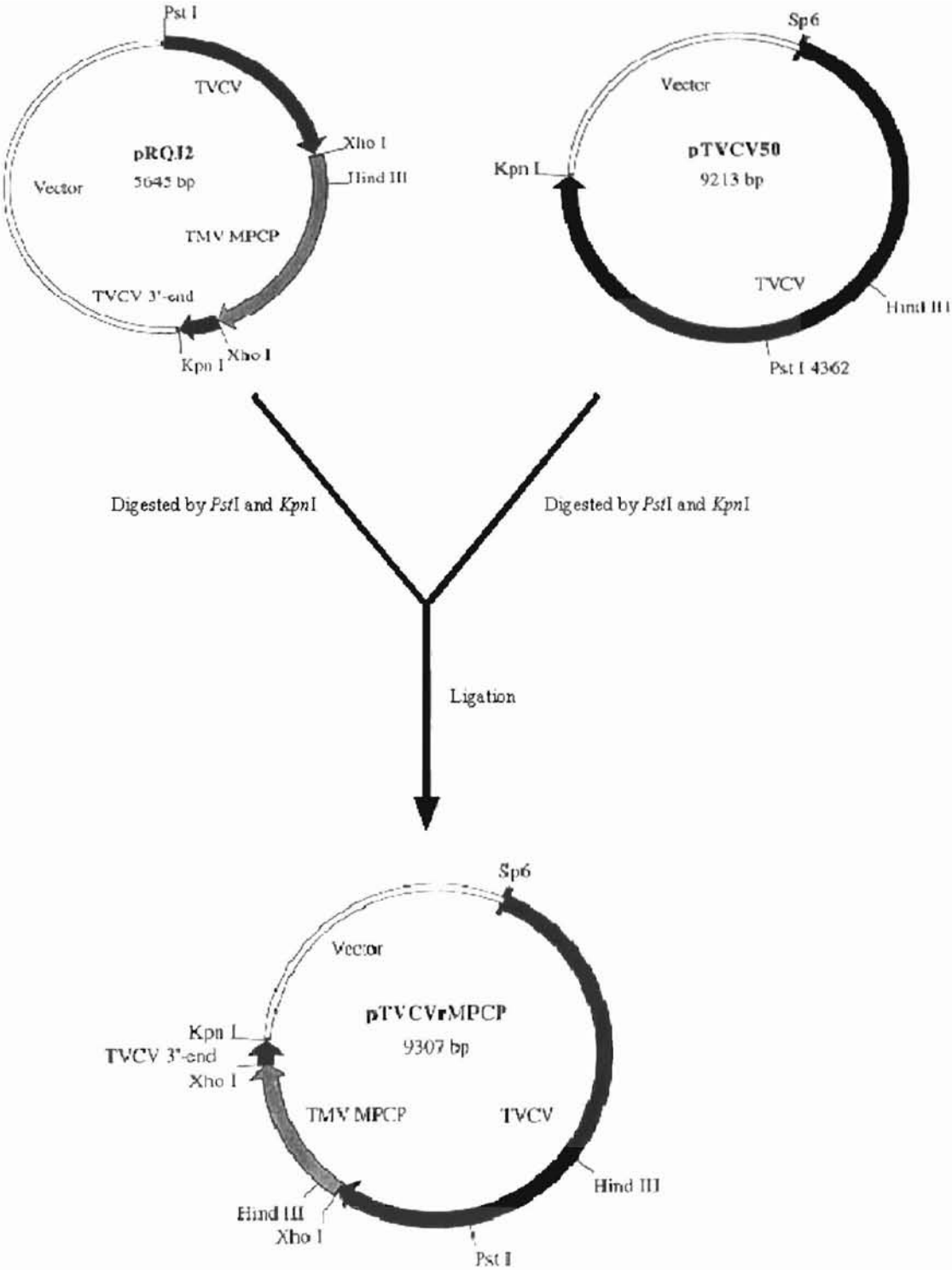


Fig 1. Construction of pTVCVrMPCP (continued 2)

Synthesis of Movement Protein Probes

Movement protein probes were used in RNA spot blotting. Two probes were synthesized by different methods. The probe for TMV was synthesized with the random hexamer method; the probe for TVCV was synthesized by a PCR method. Both probes were non-radioactively labeled by digoxigenin(dig)-dUTP (Boehringer-Mannheim).

Plasmid pMP30[25] could be used to generate the TMV MP probe. The plasmid was digested with *Pst*I and *Hin*dIII to produce the desired fragment. The digestion produced a 1.3 kb fragment, which consisted of a 0.8 kb MP segment, and a 0.5 kb vector segment. The DNA fragment was purified by gel electrophoresis and extraction. The probe was synthesized in a 20 µl volume reaction with Genius 2 kit (Boehringer-Mannheim) as described by the manufacturer. After incubation at 37 °C overnight, the reaction was stopped by adding 2 µl 0.2 M EDTA.

The TVCV movement protein probe was generated by a PCR reaction. The reaction was conducted in a 25 µl volume with 2 mM $MgCl_2$, 400 ng each primer 1392 (GGCTCTAGATGAGATGTCGATAGTCTCGTA) and primer 1393 (GCCGCTCGAGGTTAAGCATTGGTAT), 400 µM dNTPs (dig-dUTP: dTTP=1:3), 130 ng template DNA pTVCV50, and 2.5 units *Taq* polymerase enzyme. The reaction mixture was denature by preincubation of at 95°C for 5 minutes. The reaction consisted of 25 cycles of denaturation at 92 °C, 45 seconds; annealing at 52 °C, 45 seconds; and polymerization at 72 °C, 60 seconds.

Newly synthesized probes were precipitated by 1/10 volumes 4 M LiCl and 2 volumes absolute ethanol. The mixture was incubated at –70 °C for 30 minutes and centrifuged at 4 °C, 30 minutes, 14,000 × g in a tabletop centrifuge. The pellet was washed with 70% ethanol and dissolved in 50 µl TE/SDS.

Chemiluminescent Detection of RNA

This method is modified from the protocol obtained from Dr. John Cushman's lab (Department of Biochemistry, Oklahoma State University).

Samples were pipetted to a positively charged nylon membrane (MBI). The membrane was then wetted with a small amount of 2 × SSC. UV cross-linking was performed afterwards in a Stratalinker (Stratagene) with 120-mjoules energy level. For blocking, the membrane was incubated at least 2 hours at 42 °C with gentle agitation in the northern prehybridization solution (5 × SSC, 50% formamide, 0.2% SDS, 0.1% N-lauroyl sarcosine, 2% blocking reagent (Boehringer-Mannheim) in maleate buffer (100 mM maleic acid, pH 7.5, 150 mM NaCl)). Probes were denatured in a boiling water bath for 10 minutes and quickly put into ice. The membrane was then hybridized with TMV MP probe or TVCV MP probe as needed overnight at 42 °C with gentle agitation in a sealed plastic bag. Probes were diluted 1:500 – 1:1,000 in northern prehybridization solution before hybridization. After hybridization, the membrane was washed twice in

Wash I (2 × SSC, 0.1% SDS) at room temperature; twice in Wash II (0.5 × SSC, 0.1% SDS) at 65°C; and once in maleate buffer at room temperature. The membrane was then incubated with northern blocking solution (2% blocking reagent in maleate buffer). Anti-dig antibody conjugated with alkaline phosphatase (Boehringer-Mannheim) was then applied to the membrane in northern blocking solution with dilution from 1:5,000 to 1:10,000. After 30 minutes incubation, the membrane was washed twice with maleate buffer at room temperature. The membrane was then washed by AP buffer (100 mM Tris•HCl, pH 9.5, 100 mM NaCl, 100 mM $MgCl_2$). The substrate for alkaline phosphatase was either Lumi-Phos 530 or CSPD Ready-to-Use (both from Boehringer-Mannheim). After 10 minutes incubation, an X-ray film was exposed for 1 hour initially. The exposure time ranged from 30 minutes to 36 hours according to need.

Culture of BY-2 Tobacco Suspension Cell

BY-2 cells were cultured in 100 ml Tobacco BY-2 Medium (4.3 g/l Murashige & Skoog salt (Gibco BRL), 100 mg/l myo-inositol, 1 mg/l thiamine, 0.2 mg/l 2,4-dichlorophenoxyacetic acid, 255 mg/l KH_2PO_4, 30 g/l sucrose, pH 5.0) in an Erlenmeyer Flask. After 7 days of growth, the cells were transferred 1:100 into new media. The day of transference counted as day 1. The cells grew at room temperature with gentle shaking at 100 rpm.

Preparation of BY-2 Tobacco Protoplasts for Infection

BY-2 tobacco suspension cells that had grown for 5 to 8 days were used for protoplast preparation. Generally, 10 – 15 ml 7-day old BY-2 cells were used. BY-2 cells were collected by sedimentation in a clinical centrifuge at 460 × g for 10 minutes. The cells were digested in cell wall enzyme digestion mixture (0.35 mM mannitol, pH 5.0, 10 mg/ml Onozuka cellulase RS, 1 mg/ml macerase (Calbiochem)) at 30°C for about 3 hours with gentle shaking at 75 rpm. The needed amount of enzymes was weighed just before the digestion and dissolved in mannitol solution. The enzyme mixture was then forced through a 0.45 μm disposable filter for sterilization. The yield of protoplasts was checked under a microscope after 3 hour's digestion. The digestion procedure has been prolonged for an additional 30 minutes to 1 hour if the yield was not ideal (less than 50% cells became protoplasts). After digestion, the cells were forced through a cotton filter (1 cm sterile cotton packed loosely in a 5 or 10 ml syringe) to eliminate tobacco cells with cell wall residues. The protoplasts were then washed three times with protoplast culture media (tobacco BY-2 media supplemented with 0.35 mM mannitol) by sedimentation in a clinical centrifuge at 278 × g and resuspension. The protoplasts obtained from this procedure were used immediately for virus infection. Number of protoplasts were counted using a hemacytometer (Hausser scientific company).

Viral Infection of Tobacco BY-2 Protoplasts

Tobacco BY-2 protoplasts were prepared by the method described above. At least 1.0×10^6 protoplasts were collected by centrifugation for 5 minutes at 278 × g in a clinical centrifuge. Supernatants were removed by pipetting slowly. A very small amount of supernatant was left atop the pellet. In the case of single infection, 15 µg virions were then mixed with the resuspended protoplasts. In the case of co-infection with two viruses, 15 µg virions of were used for each virus. The protoplasts were then treated with 500 µl PEG solution (40% PEG (polyethylene glycol), MW=1,450, 3 mM $CaCl_2$, 3 mM MES•NaOH (2-[N-morpholine]ethanesulfonic acid) , pH 5.5) for 15 seconds. After PEG treatment, the protoplasts were incubated on ice for 15 minutes with 5.0 ml of 0.7 M mannitol. The protoplasts were then washed three times with protoplast culture media by sedimentation in a clinical centrifuge at 278 × g and resuspension. After infection, the protoplasts were cultured in 3 ml protoplast culture medium with 50 µg/ml carbenicillin in a 35 mm × 10 mm petri dish atop 1.5% agar-protoplast culture media.

Sample Collection of Infected Tobacco BY-2 Protoplasts

Samples of virus infected tobacco protoplasts were collected during a time range from 0 hour post infection to 108 hours post infection. Usually, one sample was collected each

day. For each sample, 500 µl culture medium with protoplasts were withdrawn. 2 volumes of ethanol and 1/100 volume of 2 M sodium acetate, pH 5.2, were then added into the sample. The sample was stored at –20 °C for at least 30 minutes and centrifuged at 14,000 × g for 15 minutes. The pellet was washed once with 70% ethanol, dried in a Speedvac (Savant sc110A) and dissolved in 42 µl water and 8 µl 5 × SDS-gel loading buffer (50% glycerol, 25% β-mercaptoethanol, 10% SDS (sodium dodecyl sulfate), 0.1% bromphenol blue, 0.31 M Tris•HCl). Samples were denatured in a boiling water bath for 10 minutes and quickly put into ice. The sample was then ready for SDS-PAGE (polyacrylamide gel electropheresis) gel electrophoresis and stored at –20 °C.

SDS-PAGE

Polyacrylamide gels (10.16 mm × 8.26 mm × 0.5 mm) used in the experiments had a discontinuous buffer system[26] with 6% acrylamide in the stacking gel and 15% acrylamide in the separating gel. 10 µl (1/5 of total sample volume) samples were loaded per well. The electrophoresis was performed for about 75 minutes at a constant current of 20 mA.

Western Blotting for Detection of Tobamovirus Coat Protein

To detect the coat protein of tobamoviruses, samples were prepared and separated by SDS-PAGE as described above. Samples were transferred to a PVDF membrane (either Immnu-blot or Sequi-blot from Bio-Rad) in a PolyBlot transfer system (American Bionetics). Two Whatman filter papers saturated with cathode buffer (25 mM Tris, 40 mM ε-amino-N-hexanoic acid, 20% methanol) were placed in between the cathode and the membrane. One Whatman filter paper saturated with anode buffer II (25 mM Tris, pH 10.4, 20% methanol) was placed atop the membrane on the anode side. Another Whatman filter paper saturated with anode buffer I (0.3 mM Tris, pH 10.4, 20% methanol) was placed between the anode buffer II paper and the anode. The PVDF membrane was treated with methanol and rinsed with deionized water before the transfer. The transfer was run at 2.5 mA per square centimeter of membrane area for 30 minutes. After transferring, the membrane was incubated in TBS (10 mM Tris, pH 7.4, 150 mM NaCl) with 5% skim milk at room temperature for one hour and then incubated with primary antibody solution (TBS, 1% skim milk, 0.02% azide, rabbit anti-TMV CP 1:75 or rabbit anti-TVCV CP 1:75 sera[10]) at 4°C overnight with shaking. The membrane was then washed once with TBS and twice with TBS / 0.5% Tween-20. After a brief blocking incubation in TBS / 5% skim milk for one minute, the membrane was incubated with second antibody solution (TBS, 0.125% skim milk, 1:2,500 goat anti-rabbit antibody conjugated with alkaline phosphatase (Jackson Immunotechnology)) for 2 hours at room temperature with shaking. The membrane was then washed in TBS once, TBS / 0.5% Tween-20 twice and TBS twice. All the wash steps were performed at room temperature

for 5 minutes with gentle shaking at 100 rpm. The membrane was developed at 35 °C in developing solution (AP buffer as described in chemiluminescent detection of RNA, 1:150 (v:v) 50 mg/ml nitroblue tetrazolium in 70% DMF (N,N-dimethyl formamide), 1:300 (v:v) 50mg/ml 5-bromo-4-chloro-3-indolyl-phosphate in DMF).

CHAPTER III

Results

Construction of pTVCVrMPCP

1. Digestion pattern of pRQJ1

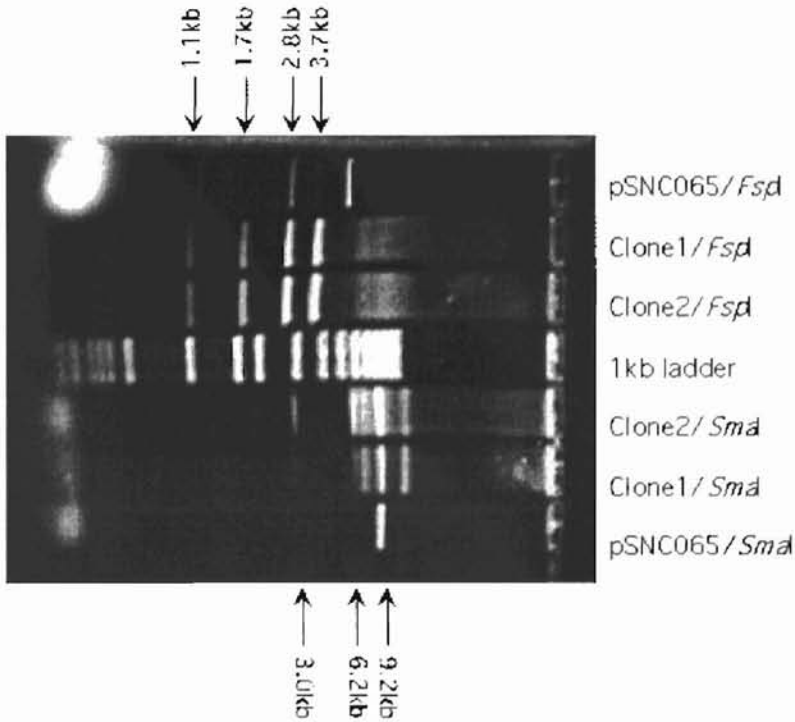


Fig 2. Digestion results of pRQJ1

Clone 1 and clone 2 were two candidate clones for plasmid pRQJ1 obtained by ligating the *Kpn*I + *Xho*I digestion fragments of pSNC065 and the PCR product from pTVCV3'H. Both clone 1 and clone 2 were the desired clone. Clone 2 was named pRQJ1 and used in the later construction. As described in the methods section, pSNC065 was digested by *Kpn*I and *Xho*I, as was the PCR product from pTVCV3'H. There was a *Fsp*I site in the PCR fragment. There were three additional *Fsp*I sites in pSNC065. Thus, pRQJ1 had four *Fsp*I sites. A digestion of pRQJ1 with *Fsp*I generated four fragments, with the size of 1.1 kb, 1.7kb, 2.8kb, and 3.7kb, respectively. Another check was performed using enzyme *Sma*I. The pSNC065 and PCR product both had one *Sma*I site. Using *Sma*I to digest pRQJ1 showed more than two bands. The smaller two bands were the 3.0 kb and 6.2 kb digestion fragments, the third band was the 9.2 kb not completely digested plasmid band. An additional fourth band was the open circle conformation of the plasmid. In contrast, digestion of pSNC065 gave only one band at about 9.2 kb.

2. Digestion pattern of pRQJ2

As shown in fig 3, all clones from clone 1 to clone 8 were desired plasmids. The pRQJ2 digested by *Hin*dIII and *Kpn*I produced two fragments, whose lengths were about 1.3 kb and about 4.3 kb. The 1.3 kb fragment was from digestion of pRQJ1 by the same enzymes. The 4.3 kb fragment was from digestion of same two enzymes of pTZ171. The pRQJ2 was also checked by digestion with *Xho*I as in fig 4.

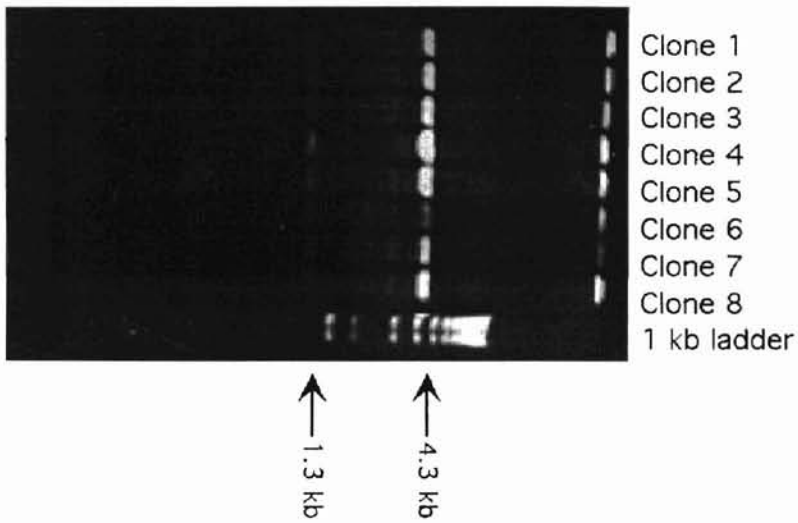


Fig 3. Digestion results of pRQJ2by *Kpn*I and *Hin*dIII

Three of the eight possible clones in the same experiment were also digested by *Xho*I. The results are showed in fig 4. All of them had the right pattern, a 1.3 kb fragment plus a 4.3 kb fragment. Clone 1 was named pRQJ2 and used in the later construction.

3. Digestion pattern of pTVCVrMPCP

The final clone, pTVCVrMPCP, was checked by the *Xho*I digestion pattern. The correct clone contains two *Xho*I sites. One was from the inserted fragment of *Pst*I-*Kpn*I digestion of pRQJ2. The other one was from the fragment of *Pst*I-*Kpn*I digestion of pTVCV50[22]. Using *Xho*I to digest pTVCVrMPCP generated two fragments, 2.7 kb and 6.3 kb. The results showed that clone 2 was not the correct clone, while clones 1, 3, and 4 were correct. Clone 1 was named pTVCVrMPCP.

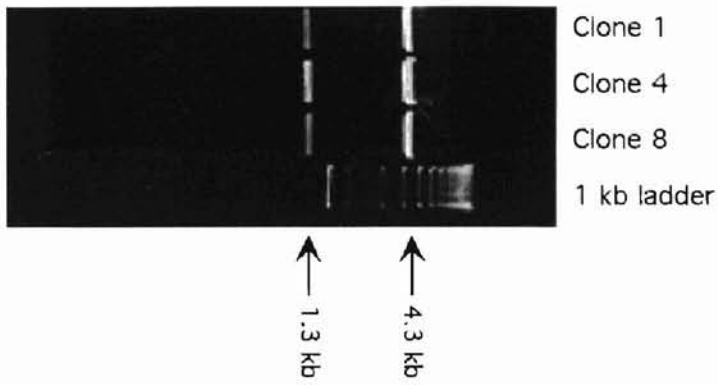


Fig 4. Digestion results of pRQJ2 by *Xho*I

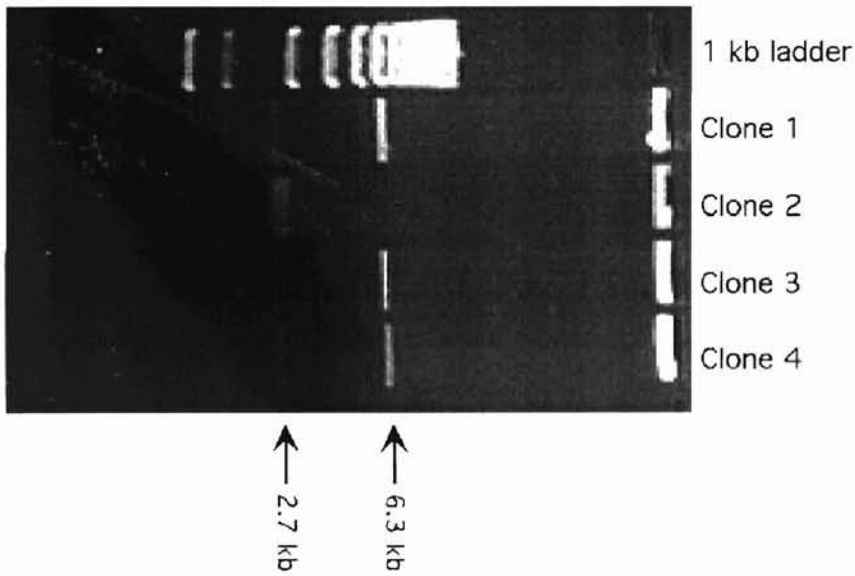


Fig 5. Digestion results of pTVCVrMP by *Xho*I

Infection of tobacco protoplasts by single virus species

1. Photograph of tobacco protoplasts made from BY-2 suspension cells

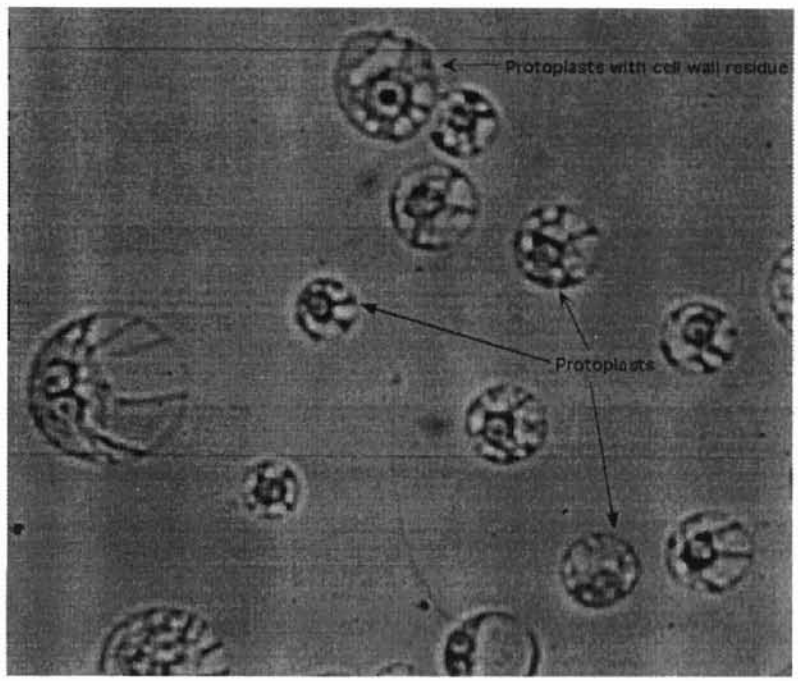


Fig 6. Photograph of tobacco protoplasts under microscope

Tobacco BY-2 suspension culture cells shown in fig 6 had been digested for 4 hours under the conditions described in the methods section. Most of the cells appeared to be fully digested. They were perfectly round. One cell at the top of the photograph was slightly irregular in shape, which was interpreted as meaning there was some cell wall residue left. The yield in this experiment was higher than in a typical experiment, in which it was about 60 – 70%. A preliminary experiment showed under the experimental condition, about 60% infected protoplasts survived by 18 hpi.

2. Western blotting results with TMV single infection

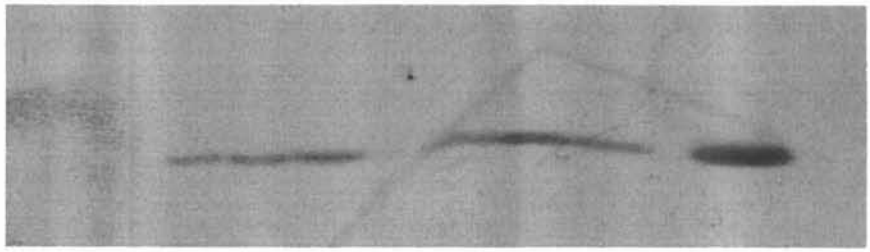


Fig 7. TMV single infection. TMV coat protein was detected by TMV anti-serum. The control (TMV) was 100 ng TMV virions. Times post infection when the corresponding samples were collected are indicated. Lane labels ending with 's' identify the samples from supernatants at a given time. The amount of coat protein used in data analysis was the sum of the amounts of coat protein in supernatant and in pellet.

The amount of coat protein accumulated when TMV infected protoplasts singly is shown in fig 8. The total amount of TMV coat protein accumulated was about 50 ng virions per 2.0×10^5 tobacco protoplasts at 92 hpi (hours post infection). The second data point which was collected at 12 hours post infection showed little accumulation of coat protein. The amount of coat protein accumulated at 12 hpi was about 5 ng virions. As shown in fig 7, there was some coat protein in the supernatants. In all the results shown in subsequent figures, coat proteins in supernatant were included in the experiment samples. The data point at 108 hpi was omitted because the sample collected had a smaller volume. Same omissions were made in the later data sets. Two separate experiments were performed.

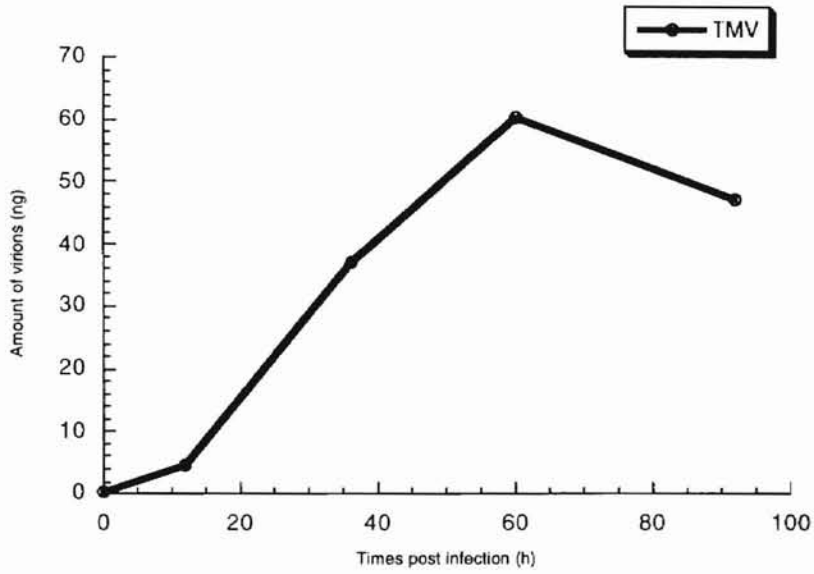


Fig 8. Amount of coat protein accumulated in TMV single infection

3. Western blotting results with TVCV single infection

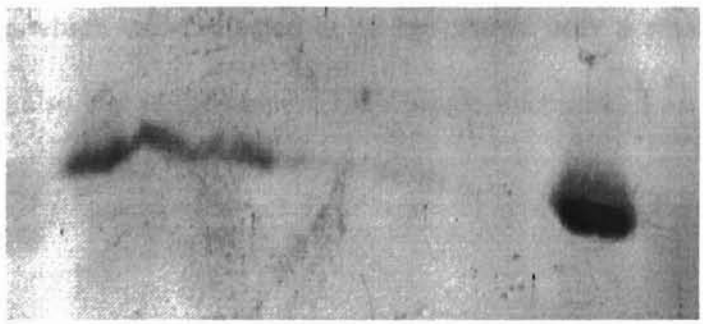


Fig 9. TVCV single infection. The TVCV coat protein was detected by western blotting using anti-TVCV serum. The positive control (TVCV) was 100 ng TVCV virions. Times post infection when the samples were collected are indicated.

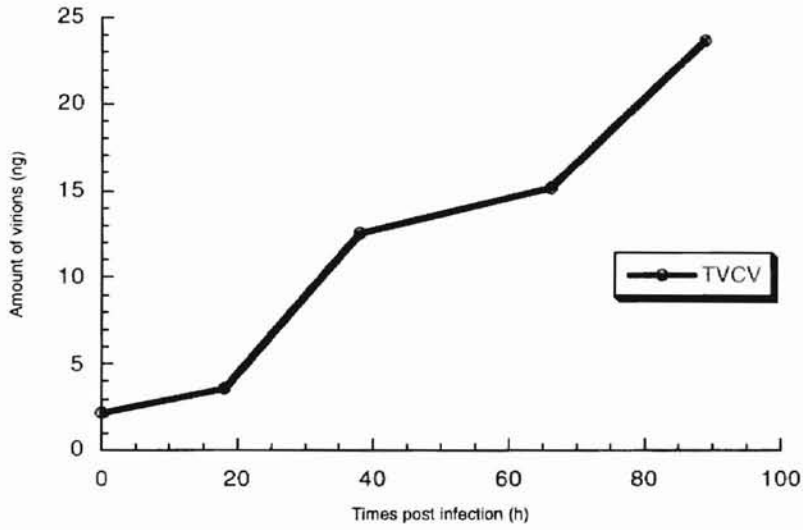
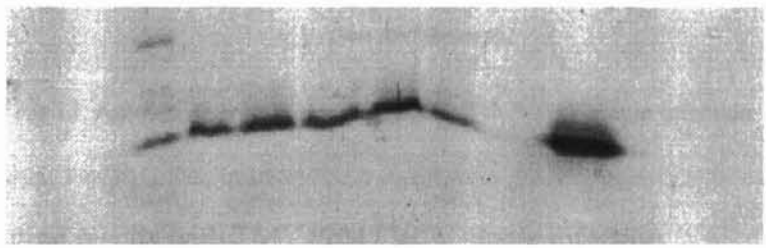


Fig 10. Amount of coat protein accumulated in TVCV single infection.

The total amount of coat protein accumulated by TVCV single infection is about 25 ng of TVCV virions. This amount is about half the amount of TMV accumulated. The second data point which was collected at 18 hpi shows only a small amount of coat protein. This was also true in the case of TMV single infection. Two experiments were performed.

4. Western blotting results with TVCVrMP single infection

The total amount of coat protein accumulated by TVCVrMP single infection was about 25 ng of TVCV virions. TVCVrMP has the TVCV coat protein gene. This amount of coat protein was roughly equal to the amount of coat protein accumulated by TVCV. Also, as noticed, the second data point which was collected at 18 hpi shows that TVCVrMP had accumulated more than 50% of its total final accumulated coat proteins. This is different from what was observed in TMV and TVCV single infection. Only one experiment was performed.



0h 18h 42h 72h 88h 108h TVCV

Fig 11. Single infection of TVCVrMP. Virus chimera TVCVrMP has the TVCV coat protein. The results were detected by western blotting using anti-TVCV serum. The positive control (TVCV) was 100 ng TVCV virions. Times post infection when the corresponding samples were collected are indicated.

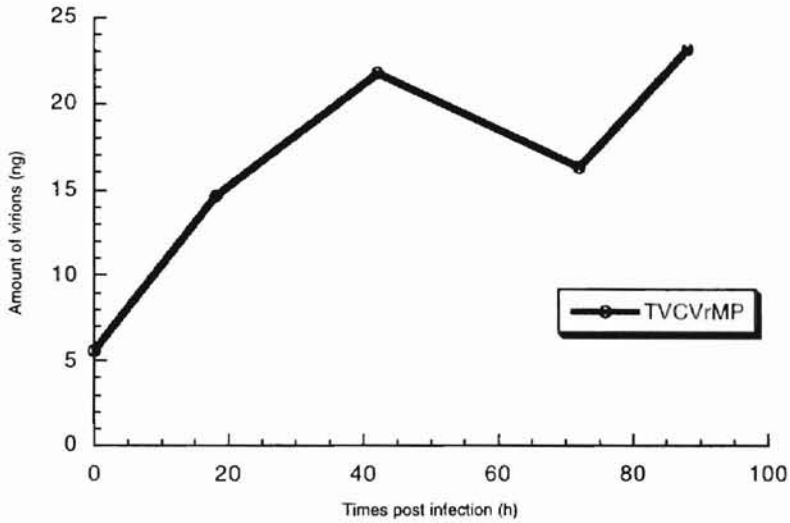


Fig 12. Amount of protein accumulated in TVCVrMP single infection

5. Western blotting results with TMV4904TVCV single infection

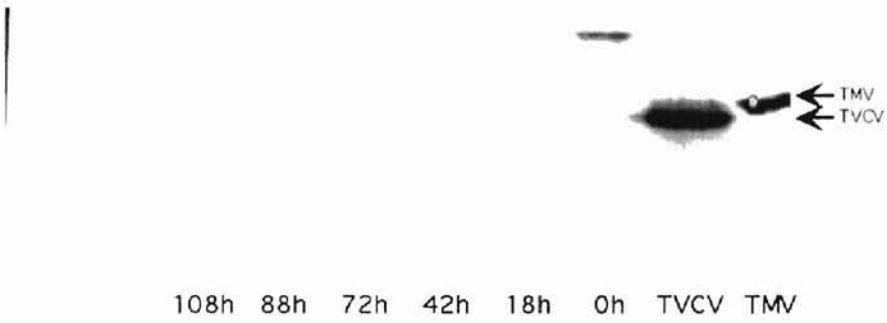


Fig 13. Single infection of TMV4904TVCV

TMV4904TVCV is a virus chimera between TMV and TVCV[22]. The number 4904 indicates the position where the two genomes were linked. From nucleotide position 1 to position 4904, the nucleotide sequence was identical with that of TMV. The gene for the126/183 kD polypeptide from TMV was located entirely in this region. The sequence

from position 4904 to the end was identical to that of TVCV. This region contains the *MP* and *CP* genes. The position 4904 is the junction between TMV *RdRP* gene and TVCV *MP* gene. An engineered *Xho*I site was used to link the two halves. The results were detected by western blotting using anit-TVCV serum. No coat protein was accumulated by TMV4904TVCV. Only one experiment was performed.

6. Western blotting results with TVCVMPB single infection

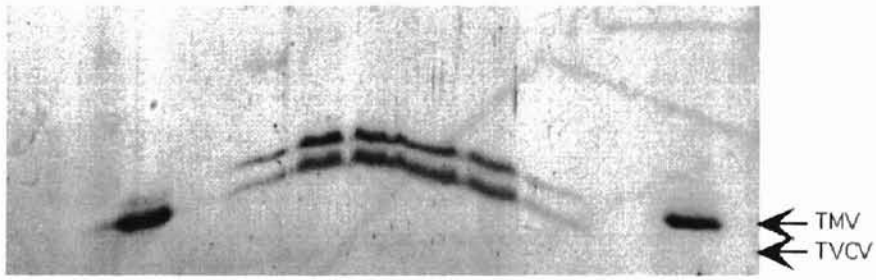
TVCVMPB is a virus based on TVCV[27]. The *MP* gene was dysfunctional because of an insertion of four nucleotides near the beginning of the *MP* gene, which caused a frame shift and a premature termination. No other mutation was introduced. The TVCVMPB has TVCV coat protein. The result was detected by western blotting using anti-TVCV serum. Very small amount of coat protein was accumulated by TVCVMPB. Only one experiment was performed.

108h 88h 72h 42h 18h 0h TVCV TMV

Fig 14. Single infection of TVCVMPB

Co-infection of tobacco protoplasts by two viral species

1. TMV and TVCV co-infection



TVCV 0h 18h 42h 72h 88h 108h TMV

Fig 15. TMV, TVCV co-infection. The results were detected by western blotting using anti-TVCV serum. The TVCV anti-serum used in this experiment has some cross reaction with TMV coat protein. "TVCV" denotes the TVCV control which was 100 ng TVCV virions. "TMV" denotes the TMV control which was 100 ng TMV virion. Times post infection when the sample was collected are indicated.

The results of TMV and TVCV dual infection are shown in fig 15. There are two bands in each experimental lane. The upper band is the TMV signal, while the lower one is the TVCV signal. The amount of coat protein in each band was determined relative to the corresponding control. In the competition infection, both TMV and TVCV began to accumulate coat protein much earlier than when they infected singly. For TMV, about 50% of total coat protein had already accumulated by 18 hpi. For TVCV, more than 80% of total coat protein had accumulated by 18 hpi. The total amount of coat protein accumulated in TMV single infection was about the amount it accumulated in

competition. This was also true for TVCV. Three experiments were performed with one focused one early stage. Although actual data point may vary, the early phase growth can be observed in all three experiments.

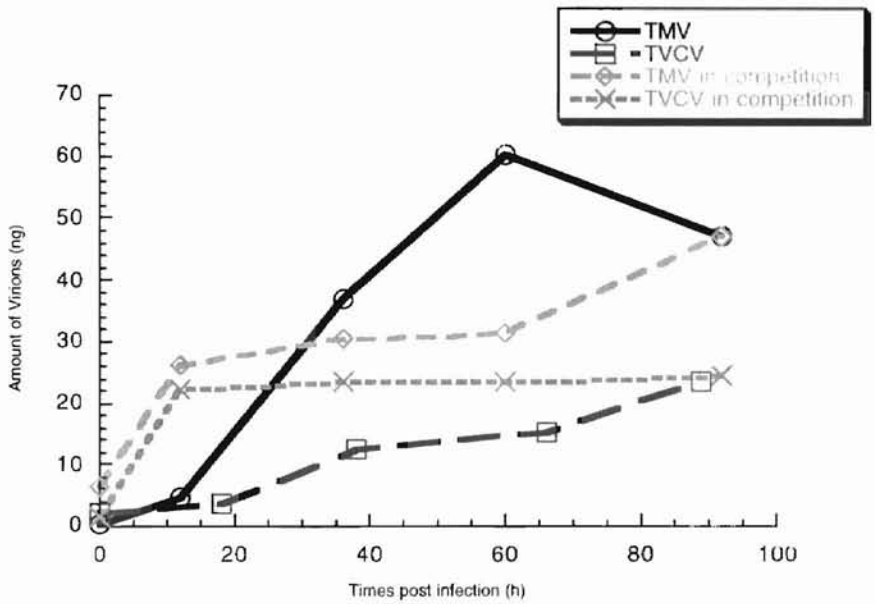


Fig 16. Amount of coat protein accumulated by TMV and TVCV

2. TMV and TVCVrMP co-infection

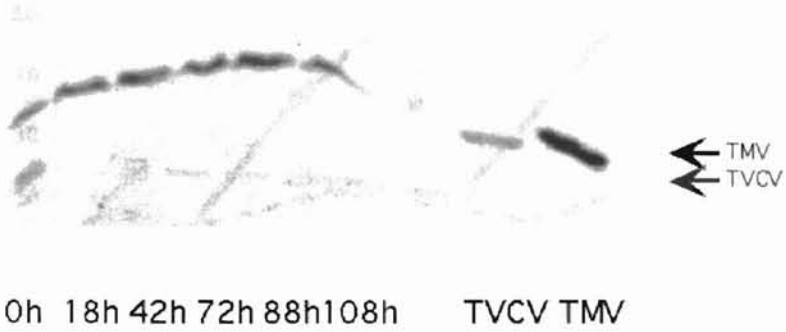


Fig 17. TMV, TVCVrMP co-infection 1. This result was detected by western blotting using anti-TMV serum. "TVCV" and "TMV" were the control lanes each contained 100 ng of virions of the corresponding type. Times post infection when the samples were collected are indicated. The amount of coat protein accumulated by TMV was calculated relative to TMV control in this result.

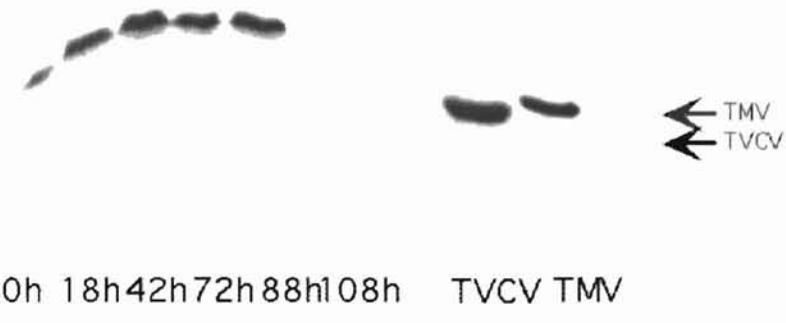


Fig 18. TMV, TVCVrMP co-infection 2. This result was detected by western blotting using anti-TVCV serum. The two positive controls (TVCV, and TMV) were 100 ng of corresponding virions. Times post infection when the corresponding samples were collected are indicated.

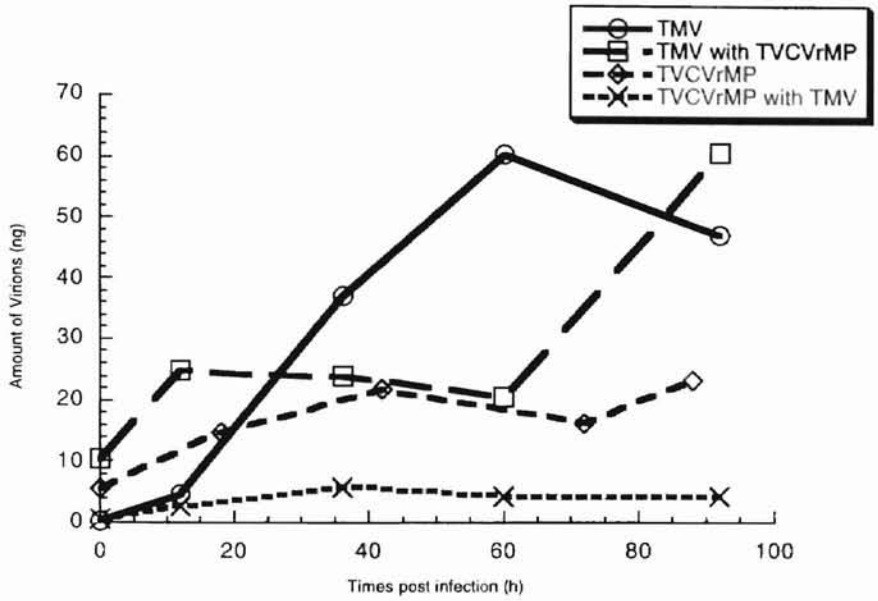


Fig 19. Amount of coat protein accumulated by TMV and TVCVrMP

The final amount of coat protein accumulated by TMV in competition with TVCVrMP and in single infection are roughly equal. An early phase accumulation of coat protein of TMV was also observed in competition with TVCVrMP. This is the same situation we found in TMV's competition with TVCV. TVCVrMP accumulated same level of coat protein as TVCV when they infected singly. In competition with TMV, the amount of coat protein accumulated by TVCVrMP was dramatically decreased by about 5 fold. Only one experiment was performed.

Detection of TMV and TVCV RNA signals

Form the results shown in fig 20, RNA signals of TMV and TVCV could be detected using TMV and TVCV *MP* gene probes, respectively. The results showed that both TMV and TVCV probes can effectively detect the corresponding RNA. There was some weak hybridization between TVCV probe and TMV RNA. The samples used in this experiment were TMV and TVCV virions.

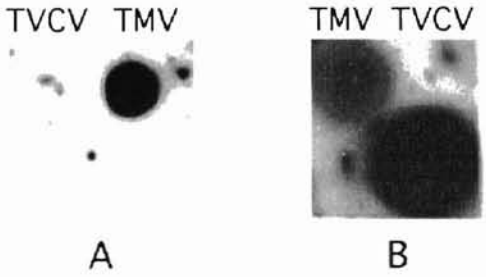


Fig 20. Detection of TMV and TVCV RNA signals. Detection used non-radioactive dig-labeled DNA probe. Experiment A used TMV *MP* gene probe. Experiment B used TVCV *MP* gene probe. X-ray films were exposed for 46 hours in both experiment.

Standard curve of western blotting

The standard curve shown in fig. 21 was detected by western blotting using anti-TVCV serum. The solid line was obtained by connecting data points. The dashed line is the linear curve fit. The R value of this linear curve fit is 0.997. This result was obtained using 80 ng virion data as standard.

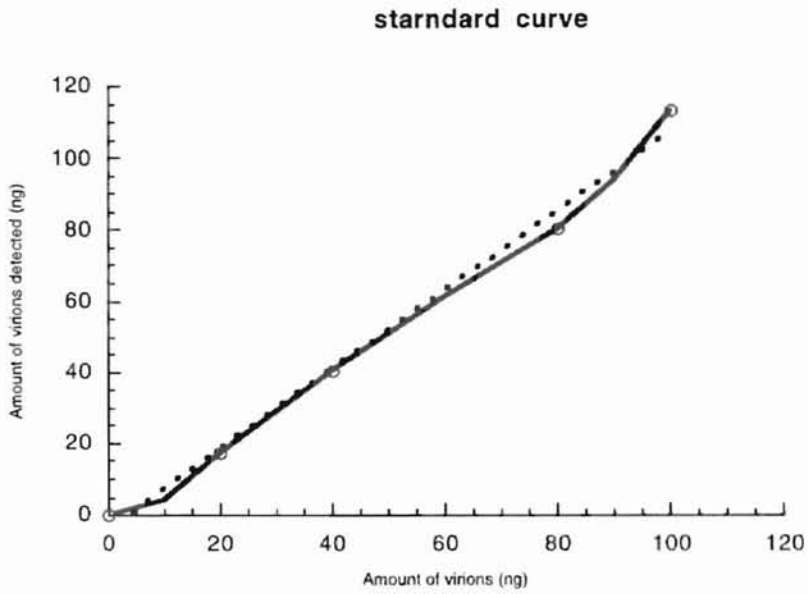


Fig 21. TVCV western blotting detection standard curve

CHAPTER IV

Discussion

Feasibility of using western blotting as a quantitative method

In this study, western blotting was used as a quantitative method. The feasiblility of using western blotting as a quantitative measurement was demonstrated by the standard curve showed in fig 21. The linear curve fit has a R of 0.997, which suggested a good fit. The standard point used in the study (100 ng virions) located in the linear range, which provided the feasibility of this method.

CP amount used as an index of viral ability of replication

In this study, CP amount was estimated by densitometry of western blot and used as an index of replication level. Coat protein is usually the most abundant protein a virus produces. Thus, a detection of CP with western blotting is one of the most sensitive methods. The amount of CP produced by a virus should be influenced by the ability of

viral replication within single cells, the amount of subgenomic RNA which serves as the template for CP synthesis, and the amount of CP synthesized per template RNA. The degradation of CP could also happen. Thus, the amount of CP accumulated can serve as an overall performance index. Some environmental factors like the illumination level, the temperature, the moisture percentage, and the pH value of the medium, could affect the culture condition of the infected protoplasts and thus affect the viral CP amount. Those factors should be consistent from one experiment to another.

Recent results suggest that the TMV replication process could be extremely rapid[28,29,30]. The MP required for cell-to-cell movement accumulates maximally before the CP in protoplasts[30]. The accumulation of CP occurs much later than the accumulation of MP. We observed that the accumulation of CP began as early as 12 hours post infection.

Surprisingly, the mutated virus TVCVMPB didn't accumulated CP significantly. TVCVMPB is a wild type TVCV with its *MP* mutated. The mutation was insertion of four nucleotides within the region of *MP* gene and causes frame-shift and premature termination of MP translation. All other genes of TVCVMPB remained intact. One might conclude that TVCVMPB should accumulate CP. In fact, a recent result showed that TVCVMPB has reasonable yield from infection of a transgenic tobacco carrying tobacco *MP* gene[32]. The lack of CP accumulation might be thought due to the defect in the *MP* gene. The origin of virion assembly of TVCV is located within the *MP* gene.

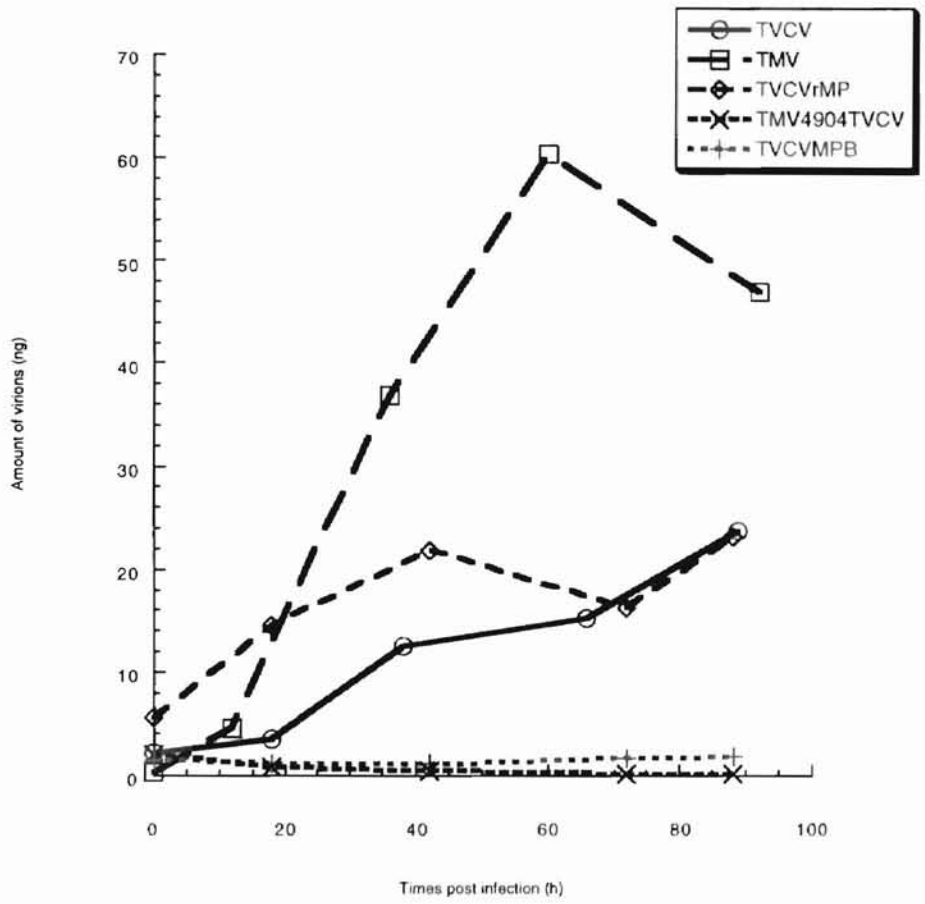


Fig 22. Amount of coat protein accumulated by single infection of different viruses

Thus, an insertion in the *MP* gene may affect the synthesis of subgenomic RNA that serves as the template of CP translation as well as the assembly process of TVCVMPB virions. These two reasons may slow the accumulation of CP with TVCVMPB.

On the other hand, both wild type viruses and TVCVrMP did accumulate CP to a certain level. Within these three, TMV accumulated the most. TVCV and TVCVrMP accumulated a similar level of coat protein, which is about half of that of TMV.

TMV4904TVCV does not replicate well in single cells

The virus chimera TMV4904TVCV has the TMV's *MT*, *Hel*, and *RdRP*. *MP* and *CP* gene for TMV4904TVCV came from TVCV. The junction between TMV part and TVCV part is at the junction of *RdRP* gene and *MP* gene. The origin of virion assembly is also intact.

TMV4904TVCV only accumulated very small amounts of CP, if any. This suggested the replication of TMV4904TVCV is weak. This phenomenon could result from the fact that virus chimera TMV4904TVCV has an incompatible replicase gene and 3' non-translated end. This result was consistent with the results from TMV4904TVCV infection of transgenic plants carrying *MP* transgene[32].

Competition between TMV4904TVCV and wild type viruses

Since TMV4904TVCV does not replicate well in single infection study, competitions between TMV4904TVCV and wild type viruses were not performed. In this study, the major motivation is to determine if a virus chimera is hazardous. The fact that virus chimera TMV4904TVCV does not replicate well by itself strongly suggesting this virus chimera is not hazardous. Thus, competitions between wild type viruses and TMV4904TVCV could not prove more. Also, since the amount of coat protein accumulated by TMV4904TVCV is very low in single infection study, it is very difficult

to determine if wild type viruses actually inhibit the replication of TMV4904TVCV in competitions.

Virus chimera TVCVrMP replicates well in single cells

Virus chimera TVCVrMP accumulated CP to a high level. The amount of CP it accumulated is comparable to that of wild type viruses. The accumulation of CP of TVCVrMP occurred a little earlier than for both wild type viruses. After an early stage of growth, the virus seemed to meet its limit of CP accumulation. In fact, there was almost no more CP accumulated at 88 hours post infection than at 36 hpi . The total amount of CP TVCVrMP accumulated was comparable to that of TVCV, but lower than the amount TMV accumulated.

The high level of CP accumulation suggested that the replication of this virus chimera was strong. It suggested that TVCVrMP was capable of replication in single cells by itself. The results were consistent with the results of infection of whole plant[22].

Competition has limited effects on wild type virus TMV and TVCV

Fig 16. summarizes the amount of CP *vs.* time of competition between TMV and TVCV. Fig 23 summarizes the amount of CP accumulated by TMV when it infected

singly, in dual infection with TVCV, and with TVCVrMP. Fig 24 summarizes the amount of CP accumulated by TVCV when it infected singly and in dual infection with TMV.

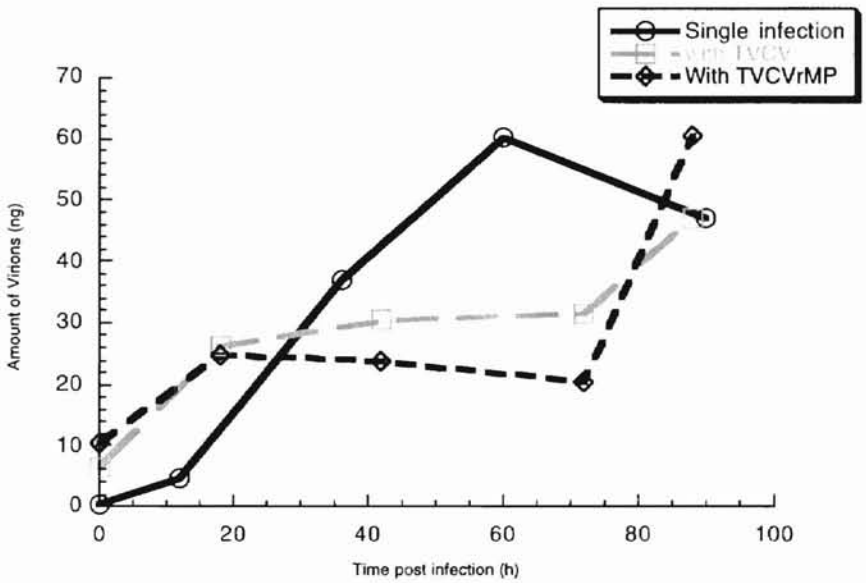


Fig 23. The amount of coat protein accumulated by TMV in single infection, competition with TVCV, and competition with TVCVrMP

The final amount of TMV and TVCV CP accumulated differed little from single infection to competition. Another effect of competition deserves notice. In competition of TMV with TVCV, both CP began to accumulate earlier than in single infections. TVCV began to accumulate coat protein as early as 12 hours post infection. For TMV, the time was about 18 hours post infection. The reason for this early phase growth is unclear. It could result from higher amount of tobamoviruses used in infection for competition work (15 µg for single infection; 15 µg each, 30 µg total for competition). Another possible reason for this early phase growth is that there exists some kinds of cooperation between TMV and TVCV when they infect protoplasts together. The

corresponding single infections with 30 μg tobamovirus virion need to be carried out in the future.

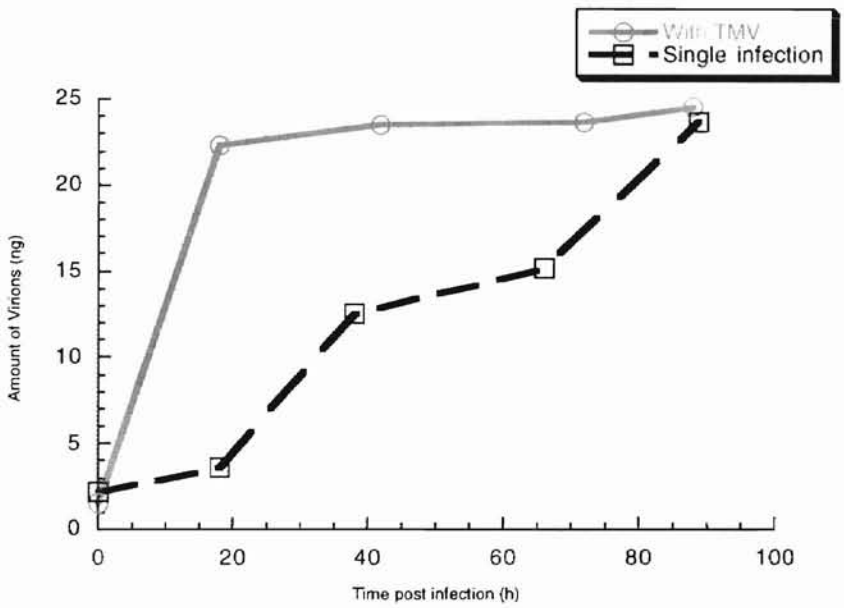


Fig 24. Amount of coat protein accumulated by TVCV in single infection and in dual infection with TMV

Competition decreased the replication of TVCVrMP

As in fig 12, the virus chimera TVCVrMP accumulated CP to high amounts when it infected alone. From fig 19 and fig 25, a dramatic decrease of TVCVrMP coat protein accumulation during competition with TMV was observed. This result suggested that TVCVrMP was not capable of competition with TMV. Compared to the results of competition between TMV and TVCV, the replacement of the *MP* gene in TVCV have certain effects on viral abilities. The actual mechanism of the inhibition of CP accumulation by TMV for TVCVrMP is unknown. Since the virus chimera TVCVrMP

appeared to be capable of accumulating CP by single infection, an inhibition by TMV in replication is possible.

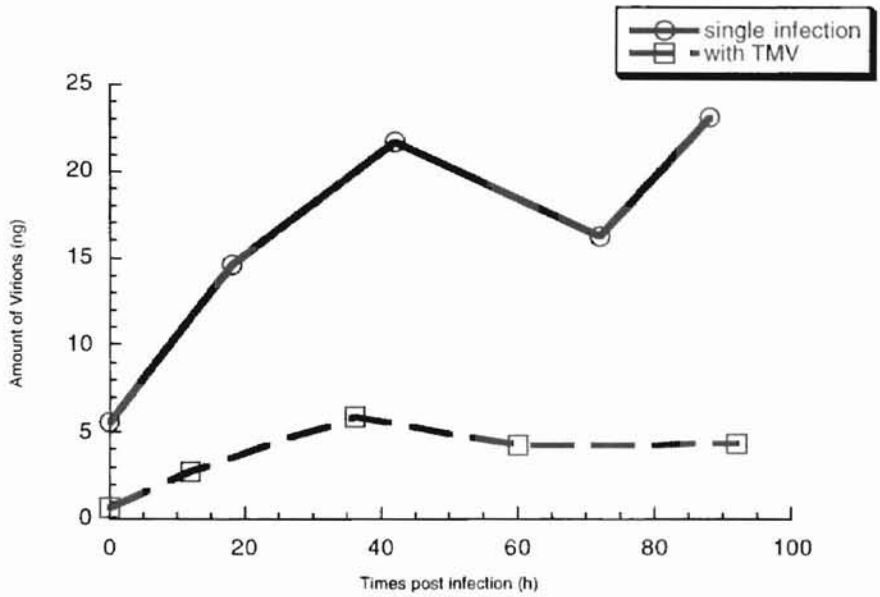


Fig 25. shows the amount of coat protein accumulated by TVCVrMP with or without competition.

Virus chimera like TVCVrMP may not be hazardous

The ability to infect plants and spread from one to another is crucial for a virus to survive in nature. The ability to compete with other viruses is also very important. Studies of the tobamovirus in Australia[31] has showed the process of one virus gradually dominated by another one.

Although TVCVrMP replicates well by itself, the inability of compete with prevalently existing TMV may limit its spread and survival in nature. Thus, virus chimeras like TVCVrMP might not be hazardous.

Bibliography

1. Lewandowski, D.J., and Dawson, W.O. (1994) Tobamoviruses. In Encyclopedia of Virology (1994). R. G. Webster and A. Granoff, eds. New York, Academic Press, 1436-1441.
2. Young, N.D., and Zaitlin, M. (1986) Plant Mol. Biol. 6:455-465. Analysis of tobacco mosaic virus replicative structures synthesized in vitro.
3. Gorbalenya, A.E., and Koonin, E.V. (1989) Nucl. Acids Res. 17:8413-8440. Viral proteins containing the purine NTP-binding sequence pattern
4. Hodgman, T.C. (1988) Nature 333:22-23. Anew superfamily of replicative proteins.
5. Rozanov, M.N., Koonin, E. V., and Gorbalenya, A.E. (1992) J. Gen. Virol. 73:2129-2134. Conservation of the putative methyltransferase domain: a hallmark of the 'Sindbis-like' supergroup of positive-strand RNA viruses.
6. Koonin, E.V. (1991) J. Gen. Virol. 72:2197-2206. The phylogeny of RNA-dependent RNA polymerases of positive strand RNA viruses.
7. Deom, C.M., Oliver, M.J., and Beachy, R.N. (1987) Science 237:389-394. The 20-kilodalton gene product of tobacco mosaic virus potentiates virus movement.
8. Meshi, T., Watanabe, Y., Saito, T., Sugimoto, A., Maeda, T., and Okada, Y. (1987) EMBO J. 6:2557-2563. Function of the 30kd protein of tobacco mosaic virus: involvement in cell-to-cell movement and dispensability for replication.

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

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

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

12. Lartey, R.T., Voss, T.C., and Melcher, U. (1995) Gene 166:331-332. Completion of a cDNA sequence from a tobamovirus pathogenic to vrucifers.

13. Aguilar, I., Sanchez, F., Martin, A., Martinez-Herrera, D., and Ponz, F. (1996) Plant Mol. Biol. 30:191-197. Nucleotide sequence of Chinese rape mosaic virus (oilseed rape mosaic virus), a crucifer tobamovirus infectious on *Arabidopsis thaliana*.

14. Lartey, R.T., Voss, T.C., and Melcher, U. (1996) Mol. Biol. Evol. 13:1327-1338. Tobamovirus evolution: gene overlaps, recombination , and taxonomic implications.

15. Li, Z.-Y., Uyeda, I., and Shikata, E. (1983) Mem. Fac. Agric. Hokkaido Univ. 13:542-549. Crucifer strain of tobacco mosaic virus isolated from garlic.

16. Beachy, R.N., Loesch-Fries, S., and Tumer, N. (1990) Annu. Rev. Plant Pathol. 28:451-474. Coat protein-mediated resistance against virus infection.

17. Fitchen, J.H., and Beachy, R.N. (1993) Annu. Rev. Microbiol. 47:739-763. Genetically engineered protection against viruses in transgenic plants.

18. Falk, B.W., and Bruening, G. (1994) Science 263:1395-1396. Will transgenic crops generate new viruses and new diseases?

19. Mellon, M., and Rissler, J. (1995) BioTechnology 13:96-96. Transgenic crops: USDA data on small-scale tests contribute little to commercial risk assessment.

20. Ding, S., Shi, B., Li, W., and Symons, R.H. (1996) Proc. Natl. Acad. Sci. USA 93:7470-7474. An interspecies hybrid RNA virus is significantly more virulent than either parental virus.

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

22. Zhang, Y. Lartey, R.T., Hartson, S.D., Voss, T.C., and Melcher, U. (1998) Arch. Virol. In press. Limitations to tabacco mosaic virus infection of turnip.

23. Li, W.-H., and Graur, D. (1991) Fundamentals of Molecular Evolution. Sunderland MA, Sinauer Associates.

24. Zhang, Y.-L., Doctoral dissertation, department of biochemistry of molecular biology, Oklahoma state university.

25. Culver, J., and Dawson, W., personal communication.

26. Laemmli, U.K. (1970) Nature 227:680. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4.

27. Melcher, U.K., unpublished.

28. Shaw, J.G., Plaskitt, K.A., and Wilson, T.M.A. (1986) Virology 148: 326-336. Evidence that tobacco mosaic virus particles disassemble contranslationally *in vivo*.

29. Wu, X., and Shaw, J. (1996) Proc. Natl. Acad. Sci. USA 93:2981-2984. Bidirectional uncoating of the genomic RNA of a helical virus.

30. Nelson, R.S., and Aart, J.E. (1998) Progress in botany 59:476-533. The mystery of virus trafficking into, through and out of vascular tissue.

31. Fraile, A., Escriu, F., Aranda, M.A., Malpica, J.M., Gibbs, A.J., and Garciaarenal, F. (1997) J. of Virology 71:8316-8320. A century of tobamovirus evolution in an Australian population of *Nicotiana glauca*.

32. Melcher, U., and Williams, A., unpublished.

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