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Molecular Characterization of Viruses Infecting Greenhouse Vegetables in Ontario

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Abstract

Greenhouse vegetable production is a very important and successful agricultural sector in Ontario. However, newly emerging viral or virus-like diseases have become a major limiting factor in greenhouse vegetable production. To determine the identity of the viral pathogens in the three major greenhouse vegetables in Ontario, we conducted in-depth sequencing of small RNAs isolated from virally infected plants used next-generation sequencing technology. Subsequent bioinformatics analyses revealed six viral pathogens including *Bell pepper endornavirus* (BPEV), *Parietaria mottle virus* (PMoV), *Cucumber green mottle mosaic virus* (CGMMV), *Tobacco streak virus* (TSV), *Pepper mild mottle virus* (PMMoV), and *Pepino mosaic virus* (PepMV). The full-length genome sequences of BPEV, PMoV, and CGMMV have been cloned and sequenced.

Since CGMMV causes the most severe economic lossess, we further characterized CGMMV and constructed an infectious CGMMV clone for the development of an attnuated virus for the control of CGMMV. We found that in the mimic greenhouse hydroponic production system, CGMMV is water-transmissible. We introduced various mutaitons into the CGMMV infectious clones and tested their infectivity. Our data demonstrated that when host plants were infected by two particular mutants MRTAL and MGVDT, no symptom appeared on the systemic leaves and viral RNA or coat protein accumulation level was low. Based on these data, we suggest that the mutants MRTAL and MGVDT have great potential to be used as attenuated CGMMV variants for cross protection against CGMMV.

Keywords

Small RNA sequencing, CGMMV, BPEV, PMoV, Water-mediated transmission, Cross protection.

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List of Abbreviations

aa	Amino acid
AgLV	Ageratum latent virus
AGO	Argonaute proteins
APLPV	American plume line pattern virus
ATP	Adenosine-Tri-Phosphate
AV-2	Asparagus virus 2
Avr gene	Avirulence gene
BaCV	Bacopa chlorosis virus
Bp	Base pair
BPEV	Bell pepper endornavirus
CTAB	Cetyl trimethylammonium bromide
CGMMV	Cucumber green mottle mosaic virus
HC-Pro	Helper component proteinase
CP	Coat protein
CPDs	Cyclobutane pyrimidine dimers
CTV	Citrus tristeza virus
DAS-ELISA	Double antibody sandwich ELISA
DCL	Dicer-like enzymes
DEPC	Diethylpyrocarbonate

DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
dsRNA	Double-stranded RNA
DTT	Dithiothreitol
EM	Electron microscopy
ELISA	Enzyme-linked immunosorbent assay
GaBRV-XL	Gremmeniella abietina type B RNA virus XL
GEB	General extraction buffer
h	Hour
Hel-1	Helicase 1
HmEV-1	Helicobasidium mompa endornavirus
hpRNAS	Hairpin RNAs
HR	Hypersensitive response
ICTV	International Committee on Taxonomy of Viruses
IgG	Immunoglobulin G
IPM	Integrated pest management
kDa	Kilo Daltons
MCS	MiSeq Control Software
Min	Minute
MNSV	Melon necrotic spot virus

MP	Cell-to-cell movement protein
MTR	Methyltransferase
NGS	Next Generation sequencing
NCBI	National Center for Biotechnology Information
nt	Nucleotides
OMAFRA	Ontario Ministry of Agriculture, Food and Rural Affairs
ORV	Oryza rufipogon endornavirus
ORF	Open reading frame
ORSV	Odontoglossum ringspot virus
OSV	Oryza sativa endornavirus
PepMV	Pepino mosaic virus
PDV	Prune dwarf virus
PEG8000	Polyethylene glycol 8000
piwiRNAs	Piwi-interacting RNAs
PEV-1	Phytophthora endornavirus
PLRV	Potato leaf roll virus
PMoV	Parietaria mottle virus
PMMoV	Pepper mild mottle virus
PNRSV	Prunus necrotic ringspot virus
PTA	Sodium (K) phosphotungstate (PTA)

PVX	Potato virus X
PVY	Potato virus Y
qPCR	Quantitative PCR
qRT-PCR	Reverse Transcription qPCR
RACE	Random amplification of cDNA fragments
RdRp	Viral RNA-dependent RNA polymerases
R gene	Resistance gene
RIs	Replication intermediates
RISC	RNA-induced silencing complexes
RNA	Ribonucleic acid
rpm	Round per minute
siRNAs	Small interfering RNAs
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SNSV	Strawberry necrotic shock virus
SpLV	Spinach latent virus
sRNA	Small Ribonucleic acid
STP	Stop Solution
TaEV	Tuberaestivum endornavirus
TEM	Transmission electron microscopy
TMV	Tobacco mosaic virus

ToNSV	Tomato necrotic spot virus
TSV	Tobacco streak virus
UGT	UDP-glucose–glycosyltransferase
UTRs	Untranslated regions
UV	Ultraviolet
UWO	University of Western Ontario
UV	Ultraviolet
VAMP	Virus-associated molecular pattern
VFV	Vicia faba endornavirus
vsiRNAs	Viral-derived small interfering RNAs
WT	Wilt type
%	Percentage

Charper 1. General Introduction

1.1 Overview

Worldwide, the major greenhouse vegetable production countries include Spain, The Netherlands, Mexico, Canada and the United States. Canada's greenhouse industry is mainly comprised of tomatoes, peppers, and cucumbers. In 2013, the total greenhouse vegetable area in Canada was over 14 million square meters, and there was a steady increase in the harvested area of greenhouse vegetables from 2008-2013 (2013 Statistical Overview of the Canadian Greenhouse Vegetable Industry). Ontario continues to be the leader in the greenhouse vegetable sector, representing approxminately 70% of the total harvested area in Canada, and has the largest square meters of greenhouse vegetables harvested. Most of the vegetable greenhouses in Ontario are centered in the Leamington and Niagara areas. In 2013, the greenhouse farm gate value (FGV) was 782.95 million, up 15% from 2012, and over 70% of our farmer's produce was exported to the United States.

The greenhouse agricultural sector is a very significant component of the provincial economy. Viral diseases have been a major limiting factor in greenhouse vegetable production. In addition to many known viruses infecting greenhouse vegetables crops, newly emerging viral or virus-like diseases also threaten greenhouse vegetable from time to time (Daughtrey *et al*., 1997; Hanssen *et al*., 2010b; Moury and Verdin, 2012; Lecoq and Desbiez, 2012). For instance, *Pepino mosaic virus* (PepMV) is a major viral disease in greenhouse tomato (*Solanum lycopersicum*) production worldwide. The damage and economic losses caused by PepMV vary greatly, not only between different production areas, but also between different infected crops within the same region (Hanssen *et al*., 2010, Hanssen *et al*., 2010a). During a viral disease outbreak on greenhouse cucumbers in 2013 in Alberta, Canada, *cucumber green mottle mosaic virus* (CGMMV) and *melon necrotic spot virus* (MNSV) were identified (Ling *et al*., 2014, Li *et al*., 2015a; Li *et al*., 2015b). Disease incidence varied from one greenhouse to another, and in some severe cases, diseased cucumber plants were widely distributed within greenhouses, resulting in 10 to 15% yield losses, based on grower's estimation. These viral diseases induce a

diverse range of symptoms on the leaves and fruits of infected plants, and most of them have a significant impact on vegetable yield, quality and marketability.

1.2 Detection and Diagnosis of plant viruses

The ability to detect viruses is basic to almost all areas of virological investagation. The detection methods depend on different properties of virus. In the past three decades, a number of methods have been reported for the detection and identification of viruses. Several methods have been adopted for routine use in diagnosis, pathology and virology laboratories. These include the two most successfully established virus detection methods: the protein-based enzyme-linked immunosorbent assay (ELISA) for either DNA and RNA viruses, and the nucleotide sequence-based reverse transcription polymerase chain reaction (RT-PCR) for RNA viruses or polymerase chain reaction (PCR) for DNA viruses (Boonham *et al*., 2014). More recently, next generation sequencing (NGS) technology has become a very powerful technology for robust detection and identification of either known or unknown viruses. Many methods derived on these technologies have been developed for particular purposes. For examples, some techniques could be used to detect and diagnose viruses in the field or resource poor locations, whereas some others are able to detect many viruses in a single test.

Looking further into the future, virus discovery and multiplex detection methods seem to converge as NGS becomes ever cheaper, easier to perform and can provide high levels of multiplexing without the use of virus specific reagents. NGS technologies has led to a revolution in virus discovery and exciting new possibilities for diagnostics; the application of massively parallel sequencing approaches, and subsequent bioinformatics analysis for viral sequences, carries the promise of routine, generic detection of viruses and other pathogens alike (Adams *et al*., 2009; Al Rwahnih *et a*l., 2009;Kreuze *et al*., 2009).

1.3 Control of plant viruses

Viral diseases have caused major economic losses for greenhouse growers. In the past, an efficient management strategy was to control insect vectors using pesticides. The demand for critical reassessment of strategies used in the viral disease management has recently revolutionized the greenhouse technology of this crop cultivation. These approaches must be

integrated into a highly specialized and effective crop management and pest control program (Karuppuchamy and Venugopal, 2016). In Ontario, the majority of diseases are currently controlled by using crop sanitation procedures and the application of pesticides. Crop sanitation procedures (Sanitation Guidelines for Management of Pests and Diseases of Greenhouse Vegetables) are intended to reduce the probability of introducing pests into a greenhouse, or to reduce the severity of a pest outbreak. However, once a viral disease is introduced into a greenhouse, growers usually initiate a pesticide program to protect the crop. In Ontario the greenhouse vegetable industry was estimated to have sprayed approximately 0.34 million kg of pesticides in 2008 (Sanitation Guidelines for Management of Pests and Diseases of Greenhouse Vegetables). Currently, the Ontario Ministry of Agriculture, Food and Rural Affairs (OMAFRA) has a mandate for the implementation of integrated pest management (IPM) programs for disease control in greenhouses. The goal of IPM is to achieve effective management of pests by using all of the tools available, in the safest manner possible and in a way that enhances the economic viability of the crop. This includes chemical, cultural, mechanical and biological tools. It means that the problem could be resolved not just relying on one technique. By knowing the viruses and their life cycles, growers can plan control programs to exploit weaknesses in virus lifecycles and use the most effective management strategy.

1.4 Research objectives

The objectives of this study were (1) to identify major viral pathogens from virus-like diseases endemic in greenhouse peppers, cucumbers and tomatoes using in-depth small RNA sequencing, (2) to characterize the important pathogens identified at the molecular level, (3) to understand which the identified virus (CGMMV) spreads from a plant to plant in the greenhouse. (4) Many efforts were also made to develop an attenuated variant against CGMMV, the most important virus identified, to establish effective management strategies so as to control the viral pathogens in greenhouse vegetables and avoid possible catastrophic losses.

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Chapter 2. Identification and characterization of major viral pathogens in greenhouse peppers, cucumbers and tomatoes

2.1 Introducton

The symptoms appearing on the leaves of the plants infected by viruses are an obvious piece of evidence for the diagnosis of disease caused by viral pathogens. However, the virus identification might be misleading if the diagnosis is based on the symptom alone. For example, virus disease in lettuce may be caused by some of 14 viruses with aphids, leafhoppers, thrips, nematodes or fungi that may act as disease vectors (Cock, 1968). Many of the causal viruses produce brown necrotic spots or bronzing on leaves, and, later, chlorotic stunting. Therefore, correct identification of a virus, or viruses, infecting a particular crop is essential in the development of effective control measures to be applied. Molecular identification of viral pathogens has so far been a prerequisite for the development of effective integrated pest management (IPM)-based strategies to control viral diseases (Karuppuchamy and Venugopal, 2016).

To date, there are a number of diagnostic tools, including physical, biological, serological and molecular approaches that have been employed to identify and characterize viral pathogens. Traditionally, viral pathogens are detected on cultured cell monolayers that exhibit cytopathic effects or plaques, or by antibody neutralization tests. However, many viral types are not cultivable in the laboratory and antibody neutralization tests depend on the availability of quality antiserum (Wang *et al*., 2002), hindering identification, discovery, and research of these pathogens. Over the past few decades, molecular methods, such as RT-PCR, and the microarray-based Virochip have been used to detect and explore uncultivable or non-isolated viruses. However, established molecular methods have two major shortcomings: (i) sequence information for the target viruses must be known, making it difficult to target and study emerging viruses; and (ii) typically, an individual analytical test must be conducted to confirm or refute each pathogen, making identification of rare or unexpected pathogens difficult, and identification of previously unknown pathogens impossible.

Major advances in DNA sequencing technology over the last decade have led to the advent of next generation sequencing (NGS) platforms, which allow for sequencing the total nucleic acid content in disease samples and, subsequently, identifying the pathogens by analysis of the NGS data using bioinformatics tools (Wu *et al*., 2015). This method offers significant advantages over all existing methods of identifying a viral pathogen, including removing the need for targeting a specific pathogen or requiring sequence information for that pathogen, identifying multiple pathogens in a single sample, and eliminating the need for costly and often ineffective culturing or antibody laboratory tests. One of powerful, robust NGS approaches for the identification of viral pathogens is small RNA sequencing (Cox-Foster *et al*., 2007; Quan *et al*., 2008; Li *et al*., 2012).

A metagenomic survey of microbes in honey bee colony collapse syndrome demonstrated the successful use of this approach in identifying a virus as the possible causal agent for this disorder (Cox-Foster *et al*., 2007). Using sRNA assembly and analysis, Wu *et al*., (2010) were able to identify several known and unknown viruses infecting invertebrate cell lines. In plant systems, NGS technologies have also been applied for virus and viroid identification (Adams *et al*., 2009; Al Rwahnih *et al*., 2009; Kreuze *et al*., 2009; Navarro *et al*., 2009; Coetzee *et al*., 2010; Hagen *et al*., 2011). Deep sequencing of sRNAs has been shown to be the most promising as a detection and identification technique with abilities to identify RNA and DNA viruses in sweet potato (Kreuze *et al*., 2009) and pospiviruses in grapevine (Navarro *et al*., 2009). Hagen *et al*., (2011) extended NGS applications to identify RNA and DNA viruses in tomato and squash. A new computational algorithm has also been developed to allow for homology-independent discovery of viroids (Wu *et al*., 2012).

The first "short read" sequencing platform was commercially launched by 2006. As mentioned in the review (Voelkerding *et al*., 2009), template DNA is fragmented into lengths of several hundred base pairs and end-repaired to generate 5'phosphorylated blunt ends. The polymerase activity of the Klenow fragment is used to add a single A base to the 3' end of the blunt phosphorylated DNA fragments. This addition prepares the DNA fragments for ligation to oligonucleotide adapters, which have an overhang of a single T base at their 3' end to increase ligation efficiency. The adapter oligonucleotides are complementary to the flow-cell anchors. Under limiting-dilution conditions, adapter-modified, single-stranded template DNA is added to

the flow cell and immobilized by hybridization to the anchors. In contrast to emulsion PCR, DNA templates are amplified in the flow cell by "bridge" amplification, which relies on captured DNA strands "arching" over and hybridizing to an adjacent anchor oligonucleotide. Multiple amplification cycles convert the single-molecule DNA template to a clonally amplified arching "cluster," with each cluster containing approximately 1000 clonal molecules. Approximately 50 $x10^6$ separate clusters can be generated per flow cell. For sequencing, the clusters are denatured, followd by chemical cleavage, leaving only forward strands for single-end sequencing. Sequencing of the forward strands is initiated by hybridizing a primer complementary to the adapter sequences, which is followed by addition of polymerase and a mixture of 4 different colored fluorescent reversible dye terminators. The terminators are incorporated according to sequence complementarity in each strand in a clonal cluster. After incorporation, excess reagents are washed away, the clusters are optically interrogated, and the fluorescence is recorded. With successive chemical steps, the reversible dye terminators are unblocked, the fluorescent labels are cleaved and washed away, and the next sequencing cycle is performed.

Regulatory small RNAs including small interfering RNAs (siRNAs), hairpin RNAs (hpRNAs), and Piwi-interacting RNAs (piwiRNAs), ranging in size from 20 to 24 nucleotides (nt), are ubiquitous components of endogenous plant transcriptomes, and are a common response to exogenous viral infections (Wu *et al*., 2012). Most viruses are RNA viruses whose genomes contain imperfect regulatory stem-loops and produce double stranded RNA (dsRNA) replication intermediates (RIs) by viral RNA-dependent RNA polymerases (RdRp). As a virus-associated molecular pattern (VAMP, a form of PAMP), these dsRNAs are siRNA precursors and represent the hallmark of RNA virus infection. VAMPs are associated with Dicer-like enzymes (DCL), which can cleave dsRNA into viral-derived small interfering RNAs (vsiRNAs) that, upon loading into Argonaute (AGO) proteins, improve antiviral defenses through RNA silencing. In principle, 'dicing' of dsRNA viral RIs would be enough to mediate antiviral silencing. It is well known that plants are infected by either DNA or RNA viruses produce vsiRNAs, as a result of virus-induced innate resistance. Despite being short sequences (21 to 24 nt in length), vsiRNAs overlap each other and cover the entire viral genome (Kreuze *et al*., 2009; Wu *et al*., 2012). Therefore, deep sequencing and assembly of total small RNAs for virus-infected samples offer a specific and apparently sensitive approach to virus discovery.

During virus–host interactions, virus-specific dsRNAs are introduced into a variety of processes, such as the production of virus-encoded RNA polymerase for viral genome replication, or imperfect folding of self-complementary single stranded viral genomic RNA (Ding and Voinnet, 2007). Host-encoded RdRp plays a crucial role in virus-induced gene silencing by producing the complementary viral RNA for secondary siRNA synthesis (Molnár *et al*., 2005; Seo *et al*., 2013). Studies in *Arabidopsis thaliana* and other plant species have revealed that the core components involved in plant small RNA biogenesis and the silencing pathways are encoded by multi-protein families (Seo *et al*., 2013). They are diverse and exhibit functional redundancy (Llave, 2010; Silva *et al*., 2011). For instance, the *A. thaliana* and rice genomes have four and eight DCLs, six and five RdRps, and ten and nineteen AGO proteins, respectively, while *Populous* has five DCLs. *A. thaliana* possesses six silencing pathways (Brodersen and Voinnet, 2006; Margis *et al*., 2006). It has also been shown that the formation of 21, 22 and 24 nt vsiRNAs is catalyzed to induce antiviral responses by DCL4, DCL2 and DCL3 in virus-infected *A. thaliana* (Blevins *et al*., 2006; Ruiz-Ferrer and Voinnet, 2009). Efficient antiviral defense in plants is conducted by 21nt and 22nt vsRNAs, which are generated by DCL4 and DCL2. Nevertheless, they exhibit functional redundancy or, cooperative interaction since formation of the 22 nt vsiRNAs happens mainly in the absence of DCL4 (Deleris *et al*., 2006; Donaire *et al*., 2008; Donaire *et al*., 2009; Ruiz-Ferrer and Voinnet, 2009). Multiple AGO proteins (AGO2, AGO5) and RdRp (RdRp1, RdRp2 and RdRp6) are also involved in antiviral defense and vsRNA biogenesis in plants (Ding and Voinnet, 2007; Takeda *et al*., 2008; Donaire *et al*., 2008; Donaire *et al*., 2009; Qi *et al*., 2009; Garcia-Ruiz *et al*., 2010). The accumulated level of vsiRNAs corresponding to infection by different viruses has been reported to be variable in different host plants (Donaire *et al*., 2009, Lin *et al*., 2010).

2.2 Materials and Methods

2.2.1 Samples collection

Greenhouse vegetable crops (Figure 2.1) including cucumbers (*Cucumis sativus* cv Picowell RZ), tomatoes (*Solanum lycopersicum*), and peppers (*Capsicum annuum* cv Healey) were collected from greenhouses in Ontario, Canada. The symptomatic leaves were collected in Sep 2014. Randonly-picked leaves and fruits were collected in Nov 2014, Feb 2015, Mar 2015 and Sep 2015.

tomato **pepper** **cucumber**

Figure 2.1 Three diseased greenhouse vegetable crops which were collected from a greenhouse in Ontario, Canada.

2.2.2 Total RNA extraction

Total RNAs were extracted from leaves or other tissues indicated using a modified CTAB protocol from Li *et al*., (2008). 100 mg of tissues were homogenized in the presence of liquid nitrogen and suspended in 1ml of CTAB buffer and 5µl β-mercaptoethanol. After a vigorous mixing, samples were incubated for 15 min at 65°C and centrifuged for 5 min at 12000 rpm and 4°C. Supernatants were transferred to new tubes and mixed with equal volumes of chloroform and centrifuged for 10 min at 12000 rpm and 4°C. Supernatants were moved to new tubes with equal volumes of isopropanol, incubated on ice for 30 min and centrifuged at 12000 rpm and 4°C. Finally, the resulting pellets were washed with 70% ethanol, dissolved in 30 µl of DEPC-treated water and then frozen at - 80ºC until required for further use.

2.2.3 Small RNA extraction and small RNA deep sequencing

Small RNAs were extracted from leaves of greenhouse crops with a mirPremier®microRNA isolation kit (Sigma-Aldrich, St Louis, MO). 5 µl of the total RNA was used in ligation with 3' adaptor. After incubation at 70ºC for 2 min and immediate cooling on ice, a mix of ligation buffer, RNAase inhibitor and T4 RNA ligase (truncated) were added and the samples were incubated at 28ºC for an hour. The reaction was stopped by adding 1 µl Stop Solution (STP) and incubation was further continued for 15 min at 28ºC. 5' adaptor was added to

the reaction mix and incubated for 70ºC for 2 min, followed by adding a combination of 10 mM Adenosine-Tri-Phosphate (ATP) and T4 ligase and further incubating for an hour at 28ºC.

Adaptor-ligated total RNA was subjected to Reverse Transcriptase PCR (RT-PCR) using 12.5 mM Deoxyribonucleotide triphosphate (dNTP), RT primer (Illumina), 5X first strand buffer, Dithiothreitol (DTT) 100 mM RNAase inhibitor and single strand synthesis II RNA transcriptase at 50ºC for one hour. PCR amplification of the library was performed using the reagents (Illumina) by heating to 98ºC, cooling to 60ºC and then reheating to 72ºC for 13 cycles. PCR-amplified products were run on a PAGE gel and ethidium bromide (the final concentration is 0.5mg/ µl for staining). Custom RNA ladder and High Resolution ladder (supplied with Illumina TruSeq small RNA kit) were used as standards for comparison of the band sizes. Band sizes between 140 bp to 160 bp were excised using a UV box. Bands were excised by packing the band-cuts on the gel to enhance the chances of acquiring as accurate as possible of the pool of miRNAs in the final concentration. The gel-purified band was subjected to further purification using Gel Breaker tubes and eluted using 10 µl of 10mM Tris-HCL (pH=8.5). Libraries were quantified using BioRad qPCR assays according to the manufacturer's protocol.

2.2.4 Next-generation sequencing setup and data analysis

Small RNA libraries were diluted to 1 nM concentration. Equal volumes of 1 nM libraries to be multiplexed were combined together. 9 µl of the multiplex pool was denatured at room temperature for 5 min using 0.5 µl 2 N NaOH and 0.5 µl of Elution Buffer (Supplied by Qiagen, Valencia, CA) to keep the final concentration to about 1 nM. Further dilution was achieved by using 5 µl of the denatured library and adding 995 µl of pre-chilled Hybridization buffer (supplied by Illumina). This was performed in order to have a final concentration for clustering at a ratio of denatured library (1 µl) for every 1pM of final concentration. The reaction mixture was vortexed and 120 µl of this dilution was aliquoted into a strip of PCR tubes. Prior to beginning the sequencing run, sample libraries were loaded onto the cartridge, which was labeled Load Samples. Using the MiSeq Control Software (MCS) interface and following the run setup steps the flow cell and reagents were loaded, and the sequencing was subsequently run. The adaptor sequences of raw reads from high-throughput sequencing were trimmed using

Trimmomatic software 0.32. After trimming, the assembled contigs were used to assemble into large contigs. Contigs (longer than 100 nt) were kept and further analyzed by Blastn.

2.2.5 Determination of the full-length genomic sequences and phylogenetic analyses

Primers were then designed to amplify PCR products spanning the gaps between the various virus specific contigs to generate overlapping amplicons covering the entire sequence. The sequences of the primers used, as well as their annealing temperature, are presented in Table 1.1. These amplicons were generated from total RNA templates by RT-PCR. The amplicons from each reaction were then cloned and 5 clones per ligation were selected and sequenced. The 5′ end and 3’ end of the genomes of the virus were determined using the random amplification of cDNA fragments (RACE) strategy. Amplifications were carried out following the recommendations of the RACE kit supplier (Invitrogen). All amplified fragments were submitted to Sanger sequencing (Eurofins) either directly or after cloning in a blunt vector (Invitrogen). The sequence of all fragments was assembled by using SeqMan Pro software. Phylogenetic analyses were performed using either RdRp and coat protein amino acid sequences using the PhyML program (v3.1/3.0 aLRT). The maximum likelihood method was implemented using the ATGC bioinformatics platform (http://www.atgc-montpellier.fr/phyml/), with SPR and NNI tree improvement and support obtained from an approximate likelihood ratio test (Anisimova *et al*., 2011).

Table 2.1 A list of the primers used for cloning and sequencing of the complete genome sequence of BPEV, PMoV, and CGMMV.

The name of primer	Sequence(5’-3’)	Annealing temperature
GCMMV_F1(270-294)	GATTAGCTTTACCGCCACCAAGAAC	58.1°C
GCMMV_R1(3174-3199)	ATCTTTCCTTTGATCTTTGTCAACTC	53.7°C
GCMMV_F2(3174-3199)	GAGTTGACAAAGATCAAAGGAAAG	53.7°C
GCMMV_R2(4947-4973)	AGGCGCTTATCACTCAAATACTTACAC	56.9°C
BPEV_F1(641-665)	ACAAAGCCATGGAATTGCCAAATG	57.2°C
BPEV_R1(2612-2634)	CATATCCACCTGTGGTTGTGCAT	57.5°C

BPEV_F2(2612-2634)	ATGCACAACCACAGGTGGATATG	57.5°C
BPEV_R2(4830-4854)	CGAATGTATTGTCTCCACTCGTTTG	56.0°C
BPEV_F3(4830-4854)	CAAACGAGTGGAGACAATACATTCG	56.0°C
BPEV_R3(5646-5666)	GTTGGCACTTCGAACTCTGGC	58.8°C
BPEV_F4(5646-5666)	GCCAGAGTTCGAAGTGCCAAC	58.8°C
BPEV_R4(6617-6643)	GAGAGCAGCCACCGAATGATATGATCA	60.1°C
BPEV_F5(6617-6643)	TGATCATATCATTCGGTGGCTGCTCTC	60.1°C
BPEV_R5((7338-7358)	GATTCATGGTAGCTCAGTGGC	55.7°C
BPEV_F6((7338-7358)	GCCACTGAGCTACCATGAATC	55.7°C
BPEV_R6(10051-10075)	CGCAATGCTCTTATATTGGCTTGC	57.1°C
BPEV_F7(10051-10075)	GCAAGCCAATATAAGAGCATTGCG	57.1°C
BPEV_R7(12289-12313)	CCCTGTTATCATACTCGAACACAGC	57.2°C
BPEV_F8(12289-12313)	GCTGTGTTCGAGTATGATAACAGGG	57.2°C
BPEV_R8(14070-14093)	GTTGCTTGACCAGTCAATCTCATG	56.3°C
PMoV-RNA1-F(756-777)	CTCAACGTTGATTGGGAAAAGG	60.8°C
PMoV-RNA1-R(2799-2818)	TGCATGCGAAACTCAGGTAA	58.4°C
PMoV-RNA2-F1(25-45)	AGATAACAACCTCCGTAATGG	58.7
PMoV-RNA2-R1(1640-1660)	GTATTACCCAAGTACGTGCA	58.4
PMoV-RNA2-F3(1640-1660)	TGCACGTACTTGGGTAATAC	58.4
PMoV-RNA2-R3(2740-2760)	GAGCWATGCTRGGWATCTTC	59.4
PMoV-RNA3-F(910-930)	GCGAAGYTTRTTGAGAAGTC	58.4°C
PMoV-RNA3-R(1640-1660)	ATAGCRTCGTTGGYATCA	55.3°C

2.2.6 Evaluation of the incidence of viral pathogen

Samples (tomato, pepper and cucumber leaves) were collected from several commercial greenhouses in Ontario. Total RNAs were extracted by the Trizol method**.** In brief, samples were homogenized with liquid nitrogen and suspended in 1000 µl of Trizol solution (Ambion, USA). After a vigorous mixing, the samples were incubated for 10 min at room temperature, and centrifuged at 13000 rpm for 10 min and 4ºC. Supernatants were subsequently transferred to new tubes and 200 ul chloroform was added, followed by vigorously mixing through shaking for 15 sec, and incubating further for 2-3 min at room temperature. Centrifugation was performed for 15 min at 13000 rpm and 4ºC to separate layers. The aqueous layer was transferred into a new 1.5 ml tube and added 500 µl isopropanol. The sample was mixed well by tilting the tubes by hand. After a 10 min incubation at room temperature, the solution was centrifuged again, at 13000 rpm for 10 min and 4ºC. The supernatant was removed. The pellet was washed with 500 µl of 75 % ethanol, then briefly vortexed and centrifuged at 7500 g and 4ºC for 5 min. The pellet at the bottom was air dried, resuspended in 20 µl volume of ultrapure RNAase-free water, and incubated in heat block at 55ºC for 15 min. The RNA samples were aliquoted and frozen at - 80ºC for use.

The primers which specially target the viral coat protein coding region were used to detect the virus by RT-PCR. 1 µg total RNA was treated with Dnase I (Invitrogen, USA) for 15 min at room temperature, followed by addiiton of superscript III (Invitrogen, USA) to produce the first strand of cDNA.

In Feb 2015, leaf smaples were collected from 90 randomly picked plants in an Ontario greenhouse (10 samples for each row) and ground with 5 ml GEB (general extraction buffer). 100 µl buffers of each sample were tested if they might harbor CGMMV by using ELASA (Agdia, USA) and an infected cucumber sample was used as the positive control.

Double antibody sandwich ELISA (DAS-ELISA) was performed according to the standard protocol. ELISA plates were coated with 100 µl of the recommended dilution of IgG in carbonate coating buffer (15mM Na_2CO_3, 35mM $NaHCO_3$, and 5mM NaN_3, pH 9.6) and incubated overnight at 4°C. Plates were washed three times with PBST buffer, and 100 µl of

plant leaf extracts was added to each well and incubated for 2 h at room temperature. After washing the plates, 100 µl of alkaline phosphatase-conjugated IgG diluted in conjugate buffer was added and incubated for 2 h at room temperature. Wells were washed and incubated with 100 µl of substrate for 1 h at room temperature, and the absorbance was determined at 415 nm using a Microplate reader (BioRad, USA).

2.3 Results

2.3.1 Identification of viruses using small RNA sequencing

In this study, next-gerneration sequencing of the cucumber, pepper, and tomato small RNA libraries generated a total of 9177495, 13226570, and 6173141 raw reads, respectively. After trimming, a total of 8254371 (89.94%), 12204833 (92.28%), and 5625459 (91.13%) clean reads (>17nt) from the cucumber, pepper, and tomato libraries, respectively, were obtained, and were used to assemble into larger contigs. Contigs (longer than 100 nt) were kept and further analyzed by Blastn. The Blast results identified six viruses in greenhouse vegetable crops. Four viruses, *Bell pepper endornavirus* (BPEV), *Pepper mild mottle virus* (PMMoV), *Parietaria mottle virus* (PMoV) and *Tobacco streak virus* (TSV) were found in pepper samples. *Cucumber green mottle mosaic virus* (CGMMV) and *Pepino mosaic virus* (PepMV) were detected in cucumber and tomato plants, respectively. To the best of our knowledge, this was the first identification of BPEV and PMoV in Canada, and of CGMMV in Ontario, Canada. We thus further cloned and determined the full-length genomic seqeuences of these three viruses.

2.3.2 Cloning and sequencing of the full-length genome sequence of BPEV

BPEV belongs to the genus *Endornavirus*, a relatively young genus in the family *Endornaviridae* (King *et al*., 2011). Endornaviruses have a single linear dsRNA genome (9.8–17.6kbp), infect plants, fungi and oomycetes and are transmitted at a high rate through seeds and spores (Gibbs *et al*., 2000; Fukuhara *et al*., 2006). They infect economically important crops, such as rice (Moriyama *et al*., 1995), beans (Wakarchuk and Hamilton, 1990; Pfeiffer 1998), barley (Zabalgogeazcoa and Gildow, 1992), cucurbits (Coutts, 2005) and pepper (Valverde and

Gutierrez, 2007), some plant pathogenic fungi and the oomycete *Phytophthora* (Hacker *et al*., 2005).

The presence of endornaviruses in phenotypically normal plants is a unique feature of particular genotypes of these crops. The full sequences of the genome of approved or putative endornavirus species from cultivated rice [*Oryza sativa endornavirus* (OSV)] (Moriyama *et al*., 1995), wild rice [*Oryza rufipogon endornavirus* (ORV)] (Moriyama *et al*., 1995), broad bean [*Vicia faba endornavirus* (VFV)] (Pfeiffer, 1998), *Phytophthora* spp. [*Phytophthora endornavirus* 1 (PEV-1)] (Hacker *et al*., 2005), *Helicobasidium mompa* [*Helicobasidium mompa endornavirus 1* (HmEV-1)] (Osaki *et al*., 2006), *Gremmeniella abietina* [*Gremmeniella abietina type B RNA virus XL* (GaBRV-XL)] (Tuomivirta *et al*., 2009) and *Tuberaestivum* [*Tuberaestivum endornavirus* (TaEV)] (Stielow *et al*., 2011) have been found in the literature.

BPEV dsRNA was also detected in all seven bell pepper cultivars in Japan. Testing for BPEV dsRNA in *Capsicum* genotypes from the USA, including bell peppers and other horticultural types, resulted in 40 additional positive genotypes. BPEV appears to be common among domesticated Capsicum species, particularly in cultivated peppers (*C. annuum, C. frutescens and C. chinense*) (Valverde and Fontenot, 1991) and in *C. baccatum*. These closely related species share a mutual ancestral gene pool (Moscone *et al*., 2007). Previous investigations showed that all tested bell peppers contained BPEV (Valverde *et al*., 1990b; Valverde and Fontenot, 1991). Bell pepper cultivars have a narrow gene pool, and, while making crosses to develop new cultivars, breeders may have inadvertently introduced and spread BPEV among bell peppers.

Based on the assembled contig sequences from the next-generation sequencing of pepper small RNA libraries, primers were designed to clone the nearly full-length cDNA of the BPEV Canadian isolate. The 5’ and 3’ end sequences were obtained by RACEs. The genome sequence of the Canadian isolate is composed of 14726 nt which has a single open reading frame (ORF) encoding a polyprotein containing viral methyltransferase (MTR), RNA helicase 1 (Hel-1), UDP-glucose–glycosyltransferase (UGT) and RdRp (Okada *et al*., 2011; Okada *et al*., 2013; Song *et al*., 2011; Okada, *et al*., 2014). A single ORF starts at nt 226 and ends at nt 14673, which encodes a polyprotein of 4815 aa (Figure 2.2). A comparison of the genome sequences of other

BPEV isolates retrieved from the NCBI database revealed that the Canadian isolate (KT149366) shares sequence identity to the other six isolates, BPEV-YW-USA1 (NC_015781.2), BPEV-Kyosuzu-Japan (AB597230.1), BPEV-Maor-Korea (KP455654.1), BPEV-Atir-Israel (JQ951943.1), BPEV-China KF (709944.1), BPEV-YW-USA2 (JN019858.1) in a range of 88 to 99% at the nt level and 92 to 99% at the aa level.

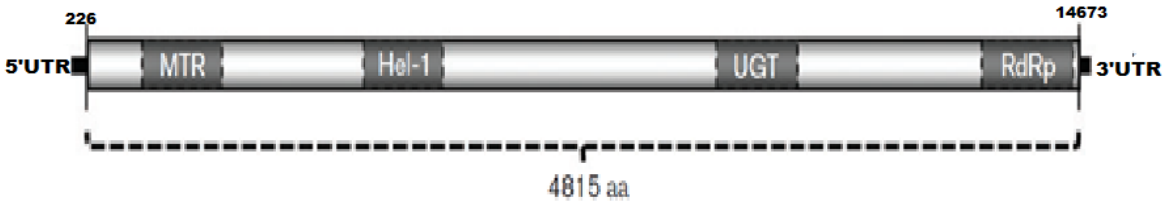

Figure 2.2 Genome organization of *Bell pepper endornavirus* (BPEV). Abbreviations: MTR=viral methyltransferase, Hel-1=RNA helicase 1, UGT=UDP-glucose–glycosyltransferase, RdRp= RNA-dependent RNA polymerase.

Genomes of all completely sequenced endornaviruses contain conserved motifs of a viral RdRp (RdRp_2, pfam00978), similar to the alpha-like virus superfamily of positive-stranded RNA viruses (Gibbs *et al*., 2000). Phylogenetic analysis using the RdRp region of the various endornavirus sequences (Figure. 2.3) generated a tree that is congruent with what is known about relationships in the BPEV group, based on isoenzyme data (McLeod *et al*., 1982). A phylogenetic analysis revealed that this Canadian isolate (BPEV-Canada-ON, GenBank no: KT149366) is distinct from the YW isolate (JN019858.1) from the USA, and the remaining four isolates from four different countries are clustered a phylogenetic group.

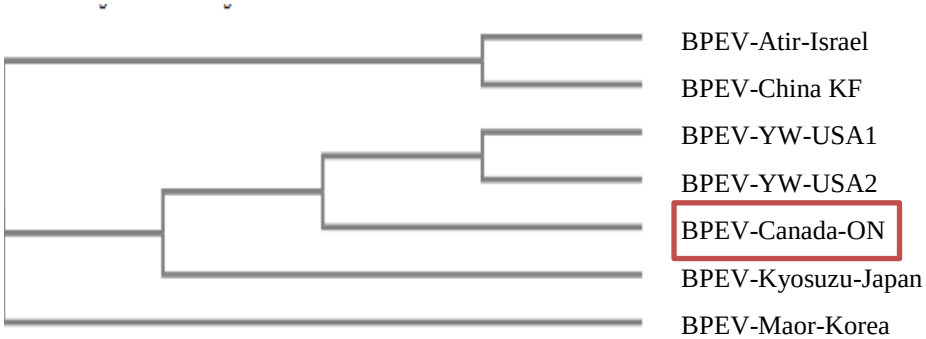

Figure 2.3 Phylogenetic tree obtained from the alignment of amino acid sequences of RdRps of published BPEV isolates.

2.3.3 Cloning and sequencing of the full-length genome sequence of PMoV

The members of the genus *Ilarvirus* are distributed worldwide and affect a large number of agronomical relevant crop species including fruit trees, vegetables, and ornamentals. Ilarvirus was first identified in the early of 1910's and has been reported in the infected woody species (King *et al*., 2012).

The genus name Ilarvirus was created as a sigla from the properties of the viruses (isometric labile particles which are associated with ringspot symptoms) and was first used as a specific reference to *Prune dwarf virus* (PDV) and *Necrotic ringspot virus* (NRSV)—now referred to as *Prunus necrotic ringspot virus* (PNRSV) (Fulton, 1968) of Prunus, but later adopted for wider use by the International Committee on Taxonomy of Viruses (ICTV) (Fenner, 1976). Ilarviruses have a reputation of being difficult to purify and discouraging subjects for the production of antibodies. Ilarviruses (the family Bromoviridae) have a positive-sense, single-stranded RNA genome consisting of three linear segments (RNA-1 to -3). The 1a (viral replicase) protein is encoded by RNA-1, and the 2a (RdRp) and 2b proteins are encoded by RNA-2. The 3a (cell-to-cell movement) protein (MP) and 3b coat protein (CP) are encoded by RNA-3, although the CP is translated from the sub-genomic RNA-4, which is derived from RNA-3 (see Figure 2.4). It is worth mentioning that the small 2b protein which has been found in some members of the genus is located toward the 3'-terminus of the RNA-2 segment and expressed through a subgenomic RNA (Xin *et al*., 1998). The function of this protein, based on similarities with the cucumoviruses in the same family, has been suggested to be involved in viral movement and gene silencing (Lucy *et al*., 2000). This latter function has been demonstrated for the ilavirus *Asparagus virus 2* (AV-2) in a recent work in Japan (Shimura *et al*., 2013). The 2b ORF is found only in subgroup 1 and subgroup 2 ilarviruses.

Searches against the NCBI database using the assembled contigs from the small RNA sequences hit the genome sequence of a virus belong to the genus *Ilarvirus* with low (73-79%) sequence similarity. Based on the contig sequences, primers were designed to clone the nearly full-length cDNA of this Canadian isolate. The complete genome is comprised of three RNAs with 3481, 2855, and 2283 nucleotides (nt) representing RNA1, RNA2, and RNA3, respectively. It contains 5 putative ORFs that are similar to the analogously positions in the

Ilarvirus genus. RNA1 consists of one ORF (position 311-3130 nt), and encodes a replication-associated protein of 939 amino acids which contains the conserved domains of viral methyltransferase (pfam01660) and viral (Superfamily 1) RNA helicase (pfam01443). RNA2 has two ORFs, coding for the 2a RdRp (position 369-1832 nt) and the 2b protein (position 1573-2175 nt), respectively. RNA3 contains two ORFs encoding a movement protein (position 277-1140 nt) and a coat protein (position1310-1861 nt). BLASTn searches to the NCBI database revealed that this virus shares a sequence identity (76%) in RNA1 to *Ageratum latent virus* (AgLV) isolate 1998 (GenBank accession: JX463340.1) (Sharman and Thomas, 2013). Similarly, RNA2 shares the lower sequence identity (72%) with PMoV (GenBank accession: AY496069.1) (Scott *et al*., 2006). For RNA3, a wide range of sequence identity (66% to 80%) was again shared with PMoV (GenBank accession: FJ858204.1) (Scott *et al*., 2006). At the amino acid level, the putative size of the replication-associated protein is 105.97kDa, which shares 82% similarity to the replicase protein of *Ageratum latent virus* (AgLV) (YP-008470969.1) (Sharman and Thomas, 2013). RdRp is composed of 487 aa, and the putative size is 55.7kDa, sharing the identity 71% with the RdRp 2 protein of PMoV (ACU44509.1) (Scott *et al*., 2006). However, the 2b protein only has the 66% similarity with TSV (ACJ38089.1) (Scott, 1998). For RNA3, coat protein shares the highest similarity (97%) with *Tomato necrotic spot virus* (ToNSV, ACI96015.1) (Sharman *et al*., 2011), but movement protein only has the 59% identify with AgLV (YP008470972.1) (Sharman and Thomas, 2013).

A representative selection of complete genome sequences of current Ilarvirus members including PMoV, AgLV, *Bacopa chlorosis virus* (BaCV), *Strawberry necrotic shock virus* (SNSV), ToNSV, *Spinach latent virus* (SpLV), AV-2 , PDV, *American plume line pattern virus* (APLPV) , and PNRSV (ICTV, 2011), were used for phylogenetic analysis using the CP aa sequences. The analysis was conducted by using the maximum likelihood method implemented in the PhyML program (v3.1/3.0 aLRT) (Sharman and Thomas, 2013).The results revealed that the recognized subgroup 1 members formed a well-supported clade that was separate from subgroup 2 and subgroup 3. The Canadian isolate belongs to subgroup1 of the genus Ilarvirus, and shares a 95% CP amino acid identity, but it is distinct from other viruses (PMoV, AgLV, BaCV) in this subgroup (Figure 2.5). So, I propose that this virus might be a new virus in the genus.

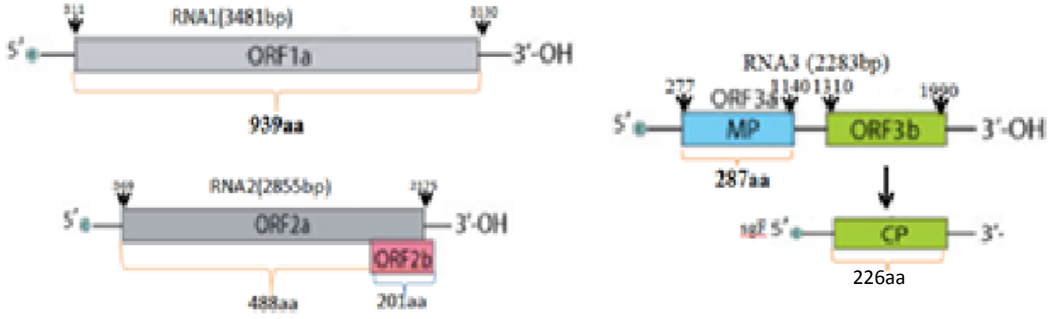

Figure 2.4 Genome organization of *Parietaria mottle virus* (PMoV). Abbreviations: ORF=open reading frame, MP=movement protein, CP=coat protein.

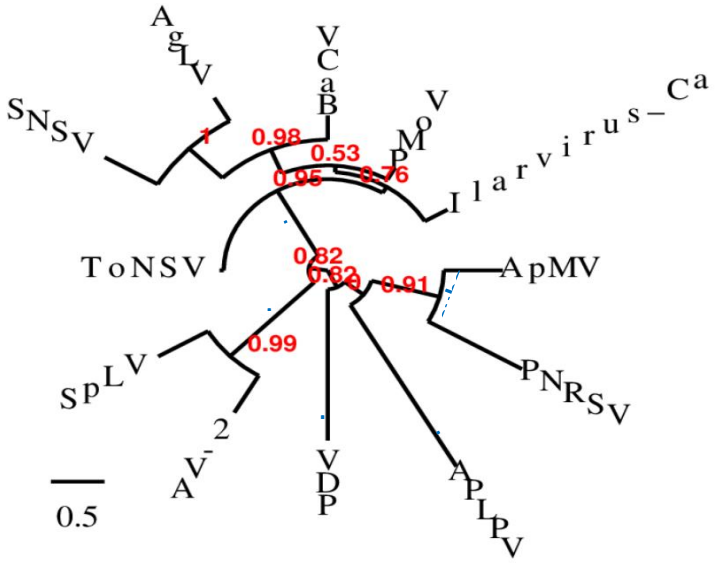

Figure 2.5 Phylogenetic tree obtained from the alignment of amino acid sequences of Coat Protein.

2.3.4 Cloning and sequencing of the full-length genome sequence of CGMMV

CGMMV was first reported in the UK in 1935 (Liu *et al*., 2015), and was found from a melon seed production field in the USA in the summer of 2013. However, the disease caused by CGMMV has long been known in Europe (ASTA, 2014), Asia (Ugaki *et al*., 1991; Liu *et al*., 2009), the Middle East (Reingold *et al*., 2016), and more recently in Canada (Ling *et al*., 2014). The complete nucleotide sequence of the genomic RNA of about 30 CGMMV isolates such as CGMMV-SH from watermelon and CGMMV-ABCA13-01 from cucumber were determined (Ugaki *et al*. 1991; Li *et al*., 2015a). CGMMV is a member of tobamovirus and has a rod-shaped, viral particle with 300 nm long and 18 nm wide. This virus is easily sap- and seed-transmissible and can survive for a long period in infected plant debris. CGMVV has a narrow range of hosts which is primarily limited to the cucurbit species, such as cucumber, melon, watermelon, squash, and pumpkin. CGMMV can cause serious problems due to its transmission mode and its stability in plant debris and soil or on greenhouse or equipment surfaces. The infected plants show severe symptoms at the early stage of infection. The infected leaves subsequently wilt and dry dead, and finally the whole plants die. The damage it causes to the host plant and fruit can be extensive, resulting in substantial yield losses. Therefore, it is a serious threat to fresh market, export and cucurbit seed industries in the areas where CGMMV is endemic.

To clone and determine the complete genomic sequence of the CGMMV isolate (CGMMV-Ca) identified in this study, primers were designed based on the virus specific contigs sequence. The complete genome sequence of CGMMV-Ca is 6423 bp long, and consists of 5'-and 3'-untranslated regions (UTRs), and three ORFs which encode the replication protein, MP, and CP (Figure2.6). Blast research against the NCBI database showed a similarity of 98% with a CGMMV isolate (NCBI accession: AB015146) at the nt level and a 98%-100% identity at the aa level. The 5'- and 3'-UTRs consist of 58 and 174 nucleotide residues, respectively. The first ORF (including the readthrough) located from nt 59 to 5005 encodes a 186 kDa replication protein and a 129 kDa non-readthrough protein (from nt 59 to 3493), and contains a viral methyltransferase domain (aa 50 to 426), a viral helicase1 domain (aa 859 to 1113) and a RNA dependent RNA polymerase domain (aa1193-1639). The other two ORFs encode MP and CP with 264 aa and 161 aa, respectively.

An alignment of the 186 kDa proteins (ranging from1645 to 1648 aa) of CGMMV-Ca (from this study) with the corresponding protein of CGMMV-W (AB369274.1), CGMMV-GX (GX strain, GQ277655.1) and CGMMV- KY (Kyuri Y strain AB015146) identified a 30 aa residue difference. To examine the evolutionary relationship of CGMMV to other tobamoviruses such as CGMMV-W, CGMMV-GX, CGMMV-KY, *Tobacco mosaic virsu* (TMV) (NC_001367), *Tomato mosaic virus* (ToMV) (NC_002692), PMMoV (NC_003630) and *Odontoglossum ringspot virus* (ORSV) (NC_001728), a phylogenetic tree was constructed to base on identities in deduced aa sequences of CP, using the PhyML program (v3.1/3.0 aLRT) (Figure 2.7). The result demonstrated that CGMMV has the closest relationship with CGMMV-GX and CGMMV-SH.

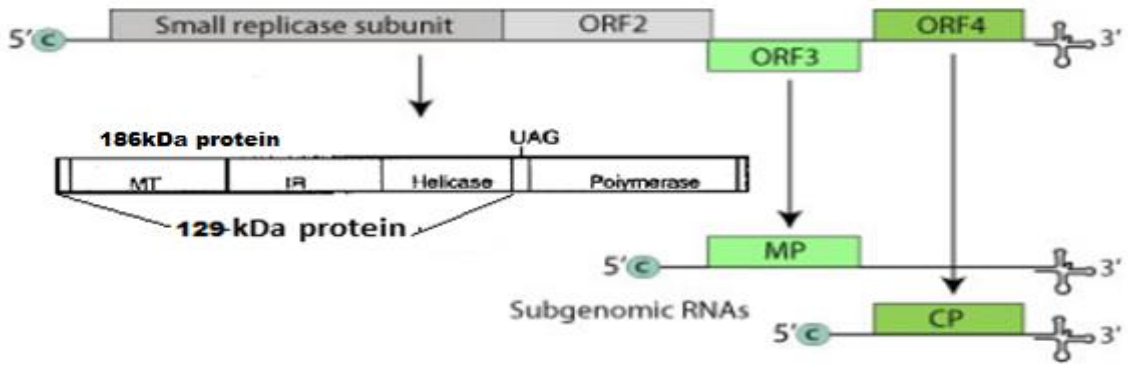

Figure 2.6 Genome organization of *Cucumber green mottle mosaic virus* (CGMMV). Abbreviations: ORF=open reading frame, MP=movement protein, CP=coat protein.

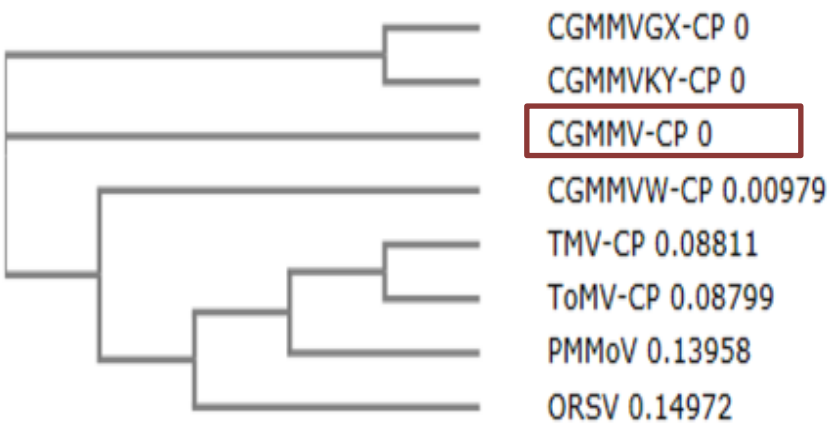

Figure 2.7 Phylogenetic tree obtained from the alignment of amino acid sequences of the coat protein (CP) of different tobamoviruses. **CGMMV-GX, *Cucumber green mottle mosaic virus* GX strain; CGMMV-KY, *Cucumber green mottle mosaic virus* kyuri Y strain; CGMMV-W, *Cucumber green mottle mosaic virus* watermelon stain; ORSV: *Odontoglossum ringspot virus*; PMMoV: *Pepper mild mottle virus*; TMV: *Tobacco mosaic virus*; ToMV*: Tomato mosaic virus*.**

2.3.5 Evaluation of the incidence of viral pathogen

Implementation of particular disease management measures relies on disease incidence and crop loss which are key factors for the assessemnt of the economic aspects of the disease. RT-PCR methodology applied in this work was reliable, resulting in a single major band of the expected size. Primer sets used in this work did not interfere with non-target viruses or nucleic acids from the host. Primers were listed in the table 1.1. No viral pathogens were determined in tomato samples. Five of six cucumber samples (83.33%) were detected to harbor CGMMV and an incidence rate of 75% was found in pepper samples for BPEV.

Due to the highest incidence rate of CGMMV, two months later, 90 leaf samples from 90 plants were collected and detected using ELISA. The results showed that all samples were CGMMV-positive. In order to confirm the infectivity of CGMMV, these leaf samples were used

as inoculum to inoculate the first truth leaf of cucumber plants. The severe mosaic symptom was observed two weeks post inoculation.

CGMMV could also be detected in different tissues of one-month-old cucumber plants, such as old leaf, newly emerging leaf, root, and fruit (Figure 2.8). Using RT-PCR CGMMV could be identified in the symptomatic leaves (100 % detection rate) and asymptomatic leaves (50 % detection rate) (Figure 2.9).

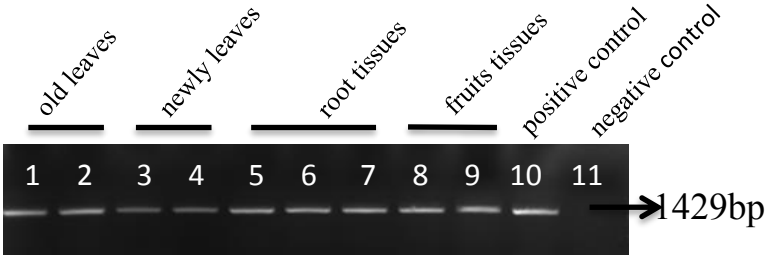

Figure 2.8 Detection of CGMMV in the different tissues of one-month-old cucumber plants by RT-PCR.

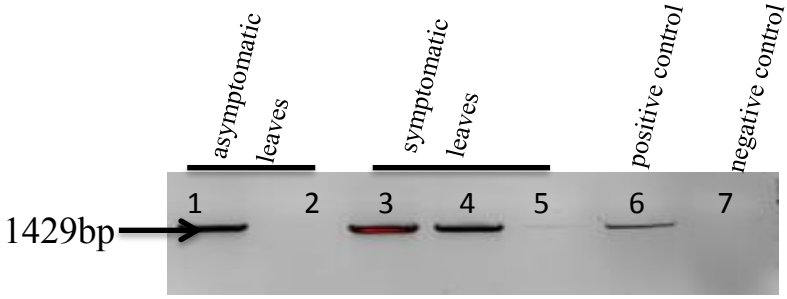

Figure 2.9 Detection of CGMMV in asymptomatic leaves, and symptomatic leaves by RT-PCR

2.4 Discussion

This chapter represents a pioneer research on greenhouse vegetable viruses in Ontario. The study contributed to the identification of the viral pathogens in the three major greenhouse vegetables, i.e., tomato, pepper, and cucumber by using a next-generation sequencing technique. This work further supports that in-depth small RNA sequencing is a powerful, robust technology for identification of viral pathogens. In principle, this technology allows for detection of both RNA and DNA viruses, from widely different families and with different tissue trophisms and intracellular replication sites, even in extremely low titer and seemingly symptomless infections. In the present study, six viruses were identified in the greenhouse vegetable crops using next-generation sequencing technology. Blast searches against the NCBI database revealed that the contig sequences obtained shared high sequence similary/identity to the sequences of BPEV, PMMoV, TSV, CGMMV, and PepMV and relatively low similarity/identify to the sequence of PMoV. This represents the first piece of evidence showing that BPEV, PMoV and CGMMV in Ontario or in Canada. I further determined the complete genome sequence of BPEV, CGMMV, and PMoV. The availability of the genome sequences of these viruses will make it possible for the development of quick diagnostic tools to monitor the incidence of these viruses, for the study of their evolutionarily relationship to other ioslates, and for the further sutdies of molecular biology of these viruses.

Incidence in epidemiology is a measure of the probability of occurrence in a population within a specified period of time. Incidence proportion (also known as cumulative incidence) is the number of newly infected plants within a specified time period divided by the size of the population initially at risk. Therefore, the assessment of disease incidence for greenhouse vegetable crops is a key factor for the economic aspects of disease management. In Israel, the high incidence (>90% infected plants) of CGMMV disease has been reported in the six commercial greenhouses in 2009 (Reingold *et al*., 2016). The DIG-labelled cDNA probe CG was used for detecting CGMMV in 166 watermelon samples which were collected from 218 fields in 12 provinces of China during 2006 and 2007. The incidence of the virus in provinces Hebei (5 samples), Guangdong (5 samples), Hubei (13 samples) and Shandong (27 samples) were 80, 100, 100 and 88.89%, respectively (Liu *et al*., 2009). In the current study, ELISA and RT-PCR

analyses of randomly-picked samples from commercial greenhouses showed 100% CGMMV and a high detection rate was present in the different parts of tissue, symptomatic leaves and asymptomatic leaves in the cucumber plants in Ontario. Such high incidences often demand an immediate action to control the spread of the disease.

However, these disease incidence assessment methods will only be accurate if performed on a representative sample of the crop. Samples of crop units (plants, leaves, fruit etc.) can be taken randomly from a crop, and can be placed in the crop and all plants within the quadrat assessed. To determine how many samples need to be taken, it is possible to sample a number of times with progressively more samples, and find the point at which the standard error is low. A disease that is uniformly spread throughout a crop will require fewer samples for an accurate assessment than a disease that has a patchy distribution throughout the crop.

2.5 Reference

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Chapter 3. Understanding CGMMV plant-to-plant transmission in a hydroponic system

3.1 Introduction

Greenhouse vegetables in Ontario, Canada are mainly grown in hydroponic systems. The use of circulating nutrient solutions in hydroponic systems might facilitate the rapid spread of water-transmissible plant pathogens throughout a whole crop, which can increase the chances of an epidemic if this is not managed appropriately (Stewart-Wade, 2011). Several recent reviews have discussed the possible source of plant viruses and their survial in water and the virus survival, the possibility of plant infection with water-borne viruses, and possible control strategies for prvention of virus spread (Mehle *et al*,. 2012, Mehle *et al*., 2014).

Viruses from infected plants, released into the nutrient solution in a hydroponic system by the roots, can infect other plants through the roots, without any contact or mechanical spread from upper plant parts, without the help of vectors. This has been demonstrated for ToMV infected tomatoes. Pares *et al*., (1992) investigated the transmission of ToMV in a recirculating nutrient solution. ToMV was identified in the nutrient solution three days after leaf inoculation of tomato plants. Healthy tomato plants, with no contact with infected plants, became systemically infected as early as ten days after planting into nutrient solution containing infectious virions.

In early 2013, greenhouse cucumber growers in Alberta, Canada, observed virus-like disease symptoms on mini-cucumber (*Cucumis sativus*) crops (e.g., 'Picowell'). The strong sequence identity to the CGMMV Asian isolates suggests that the Canadian CGMMV isolate identified in Alberta was likely to be of Asian origin (Ling *et al*., 2014). In 2014, the cucumber leaves collected in an Ontario greenhouse were found to be CGMMV-positive with an incidence of 100%. As metioned in chapter 1, CGMMV is apparanetly the most devastating viral pathogen that limits greenhouse cucumber production in Canada. ToMV and CGMMV both belong to the genus *Tobamovirus*, and it is very likely that they share the same transmission mode via the recirculating nutrient solution. However, it is not clear whether CGMMV is transmissible via

hydroponic solutions. This information is necessary for the effective prevention of the spread of diseases to reduce the economic losses, and also essential for greenhouse vegetable growers to decide if hydroponic nutrient solutions can be reused. Recycling of hydroponic solutions is not only important for cost savings but also for environmental protection.

In this chapter, I tested the possibility that the recirculating nutrient solution is a source of infection of the cucumber crop using wild-type CGMMV.

3.2 Materials and Methods

3.2.1 Plant materials, growth chambers and virus source

Cucumber cultivar Straight 8 seeds were germinated in a growth chamber set at 21-23 °C . The CGMMV isolate used in this study was obtained and identified as described in Chapter 1.

3.2.2 Experimental hydroponic system

The cucumber seedlings (Straight 8) were transplanted in two containers sharing a mini recirculating hydroponic system (Figure 3.1). After one week of co-hydroculture, CGMMV was inoculated to the upper leaf of cucumber seedlings in one container. The bait plants were placed into the other separate container. The nutrient solution (see Table 3.1) was recirculated by a mini pump for 24 h every day. The pH value of the nutrient solution was monitored daily and keep the value about 5.5-6.0.

Figure 3.1 Experimental hydroponic systems

Table 3.1 The ingredients of the hydroponic nutrient solution.

Chemical Compound	g/1000 liters
Magnesium sulfate (MgSO4)	500
Phosphate (KH2PO4)	270
Potassium nitrate (KNO3)	200
Potassium sulfate (K2SO4)	100
Calcium nitrate (Ca(NO3)2)	500
Chelated iron(Fe3+)	25
Boric acid (H3BO4)	4.17
Manganous chloride (MnCL2)	3.75
Cupric chloride(CuCL2)	0.206
Molybdenum trioxide (MoO3)	0.083
Zinc sulfate (ZnSO4)	0.656

3.2.3 Virion purification and transmission electron microscopy

Different tissue parts (leaves, stems, and roots) were collected from the CGMMV-inoculated plants and bait plants. The harvested tissues were ground in a mortar with 0.1 M phosphate buffer (pH 7.0), containing 0.1% β -mercaptoethanol (3 ml/g tissue), added with chloroform (2 ml/g tissue), mixed for 5-10 min and centrifuged at 8000 g for 20 min and 4 °C. Then the supernatant was filtered through a 40 μm nylon cell strainer and ¼ volume of 40% PEG8000/1M NaCl was added. The mixed suloution was agitated for 1 h at 4 °C, centrifuged at 8000 g for 20 min and 4°C. Finally the resulting pellet was re-suspended in 50-100 μl 0.01M PBS buffer and centrifuged at 10000 g for 20 min and 4°C. The supernatant containing partially purified virions was retained for analyses by transmission electron microscopy (TEM), Western blot and RT-PCR (extract RNA first). The same protocol was essentially used to purify CGMMV virions from the nutrient solution in the hydroponic system.

For negative staining, 20 µl purified solution containing CGMMV virions was added to a grid and incoubated for 60 sec. After removel of excess liquid with filter paper, the grid was placed on the filter paper (sample side facing up) and dried for 5 min. Subsequently, one drop of 2% PTA (pH: 7.0) was placed on the grid and incubated for 60 sec, followed by removal of liquid using filter paper. Finally, the grid was placed on the filter paper (sample side facing up), and dried for 5 min before observation by TEM.

3.2.4 RNA extraction of purified virion and western blot

The purified virions were dissolved with DEPC-treated water, vortexed, spun briefly, and incubated at 95°C for 10 min and chilled immediately on ice for 5 min. RT-PCR and qRT-PCR was done by using the CP primers (CP–F: 5'- AAGGTACCGCCTTCCAGACT-3'; CP-R:5'-GACCGTTGAGGAAAGCGTAA-3') as described previously (Wu *et al*., 2014).

10 µl purified virions solution was mixed with the same volume of loading buffer and incubated in 99°C for 15 min and chilled immediately on ice for 10 min. The solution was visualized by 12% SDS-PAGE. Western blotting was carried out using the protocol essentially as previously described (Song *et al*., 2011). The separated protein on gel was electroblotted onto a PVDF membrane. Then, the membrane was blocked overnight at 4°C, incubated in CGMMV special antibody (Agdia) and followed by incubation with anti-rabbit IGG (Sigma).

3.3 Results

3.3.1 Observation of CGMMV virions were in the inoculated plants by TEM

Ever since TMV was first visualized by electron microscopy (EM) in 1939, EM has been a valuable tool in plant virology. TEM is extremely useful in virology studies such as observation of purified viral particles and modifications of organells in infected cells. The bait plants and inoculated plants were co-cultured in recirculating solution 4 weeks after inoculation. TEM showed that CGMMV virions were present in the leaf or root tissues of inoculated plants (Figure 3.2). The virion is almost a rod-shaped 300 nm long and 18 nm wide. However, the virions were not detected in the stem tissues of inoculated plant, all parts of tissues in bait plants

and nutrient solutions. This is probably because the viral titre was too low in the stem of inoculated plants, in the bait plants and in the nutrient solution.

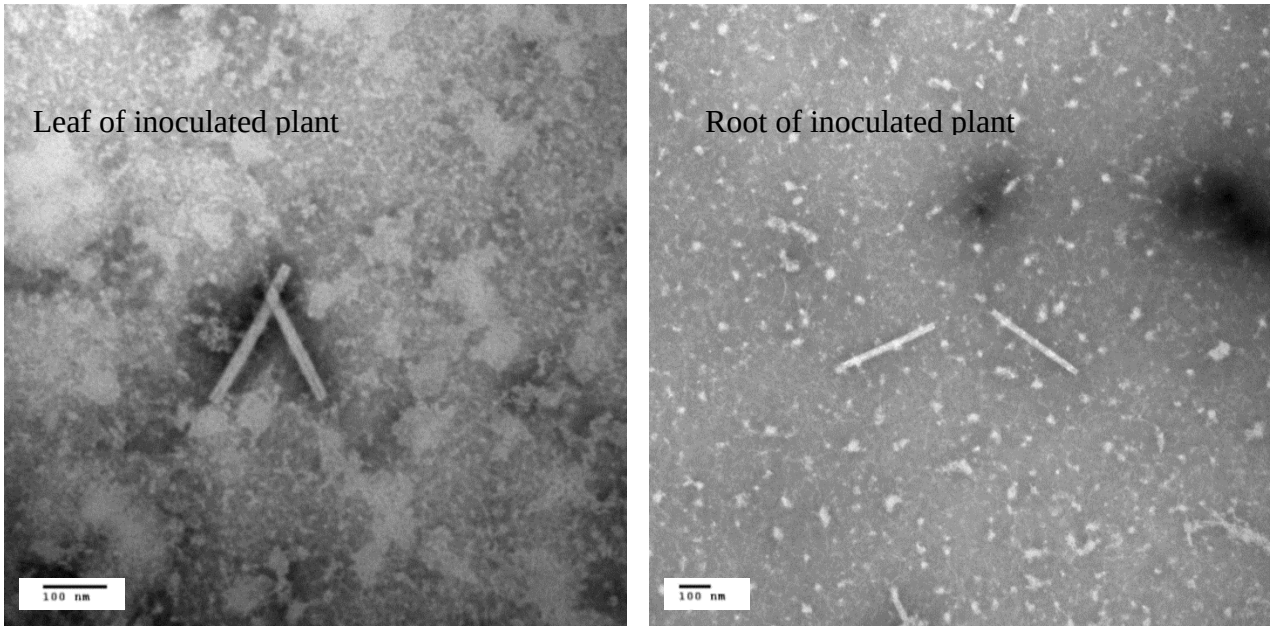

Figure 3.2 TEM of CGMMV particles purified from the leaf and root tissues of CGMMV-infected cucumber plants. Scale bars = 100 nm.

3.3.2 Occurrence of CGMMV viral RNA in the different tissues of inoculated and bait plants, as well as nutrient solutions

RT-PCR is a very sensitive method for the detection of low titres of RNA viruses. We thus conducted RT-PCR to detect CGMMV in different tisssues of inocuated and bait plants as well as in the nutrient solutions. Our RT-PCR data clearly revealed the presence of CGMMV in all the three tissues in the inoculated and bait cucumber plants except for the stem tissue of bait plants (Figure 3.3A). Moreover, in the nutrient solution (Figure 3.3A). The qRT-PCR result further confirmed that the viral accumulation level in the root tissues was highest among the three tissues in both inoculated and bait plants (Figure 3.3B). The viral accumulation level in stem tissues was 20 fold lower than that of root tissues in the inoculated plant (Figure 3.2B).

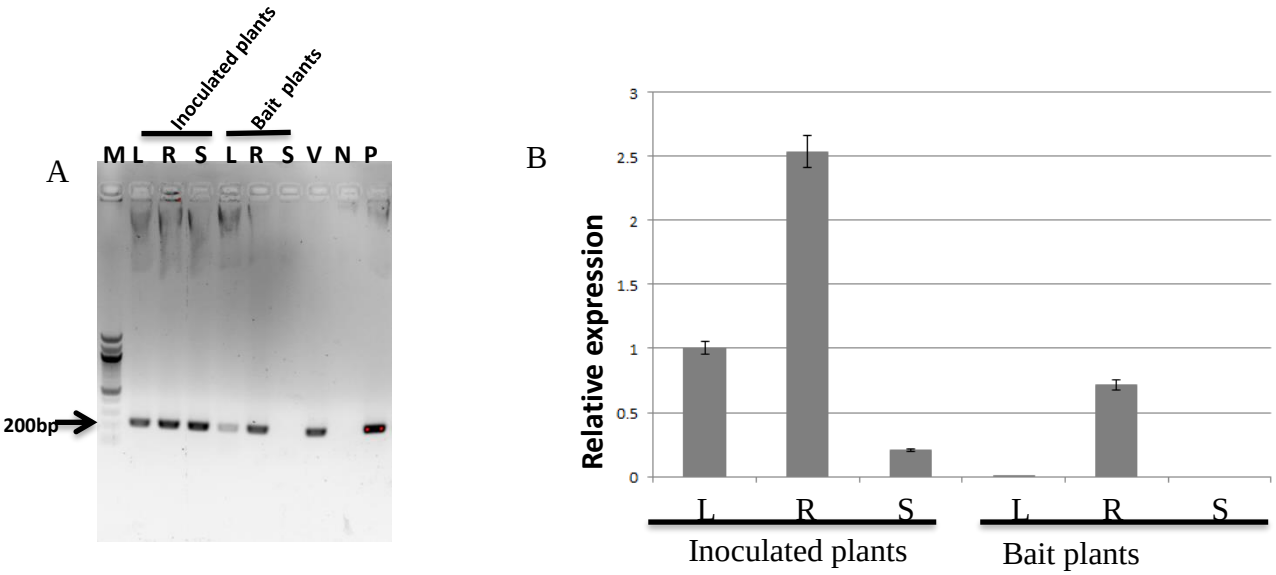

Figure 3.3 CGMMV viral RNA in the leaves, stems, roots of inoculated and bait cucumber plants was detected by RT-PCR (A) and quantified by qRT-PCR (B) using CP-specific primers. M, 100bp DNA ladder; L, leaves; R, roots,; S, stems; V, virion isolated from the hydroponic solution; N, negative control (distilled water); P, Positive control (WT CGMMV).

3.3.3 Presence of CGMMV CP in the different tissues of the inoculated and bait plants

CGMMV CP is composed of 161 amino acids with a predicted molecular mass of 17.4 KDa. To analyze the accumulation level of CP in the different parts of the inoculated plants and bait plants, Western blots were performed to detect CP from the samples of purified CGMMV virions. CP was detectable in all parts of the inoculated plants (Figure. 3.4). However, in the bait plants, a small amount of CP was only detected in the root tissue, but not detectable in the stem and leaf samples. Since Western blot is a protein-based technology, it is much more sensitive than TEM but less sensitive than RT-PCR and qRT-PCR. Therefore the Western blot data are consistent with results of TEM, RT-PCR and qRT-PCR analyses.

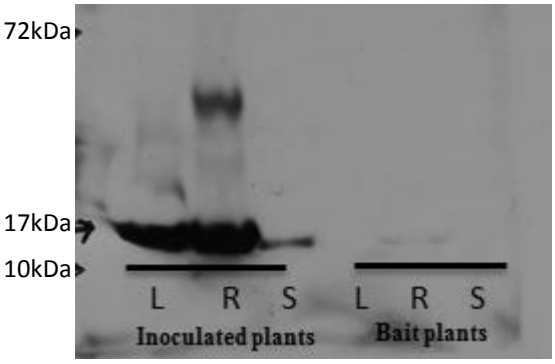

Figure 3.4 Western blot detection of CGMMV CP protein in different tissues parts of inoculated and bait cucumber plants. L: leaf tissue; R: root tissue; S: stem tissue.

3.4 Discussion

Soil-less culture has provided an alternative to plant growers facing soil-related problems, but the use of recirculating nutrient solutions in hydroponic systems may facilitate the rapid spread of water-transmissible plant pathogens throughout the whole crops. Although numerous plant viruses have been observed in water environments, their survival in water and the possibility of their direct transmission through nutrient solution are still unknown. The first report of viruses in irrigation water as a possible cause of crop infection was in 1969 (Van Dorst, 1969). In this study, I found that the bait plants physically separate from CGMMV-infected plants were CGMMV-positive after sharing a hydroponic nutrient solution. We also discovered that high levels of CGMMV were found in the root tissues in the inoculated plants and relatively low levels of CGMMV were present in the nutrient solution. Therefore it is reasonable to suggest that CGMMV from the inoculated plants could be released to and transmitted through the nutrient solution to infect the root system of the bait plants. As a result, the healthy plants could be infected by the virus. Then the quesiton is how the virus is released from and enters into the root system of the plants. It is possible that the nutrient circulating system might damage roots of hydroponic plants. Damaged root tissues would not be expected to support greater virus replication than the healthy tissue but would be expected to allow greater leakage of virions,

either alone or in sloughed off infected cells, into the nutrient solution (Pares *et al*., 1992). The possible mode of entry for the virus into the root tissues would be either from the damaged root or through lateral root. When a lateral root is initiated and the root primordia increase in length, cortical cells are deformed and pushed aside causing a lack of connection between the emerging lateral root and the cortex of the parent root leaving an opening into the interior of the parent root that may serve as an entry for pathogens (Esau, 1977). While soiless growing conditions offer many advantage for crop production, and viruses have so far not been a major problem, my work shows that virus diseases could be a serious problem particularly when solutions are recycled.

However, the infectivity of pathogens in water also depends on the concentration of the pathogen, which is usually low. This also explains why CGMMV is undetectable in the nutrient solution by TEM and Western blot. Our trasmission assay demonstrated that the nutrient solution could be a path for CGMMV to spread in hydroponic systems. The aqueous environment has been neglected as a pathogen infection pathway, most probably due to the low concentrations of pathogens. It is also possibily owing to the apparantly inefficient water-mediated plant infection in comparison to insect vector-mediated transmission between the green parts of plants. In hydroponic systems, plants like cucumbers, tomato, peppers have a long culture and harvest season which is ususally in a range of 8 to 16 months. Therefore, the pathogens in the infected plants may have time to build up, release into the aqueous system and further spread to the healthy plants via root systems. In view of the fast growth of the greenhouse vegetable sector, more studies are highly demanded to advance knowledge in understanding the mechanism underlying the release and transmission of pathogens via the root system and develop technologies to monitor and control pathogens.

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Chapter 4. Generation of an attenuated variant against CGMMV

4.1 Introduction

Viruses cause considerable losses and there is a range of control measures aimed at mitigating these losses. The use of fungicidal chemicals is an important approach to protect crop plants from fungal pathogen infection and to minimize losses. However, no such chemicals are available for the control of viral diseases are available. Most commonly used methods have focused on reducing sources of infection inside or outside the crop, minimizing the transmission of viruses, and the deployment virus-resistant crop varieties (Hull, 2002; Anderson *et al*., 2004). Generally speaking, such measures do not offer a permanent solution to a virus disease problem, in a particular area. Control of viral diseases is usually a running battle, in which the organization of control procedures, implemented by individual growers, and co-operation between them are necessary, from year to year. Several measures have been tried for the control of viral diseases, such as modified planting and harvesting procedures that introduce breaks in the infection cycle, changing of planting dates, widely increasing plant spacing and destroying the aerial parts of the plant. To manage mechanically transmitted viruses, one of the most successful controls has involved the development of virus-free vegetative stocks (Fraser, 1992; Murphy, 2006).

Most plant viruses rely on vectors for their transmission, and this is an essential part of virus dispersal mechanisms. New methodologies have been developed to discover vector receptors, host and vector resistance genes, and molecular details of host-virus-vector interactions. Current control strategies that focus on interference of virus vectors include chemical control methods, integrated management approaches, and the breeding of crops with resistance to vectors or low vector preference (Bragard *et al*., 2013).

Where a virus is transmitted through seeds or vegetative materials for vegetative propagated crops, such transmission could be an important source of infection, as it introduces the virus into the crop at a very early stage, allowing infection to be spread to other plants while they are still young. Chemotherapy and thermotherapy methods may be used for the control of such viruses. For example, hot water or dry heat and several chemicals have been shown to

reduce or completely eliminate PepMV infectivity in tomato (Ling *et al*., 2010). The effectiveness of thermotherapy, at different temperatures, and chemotherapy, with different concentrations of ribavirin, along with combinations of these two methods was also evaluated on the elimination of three viruses in apple (Hu *et al*., 2015). A treatment combining ribavirin (25 μg/ml) and thermotherapy at 36 °C (R25 + T36) resulted in a high virus eradication efficiency of 95.0% (Hu *et al*., 2015).

4.1.1 Natural genetic resistance to viruses in plants

The use of genetic resistance has been considered the most effective and sustainable approach to the control of plant viral pathogens as it is environmentally-friendly and target-specific, and provides reliable protection without additional labor or material costs during the growing season (Kang et al., 2005; Wang and Krishnaswamy, 2012). Plant resistance to disease can be genetically inherited, which was recognized over a century ago (Biffen, 1905). The success of conventional breeding strategies based on a plant's natural defense system, lies in the fact that dominant monogenic resistance (*R*) genes are good sources of protection against viruses (Fraser, 1990). As pointed out by Fraser (1988, 1992) when considering the complexity of the host population involved, there are three main types of resistance and immunity to a particular virus: (1) immunity which is related to the host range of virus; (2) cultivar resistance which describes that one or more cultivar, or breeding lines, within a species show resistance while others do not; (3) acquired resistance which is present in an otherwise susceptible individual plant, following inoculation with a virus.

In regard to the response of plants to a viral pathogen, the strong defense response that results from the incompatible plant–virus interaction is frequently accompanied by localized programmed cell death, termed the 'hypersensitive response' (HR). HR is important in agriculture and horticulture as the basis for field resistance to viral infection, when the localization of viral replication in tissues is near the site of infection. These phenomena have been the subject of many studies for many years (Ponz and Bruening, 1986; Culver, 1997). In the absence of specific recognition, a basal innate defense response also occurs; this does not prevent host from invasion by the pathogen, but can limit the extent of the invasion.

There is an advantage to vegetative propagation: maintenance of cultivar genotypes is simple (Peloquin and Hougas, 1958, Barrell *et al*., 2013). Conventional breeding takes advantage of heterotic genotypes in order to develop new cultivars, while maintaining high yields (Phumichai *et al*. 2005). Although crosses between highly heterozygous individuals allow breeders to meet market demands for superior commercial cultivars, more precise breeding of particular characteristics, of market importance, is more difficult as the breeding materials lack homozygosity (Peloquin and Hougas, 1958). Transgenic approaches have significantly reduced the breeding challenges in vegetative propagated crops (Cavatorta *et al*. 2011). An example of this is the use of inducing gene silencing to develop banana plants with resistance to *Banana bunchy top virus* (Shekhawat *et al*. 2014). This approach is limited to precise pairings of hosts and pathogens, however, as it relies on the allele-specific genetic interaction between a host R gene and a virulence (Avr) gene of its pathogen (Flor, 1971).

However, it is difficult to effectively predict the timing, and extent, of a virus outbreak, and no antiviral products are currently available for the control of plant viral diseases. The introduction of genetic resistance into host plant represents the most effective and sustainable approach to the viral disease control, as it is environmentally-friendly, target-specific, and provides reliable protection without additional labour or material costs during the growing season (Wang, 2015). Unfortunately, genetic resistance sources in nature are rare and there are still a number of barriers for the use of GM crops resistant to viruses. In addition, the difficulties involved in the transformation of important plant species are many but are expected to be overcome (Fermin *et al*., 2005). A very promising but currently underutilized alternative for plant virus management strategy is cross protection.

4.1.2 Definition, history and applications of cross protection

Cross protection describes the phenomenon in which infection with a mild or attenuated virus strain can induce the development of resistance against infection by a more virulent related strain. In general, the protective strain is non-aggressive and/or does not produce symptoms, but has the ability to inhibit pathogenic effects which are normally induced by the detrimental strain (Doods *et al*., 1985; Gal-On and Shiboleth, 2006; Gonsalves and Garnsey, 1989; Hull, 2002; Ziebell, 2008; Sherwood, 1987a; DaPalma *et al*., 2010; Ziebell and Carr, 2010).

The phenomenon of cross protection was discovered by Mckinney (1929) as early as 1929. While working with TMV, he observed that tobacco plants systemically infected with a strain of TMV that induced "greenish" mosaic symptoms were protected against a subsequent inoculation with a TMV strain that induced yellow mosaic symptom. It was also shown by Salaman (1933) that tobacco plants inoculated with a mild strain of *Potato virus X* (PVX) were immune to subsequent inoculation with severe strains of the virus, even within only 5 days after primary inoculation. Nevertheless, they were not immune to infection by unrelated viruses, TMV and *Potato virus Y* (PVY). Later, more research was publish to report virus strains that protected against closely related severe strains (Bodine, 1942; Caldwell, 1935; Thung, 1935). Different terminologies have been introduced over the last century, which include acquired immunity, antagonism, cross-immunization, dominance, induced immunity, induced resistance, interference, premunity, prophylactic inoculation, and protective inoculation etc. (Bennet, 1951; Bozarth and Ford, 1989; Fulton, 1986; Pennazio *et al*., 2001; Sherwood, 1987b; Urban *et al*., 1990). Cross protection was a useful tool for determining the relatedness of viruses. The experiment carried out by Webb and colleagues (1952), is a good example in the study of the relationships of different *Potato leaf roll virus* (PLRV) strains. The first commercially grown crops which used cross protection as a method to protect the host from viral (*Citrus tristeza virus*, CTV) infection, were citrus plants in South Africa during the 1950s. CTV is the casual pathogen of two citrus diseases that have caused devastating losses in global citrus production (Bar-Joseph *et al*., 1989; Batista *et al*., 1996; Garnsey *et al*., 1998; Roistacher and Moreno, 1991). To date, the greatest success story for cross protection has been in combating CTV.

4.1.3 Mechanisms of cross protection

Several hypotheses have been proposed to explain how infection with a primary virus can prevent infection by a second virus: (1) pathogen-derived resistance, (2) RNA silencing, (3) cell exclusion/spatial separation, (4) protection phenomena involving subviral agents, such as a satellite virus or satellite RNA, and (5) the formation of "antibodies" in plants, which would prevent a secondary virus infection (Ziebell and Carr, 2010; Nishiguchi *et a*l., 2011). However, only RNA silencing and cell exclusion explanations have widely been accepted.

For RNA silencing (Moore *et al*., 2001; Voinner, 2001), it has been suggested that upon entry of the protective virus strain into the initially infected plant cell, the virus uncoats and its RNA is translated into viral proteins including RdRp, CP(s), MP(s), and RNA silencing suppressor(s). RdRp actively replicates the viral genome via the double-stranded replicative RNAs that are recognized by plant Dicer-like enzymes (DCLs). One, or more, silencing suppressors are encoded by most viruses and the early production and utilization of virus-specific siRNAs are silenced, which results in antiviral silencing being limited. Thus, a "race" occurs between the infectious viral RNA and siRNAs derived from it, which are attempting to prevent the viral RNA from entering neighboring cells and tissues. If the levels of infectious viral RNA in a primed cell exceed the capacity of RNA-induced silencing complexes (RISC), which are activated by viral-derived siRNA, then severe symptoms may be induced in those infected plants. Currently, many successful examples support the involvement of RNA silencing in cross protection. For instance, an amino acid change in HC-Pro in a PVA mutant enhanced its ability to overcome cross protection. HC-Pro is an RNA silencing suppressor, and thus, the mutation in HC-Pro apparantly enhanced the silencing suppression activity.

From an evolutionary standpoint, superinfection exclusion can be a powerful strategy that determines the genetic structure of a virus population. Superinfection exclusion protects the virus from a related, competing secondary virus targeting the cell that has been successfully infected by the primary virus. Besides the elimination of competition for host resources, the mechanism of cell exclusion could function as a means to maintain the stability of viral sequences. Because cell exclusion prevents replication of two or more viral genomes in the same cell, it thus reduces the likelihood of the recombination or reassortment of viral genes, which is of particular importance for the evolution of segmented viruses. More recently, Gonzalez-Jara *et al* (2009) investigated the multiplicity of infection by TMV using different fluorescent reporter genes, and demonstrated that only a fraction of infected cells were infected simultaneously by two TMV genotypes, and infection of one cell by one TMV genotype prevents superinfection from another.

4.1.4 Selection, isolation and induction of attenuated viruses

The most commonly used attenuated virus strains have been isolated from naturally occurring variants that cause attenuated symptoms, or are obtained by random or direct mutagenesis of wild, severe strains using ultraviolet (UV) and nitrous acid. For example, a CGMMV isolate SH33b obtained from the UV-irradiated RNA samples of its parental isolate SH confers protection of greenhouse muskmelon against wild CGMMV (Ali *et al.* 2015). Nitrous acid deaminates nitrogenous bases and replaces the amino group with a keto-group (RC(=O)R'). This causes mismatches in the nucleic acid. Nitrous acid acts on adenine, guanine, and cytosine; adenine is converted to hypoxanthine, cytosine is converted to uracil, and guanine is converted to xanthine. A hallmark of UV irradiation (UVC, UVB) mutagenesis is the high frequency of transition mutations at dipyrimidine sequences containing cytosine. Cyclobutane pyrimidine dimers (CPDs) in DNA, as well as RNA (Bergeon and Sarasin, 2005), form preferentially at dipyrimidines containing 5-methylcytosine when cells are irradiated with UVB or sunlight.

Cross protection virus variants have also been obtained by growing virus infected plants at high (35°C) and low (15°C) temperature (Kosaka and Fukunishi, 1993). For example, to cross-protect cucumber plants from *Zucchini yellow mosaic virus* (ZYMV), an attenuated isolate ZYMV-2002 was obtained from a virulent ZYMV isolate after low temperature treatment at 12.5°C to 15°C, followed by five cycles of single-plant transfer. The isolate produced very mild or no symptoms on cucurbit plants. In addition, inoculated cucumber plants had very similar fruit productivity to healthy control plants under field conditions (Kosaka *et al*., 2006). With the development of molecular biology, many attenuated strains have been reported through site-directed mutagenesis or random mutagenesis. An attenuated strain of ToMV, $L_{11}A$, has been in practical use for over 30 years, to protect tomato from severe strain infection in greenhouses (Oshima, 1981). Tomato cultivars carrying resistance genes have been developed, and the frequency of pre-inoculation with the attenuated strain has decreased. Nevertheless, an attenuated virus strain is still required when a resistant variety is not available.

4.1.5 The properties of the attenuated virus

Although several attenuated viruses have been used for disease control in commercial fields, their practical use is still under debate. Unfortunately, there is no guarantee that such mild strains will perform well in the field, despite the time and energy expended in virus isolation, due to a number of limitations: (1) the genetic instability of the protector virus may generate a new pathogenic virus by recombination with other viruses; (2) there is some level of yield reduction and risk as a virus pool for other crops; (3) protection resulting from the presence of the mild strain might cause the serious damage from mixed infection with other viruses; and (4) naturally occurring mild strains with cross protection potentials are rare.

Nonetheless, cross protection is still considered a practical measure for plant virus disease control, particularly in cases where no other protective means are available. The criteria required for an acceptable attenuated virus strain, to be used for future development, have been proposed: (1) no effects on the quality and quantity of the crop products, and no symptoms or very mild symptoms are induced in the cultivar host; (2) it should be highly stable, genetically, without a chance of mutating into a severe phenotype; (3) most host tissues are fully infected systemically, since the efficiency of cross protection depends on the presence of the attenuated strain; (4) it should be difficult to transmit by vector, in order to limit non-intentional spread to other fields or crops; and (5) it should be easy to produce and inoculate. Of these criteria, the genetic stability of mild or attenuated strains should be considered as the most important factor, in regards to their practical use.

4.2 Materials and Methods

4.2.1 Plant materials, growth chambers and virus source

Nicotiana benthamiana and cucumber (Cultivar Straight 8) were germinated in a growth chamber set at 21-23 °C temperature, and grown in greenhouse rooms under ambient conditions (20-24°C). The CGMMV isolate used in this study was obtained from greenhouse cucumber in Ontario, Canada (see Chapter I). A CGMMV infectious clone was previously generated in our lab (see sequence in Chapter I). The full-length cDNA of CGMMV (Figure 4.1) was inserted

into a modified pCB301 vector containing a double 35S promoter and the ribozyme sequence cassette (Rz) from pCass4-RZ vector (Cui *et al*., 2013).

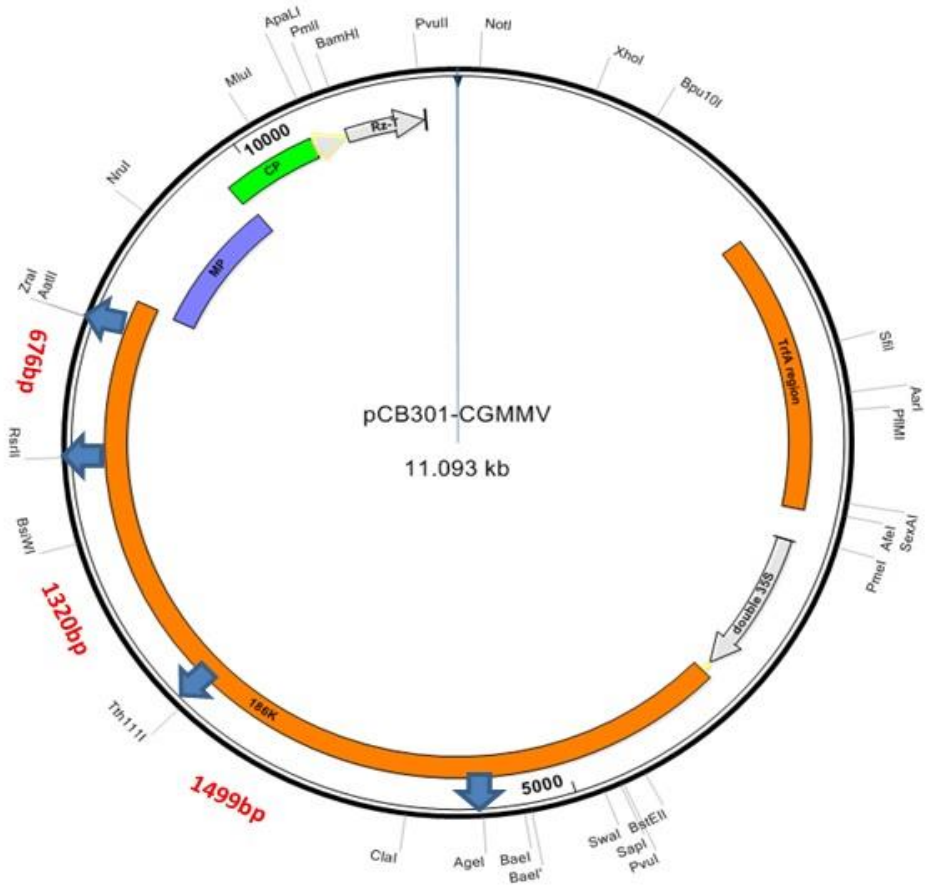

Figure 4.1 Map of the infectious clone pCB301-CGMMV.Abbreviations: Rz= ribozyme sequence cassette, CP=coat protein, MP=Movement protein, 186k= 186k Da replicase, double 35s = double 35s promoter.

4.2.2 Generation of CGMMV variants using site-directed mutagenesis

Quick Change Site-Directed Mutagenesis (Agilent technologies, USA) was carried out to generate single, double, triple and quadruple CGMMV mutants at up to four positions identified in the 129-kDa/186-kDa replicase region. Four aa residues were identified based on the attenuated strain, CGMMV SH33b, which has been shown to confer corss protection against CGMMV in greenhouse melon producitonin Japan (Ali *et al*. 2015). These include one in the intervening region, two in the helicase encoding region, and one in the polymerase encoding region (Ali *et al*. 2015). Mutations at these positions have been shown to be associated with various levels of symptom expression in infected plants (Ali *et al*., 2015). Based on sequence comparison of related tobamoviruses (PMMoV, TMV, ToMV), four other mutations were designed in the intervening region. They are located at amino acid positions 546 (Lys-Arg), 557(Val-Thr), 651(Val-Als), 762(Ser-Leu) (See Figure 3.2). To facilitate introduction of mutations into the replicase ORF and intervening region in the pCB301-CGMMV infectious clone, I took advantage of four unique restriction enzyme sites (AgeI, Tth111I, RsrII, AatII) indicated in the map of the infectious clone (Figure 4.1) by subcloning the three fragments (676,1320 and 1499 bp in length, respectively) into the Zero blunt Vector (Invitrogen) for mutagenesis.

To obtain each virus variant, two complimentary oligonucleotides, containing the desired mutations, were synthesized and flanked by an unmodified nucleotide sequence. Oligonucleotide primers were purified prior to use in the following reaction: 2.5 µl of 10× reaction buffer, 2 µl (25 ng) of dsDNA template, 0.45 µl (10 µM) of oligonucleotide primer #1, 0.45 µl (10 µM) of oligonucleotide primer #2, 0.5 µl of dNTP mix, ddH_2O to a final reaction volume to 25 µl. Then added 0.5 µl of *PfuUltra* HF DNA polymerase (2.5 U/µl). The reaction was performed using the following program: an initial 95°C melt for 30 sec; then cycles of 95°C melting for 30 sec, 55°C annealing for 30 sec, 68°C extension for 7 min, repeated for 19 cycles; finally, a 68°C extension for 10 min. One µl of the *Dpn* I restriction enzyme (10 U/µl), was added into each reaction system and mixed gently and thoroughly, spun down in a microcentrifuge for 1 min, and immediately incubated at 37°C for 1 hour to digest the parental, supercoiled dsDNA. Subsequently, 1 µl of the *Dpn* I-treated DNA from each reaction was transformed into separate 50-µl aliquots of Topo 10 competent cells. All mutants were sequenced to confirm their

identities. Finally, six mutants were screened, identified and labelled as MG, MGVD, MGVDT, MRT, MRTA, and MRTAL.

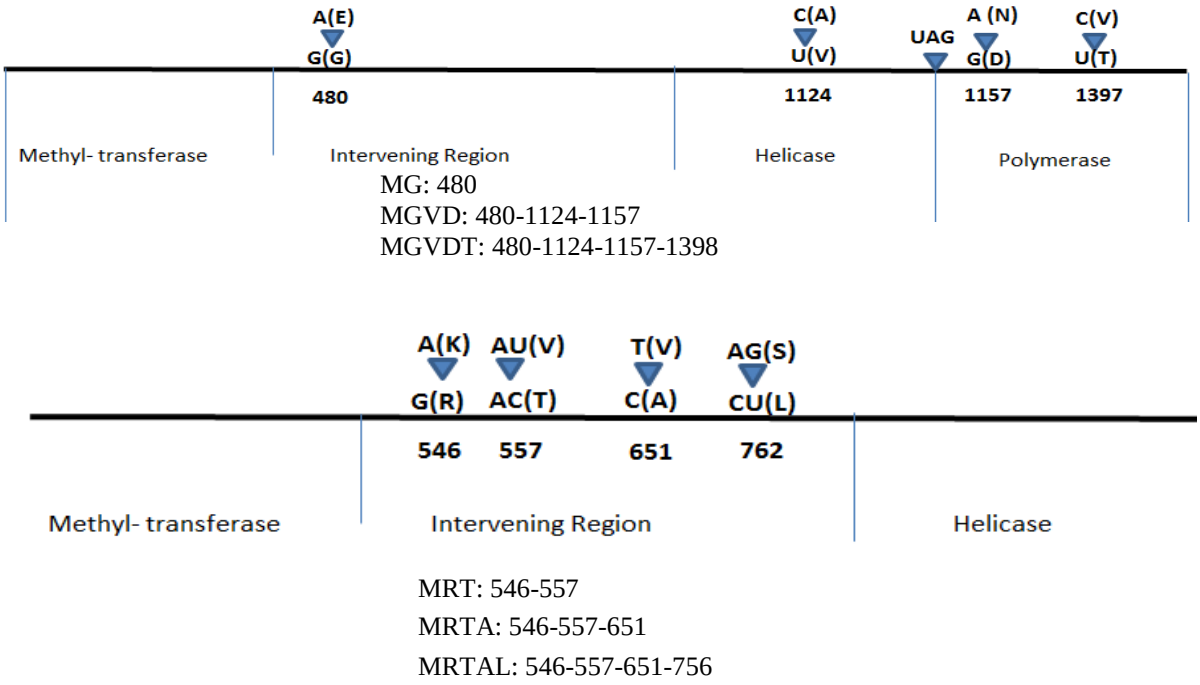

Figure 4.2 Mutations in the genome of the isolate CGMMV-Ca. Closed arrowheads indicate nucleotide mutations that caused amino acid changes. Positions of mutated nucleotides are shown below the horizon lines.

4.2.3 Evaluation of the CGMMV variants in plants.

To evaluate whether CGMMV mutants are the attenuated versions, each of mutants was transformed into *Agrobacterium tumefaciens* GV3101, via electroporation, and then introduced into *N. benthamiana* and cucumber plants (var. Straight 8), via agroinfiltration, essentially as described by Cui *et al*., (2013). To maintain a uniform concentration of *Agrobacterium*, the OD600 of the final mixture in co-infiltration experiments was adjusted to 0.5. The bacteria were

kept at room temperature for at least 3h without shaking. These cultures were infiltrated into the abaxial surface of fully expanded N. *benthamian*a and cucumber leaves using a 1-cc syringe without a needle. Symptoms induced by these mutants were compared with those caused by wild type CGMMV. The virus accumulation in cucumber plants infected with CGMMV mutants was compared to those in plants infected wild type CGMMV-Ca. Double-antibody sandwich ELISA (DAS-ELISA) was used to determine the CP accumulation levels of CGMMV and its mutants in systemically infected plants. The titers of mutants in the infected *N. benthamiana* and cucumber plants were monitored at 7, 14, 21 and 28 dpi.

Mutant(s) showing attenuated symptoms were tested for genetic stability over multiple passages in plants. To determine the stability of mutant(s) in maintaining both mild symptoms and engineered mutation(s) in the infected plants, the mutant(s) were passaged in *N. benthamiana* for five passages by using the leaf extract of the infected plants to inoculate five new plants every 28 days. The symptoms were observed on all inoculated plants. Additionally, the infected tissues from the mutant inoculated plants were identified and monitored for the viral RNA titre during each passage. The genetically stable CGMMV mutants that showed attenuated symptoms or low viral accumulation were subjected to cross-protection assays.

4.2.4 Cross-protection assay

The attenuated CGMMV mutants MGVDT and MRTAL did not cause severe symptoms on cucumber plants. Their ability to protect the cucumber plant against challenge with by the severe CGMMV-Ca strain was studied under temperature-controlled (22°C) greenhouse conditions.

Cucumber seedlings at the cotyledon stage were mechanically inoculated with mutants MGVDT and MRTAL using 0.5 g of infected cucumber leaves ground with 2 ml of 0.01M potassium phosphate buffer (pH7.2). After 7 days from the first protective inoculation, the fully expanded leaves of the plants were mechanically challenged with the wild-type virus CGMMV-Ca. The protection effect was evaluated by symptom development on the plants. The presence of the challenging virus was monitored by semi qRT-PCR at 4, 8, 12, 16, and 20 days after the inoculation of the wild-type virus.

Real-time qRT-PCR was conducted to quantify the CP accumulation level in the host cucumber plant after 4 weeks secondary inoculation and analyzed with the CFX96 Real-Time PCR Detection System (Bio-Rad) following the manufacturer's instructions. For each primer set, gel electrophoresis and melting curve analysis were carried out to ensure that only a single expected PCR product and melting temperature were generated. Each reaction contained 40 ng of cDNA template, 5 μM of primer mix, and 1X SsoFast™ EvaGreen® Supermix (Bio-Rad) in a total volume of 10 μl of reaction solution. Relative transcript abundances were calculated using CFX Manager Software (Bio-Rad). Expression of cucumber α-tubulin was used as a reference gene to normalize the data and to calculate the relative mRNA abundance levels. For each sample analyzed, three biological replicates were included and for each biological replicate, three technical repeats were carried out. All results are shown as means of biological replicates with corresponding standard errors.

4.3 Results

4.3.1 CGMMV-Ca mutants are infectious and induced attenuated symptoms in both *N. benthamiana* and cucumber plants..

To examine the involvement of these amino acids in symptom development, individual cDNA fragments containing the mutated amino acids were used to replace the corresponding fragments in the infectious CGMMV-Ca clone pCB301-CGMMV. A total of six infectious clones containing single (MG), double (MRT), triple (MGVD, MRTA), and quadruple (MGVDT, MRTAL) mutations were constructed.

Six mutants were used to infect *N. benthamiana* and cucumber plants by agrobacteria infiltration, WT CGMMV-Ca was used as positive control. As shown in Figure 4.3A, B and Figure 4.4 A, B, all mutants could develop visible mosaic symptoms in both *N. benthamiana* and cucumber plants at the different levels. However, the time point at which symptoms became visible differed from mutant to mutant. The plants infected with WT CGMMV-Ca and mutant MG started to show the yellowish symptom at 14 dpi in *N. benthamiana* and cucumber. In contrast, the plants infected with mutants MGVD, MGVDT, MRT, MRTA, MRTAL remained asymptomatic until 28 dpi. Infection by WT CGMMV-Ca caused severe symtoms inclduing leaf

mosaic, necrosis and deformation, and stunting in compasition with buffer-treated plants (Figure 4.3 and Figure 4.4). All the 6 mutans induced relatively light symtoms, comapred to WT CGMMV-ca, suggesting all of them are attenuated CGMMV variants. Overall, infection by mutants MGDVT and MTRTAL showed the mildest symtoms.

4.3.2 Accumulation of CP of the mutants in the infected *N. benthamiana* and cucumber plants

The titers of the mutants in the infected *N. benthamiana* were monitored at 7, 14, 21, and 28 dpi by ELISA (Figure 4.3 C and Figure 4.4 C). In order to minimize the variation on each leaf of a plant, three leaf disks from upper leaves of each infected plant were combined and ELISA reading was averaged from the plants tested.

After inoculation, the CP level of mutants MG, MGVD, and MGVDT quickly accumulated in *N. benthamiana* from 7 to 21 dpi, comparable to that of WT CGMMV-Ca, and suddenly dropped off at 28 dpi while WT-CGMMV-Ca remained the high CP level (Figure 4.3). No significant CP accumulation was found for the mutants MRT, MRTA, and MRTAL until 21 dpi, and the virus accumulation level increased at 28 dpi, but significantly lower than that of the WT virus. At 28 dpi, the accumulation level of all mutans was at least 50% lower than that of CGMMV-Ca. A similar trend of the CP accumulation pattern was found in the cucumber plants after inoculation with WT CGMMV-Ca and the mutants. A difference was that for mutants MG, MGVD, and MGVDT, their CP accumulation level started to decrease after 14 dpi. From 21 to 28 dpi, the mutant MGVDT remained the lower but stable level of CP (Figure 4.4). For two mutants MRT, and MRTA, their CP levels in cucumber plants were detectable until 21 dpi and then droped down to the undetectable level. Interstingly, the mutant MRTAL showed a very low but relatively stable CP level within four weeks of the monitoring period (Figure 4.4).

In order to confirm the mutations in those six mutants, total RNAs of the cucumber plants infected individually with six virus mutants were amplified by RT-PCR. The RT-PCR products obtained were analyzed by sequencing directly. Sequencing data confirmed that all the mutants were stable. Notably, both of mutants MGVDT-infected and MRTAL-infected plants didn't show any visible symptoms at 28 dpi, and the ELISA results also indicated that mutants

MGVDT and MRTAL were able to replicate in *N. benthamiana* and cucumber cells and spread systemically, making those mutants the strong candidates for inducing cross protection.

4.3.3 Consistent low viral RNA levels of the mutants MGVDT and MRTAL in systemic leaves of infected plants

The viral RNA level of WT CGMMV-Ca and the mutants in infected cucumber tissues was quantified using semiqRT-PCR at time point 4 weeks post inoculation (Figure 4.5). The same amount of RNAs for each sample was used to reverse transcription and then the same volume of cDNA was applied into PCR reaction with the CP special primer. Interestingly, In comparison with that of the WT virus and other mutans , the viral RNA level of two mutants MGVDT and MRTAL maintained relatively low. The low viral RNA levels of the mutants MGVDT and MRTAL in the systemically infected leaves were consistent with the mild symptom caused by those mutants. Together with the ELISA results, the mutants MGVDT and MRTAL were selected for further test as the attenuated CGMMV variants for cross protection.

4.3.4 Effective protection by inoculation with the mutants MGVDT and MRTAL against subsequent infection of WT CGMMV in *N. benthamiana*

The potential of the mutants MGVDT and MRTAL to cross-protect host plants against the secondary infection by WT CGMMV was evaluated. *N. benthamiana* was first inoculated with the mutants MGVDT and MRTAL, and one week later, the plants were challenged with WT CGMMV and observed on a daily basis for symptom development. As shown in Figure 4.6 and 4.7, WT CGMMV applied into either as the primary inoculum or secondary inoculum, caused severe yellow mosaic symptom and stunting in the infected plants.

Using the mutants MGVDT or MRTAL as the primary inocula, the infected plants (without second inoculation) were almost indistinguishable from mock inoculated plants in sizes and without any mosaic symptoms appearing on the newly development leaves. The plants with the primary infection by the mutants MGVDT or MRTAL, followed by the second inoculation by WT CGMMV-Ca, showed the mild yellow mosaic symptom on 7 days post the secondary inoculation. However, no yellow mosaic patch or other visible symptoms were present on the

newly developed leaves at 21 days post the secondary inoculation. The size of these plants seemed slight smaller than mock treated plants.

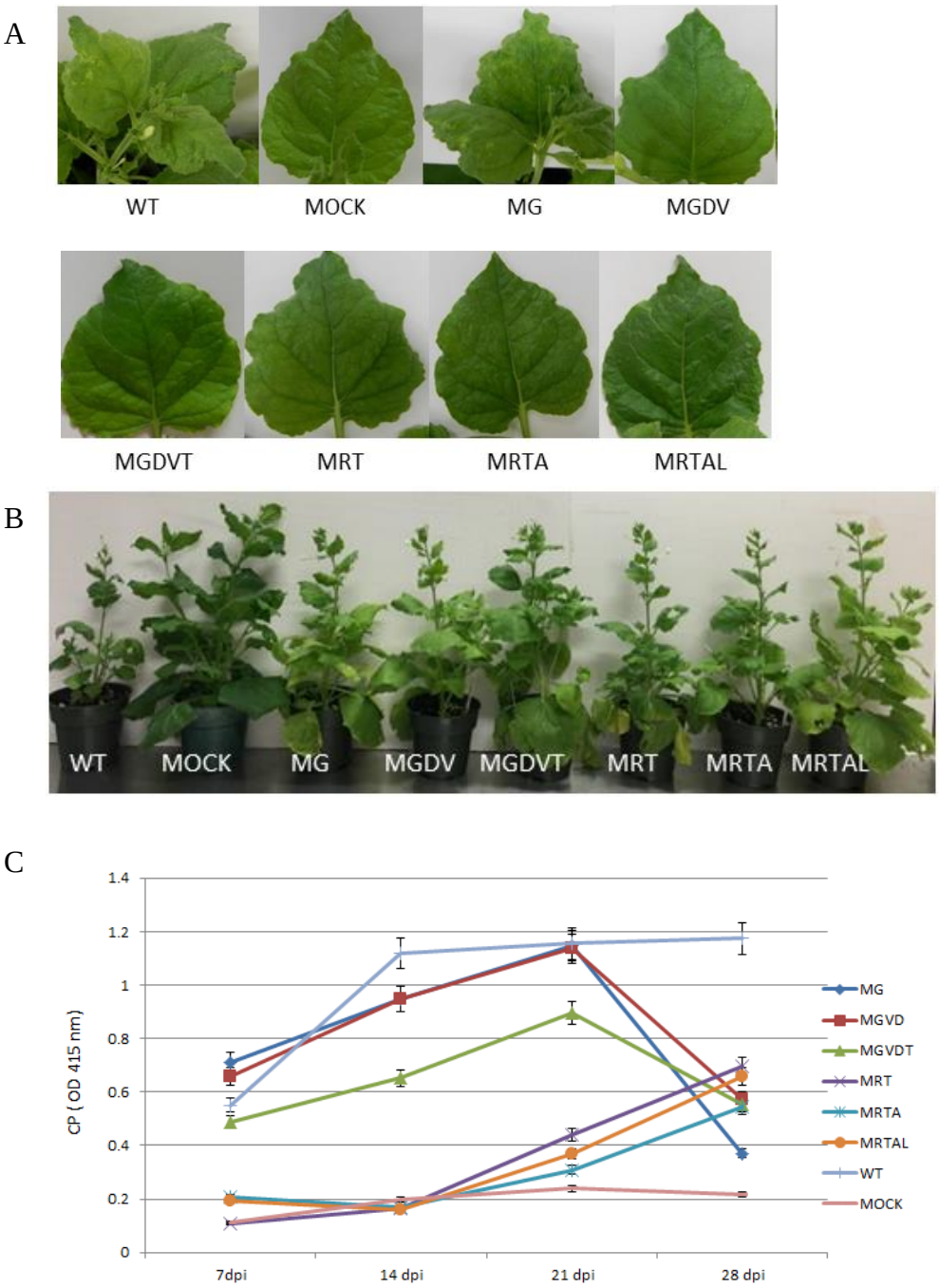

Figure 4.3 Infectivity of six CGMMV mutants with symptoms and the accumulation level of coat proteins (CPs) in *N. benthamiana* plants. A, Systemic symptoms induced by the mutants in the systemic leaf of *N. benthamiana* plants. B, The growth condition of whole plant infected with mutants. For A and B, pictures were taken 28 days post inoculation C, Accumulation level of CPs expressed by six mutants of CGMMV in *N. benthamiana* plants as determined by enzyme-linked immunosorbent assay (error bar is standard deviation, n=10, the experiment was repeated 3 times).

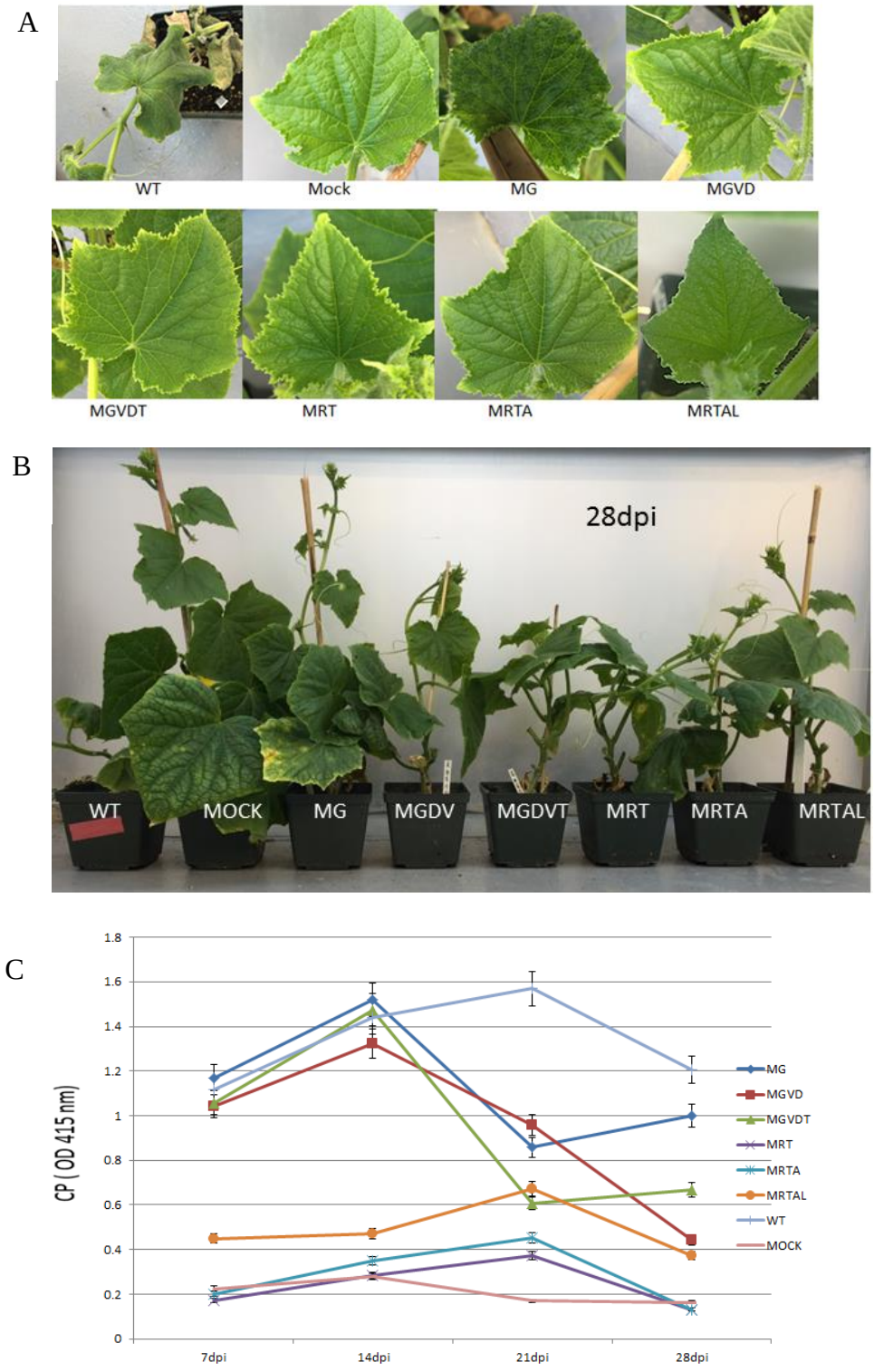

Figure 4.4 Infectivity of six CGMMV mutants with symptoms and the accumulation level of coat proteins (CPs) in cucumber plants. A, Systemic symptoms induced by the mutants in the systemic leaf of cucumber plants. B, The growth condition of whole plant infected with mutants. For A and B, pictures were taken 28 days post inoculation C, Accumulation level of CPs expressed by six mutants of CGMMV in cucumber plants as determined by enzyme-linked immunosorbent assay (error bar is standard deviation, n=10, the experiment was repeated 3 times).

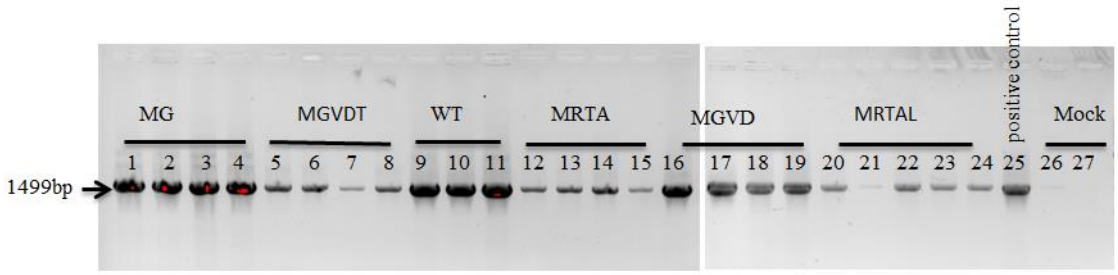

Figure 4.5 The level of mutant viral RNA in infected cucumber was quantified using semi qRT-PCR at 4 weeks post inoculation.

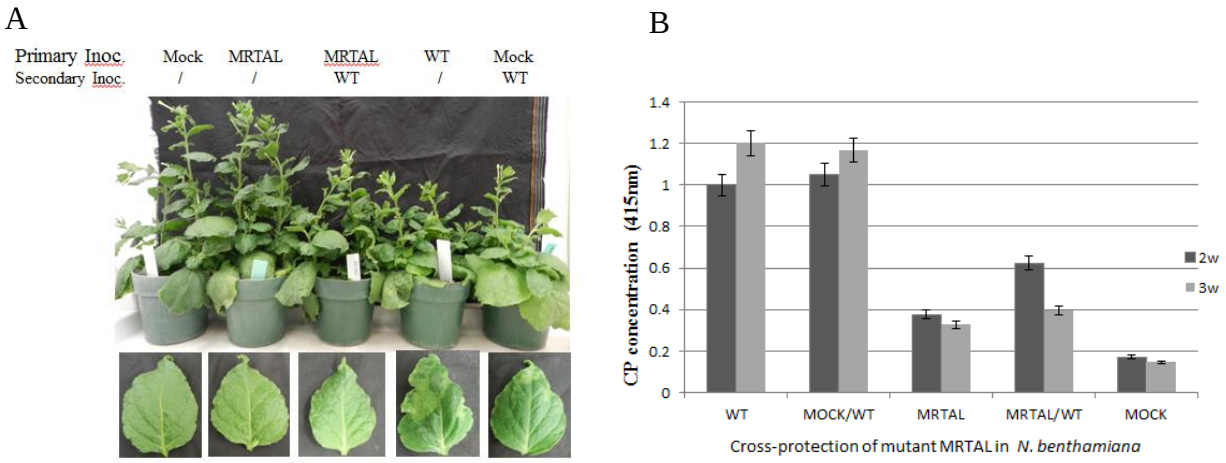

Figure 4.6 Mutant MRTAL induced effective cross protection against the wilt type CGMMV in *N. benthamiana* plants. A, Cross protection of *N. benthamiana* with the lab attenuated CGMMV virus variant (MRTAL), against challenge inoculation with the severe wild type CGMMV after 3 weeks secondary inoculation. Mock, a buffer-inoculated plant or negative control; MRTAL, a plant inoculated with the lab attenuated mild strain MRTAL only; MRTAL/WT, a plant initially inoculated with the lab attenuated mild strain MRTAL then challenged with the wild type severe CGMMV. WT, a plant inoculated with the wild type severe CGMMV; Mock/WT, a plant first inoculated buffer only then challenged with the wild type severe CGMMV.B, CP concentration in challenged compared to unchallenged plants monitored by ELISA at 2 and 3 weeks after secondary infection (error bar is standard deviation, n=5, the experiment was repeated 3 times).

The symptom observations above were further corroborated with ELISA results. As shown in Figure 4.6 and 4.7, the CP level in plants infected with WT CGMMV alone was consistently high. In contrast, the CP level remained low in the plants with the primary infection by MRTAL or MGVDT followed by the secondary infection by the WT virus within three weeks of the monitoring period . These results strongly suggested that the presence of the mutants MRTAL and MGVDT in *N. benthamiana* inhibited replication of the WT CGMMV virus, thus repressing the severe symptoms caused by the WT virus.

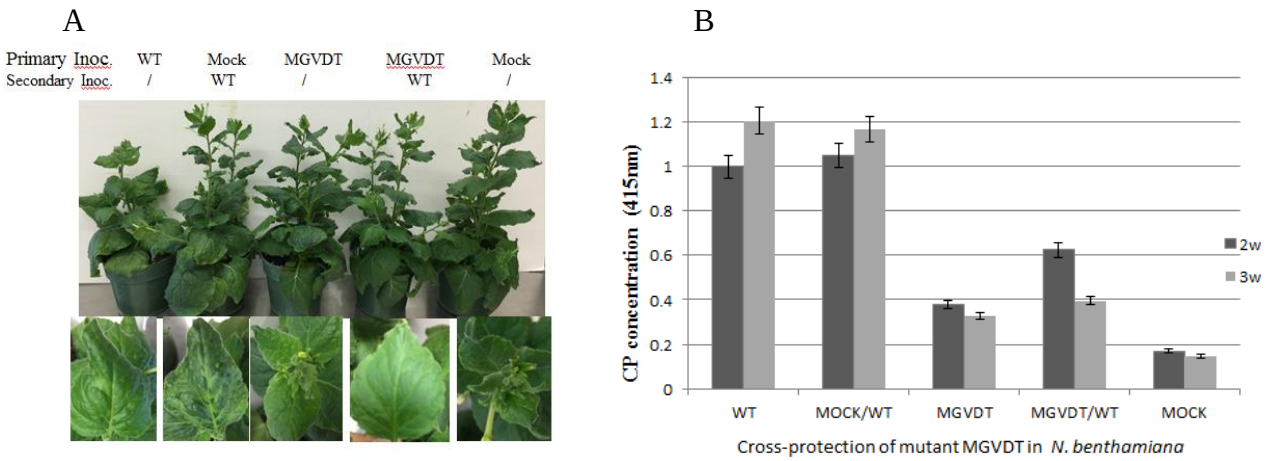

Figure 4.7 Mutant MGVDT induced effective cross protection against the wilt type CGMMV in *N. benthamiana* plants. A, Cross protection of *N. benthamiana* with the lab attenuated CGMMV virus variant (MGVDT), against challenge inoculation with the severe wild type CGMMV after 3 weeks second inoculation. Mock, a buffer-inoculated plant or negative control; MGVDT, a plant inoculated with the lab attenuated mild strain MGVDT only; MGVDT/WT, a plant initially inoculated with the lab attenuated mild strain MGVDT then challenged with the wild type severe CGMMV. WT, a plant inoculated with the wild type severe CGMMV; Mock/WT, a plant first inoculated buffer only then challenged with the wild type severe CGMMV.B, CP concentration in challenged compared to unchallenged plants monitored by ELISA at 2 and 3 weeks after secondary infection (error bar is standard deviation, n=5, the experiment was repeated 3 times).

4.3.5 Maintenance of cross-protection capacity in the cucumber plants infected with Mutants MGVDT and MRTAL

To further test the ability of those mutants to induce cross protection against CGMMV, cucumber plants (cultivar Straight 8) were inoculated with WT, MRTAL, MGVDT and the buffer (mock). One week after inoculation, WT CGMMV-Ca (as the secondary inoculum) was introduced into the upper leaves of the plants. The symptoms were observed at 4, 8, 12, 16, and 20 days after secondary inoculation with mutants MRTAL and MGVDT. The results are shown in Figure 4.8. The severe symptoms were observed on the plants inoculated with the WT virus alone or Mock followed by the second inoculation with the WT virus from 8 days to the end of the observation period. However, no symptoms developed on the plants inoculated with MRTAL and MGVDT up to 20 days aftter the secondary inoculation. A delayed development of mosaic symptoms was found in MRTAL-protected (MRTAL/WT) and MGVDT-protected (MGVDT/WT) plants between 8 and 12 days, and the mosaic symptoms were completely recovered from 12 days to 20 days after the secondary inoculation.

In order to verify the accumulation levels of viral RNA in systemic leaves of the infected plants with the mutants MRTAL and MGVDT, the viral RNA levels of the mutants in infected tissues were assessed using semi qRT-PCR at time points: 4, 8, 12, 16, and 20 dpi with CP-specific primers (Figure 4.9). At 4 days after the secondary infection, viral RNA was detected in the plants infected with WT, and barely observed in the plants inoculated with MRTAL/WT and MGVDT/WT. In the plants infected with MRTAL, MGVDT and Mock/WT, viral RNA was undetectable. Up to 12 dpi, viral RNA was easily examined in all plants except for those infected with the buffer (Mock). Notably, the accumulation level of viral RNA in the plants infected with MRTAL/WT and MGVDT/ WT significantly increased, but the viral RNA level in the plants infected with MRTAL and MGVDT remained the lower level. Until to 20 dpi, the viral RNA in some of the plants infected with MRTAL/WT and MGVDT/ WT was nearly undetectable or remained at extremely low levels in the other infected plants. By this time point, the viral RNA level in the plants treated with MRTAL and MGVDT maintained at low level compared with those plants infected with WT.

Finally, qRT-PCR was used to evaluate the relative viral RNA expression after 4 week secondary inoculation (Figure 4.10). The results showed a remarkable reduction in the relative expression level of viral RNA in the MRTAL-protected plants and MGVDT-protected plants. my data indicated WT CGMMV was present abundantly in the unprotected plants but barely detectable in the protected plants (about 3-4 fold lower than in the plants infected WT CGMMV alone). The consistently low level of the viral RNA of the mutants MRTAL and MGVDT in systemically infected leaves after 4 wpi was in agreement with the very mild or undetectable disease symptoms caused by those mutants, further suggesting mutants MRTAL and MGVDT could serve as attenuated CGMMV variants for cross protection.

4.3.6 Mutant MRTAL and mutant MGVDT are stable for at least five passages in *N. benthamaina*

One of the criteria for a good attenuated virus strain for cross protection is its genetic stability. To determine the stability of mutants MRTAL and MGVDT, both the mild symptoms and the engineered mutations in infected plants were tested. Both the mutants were inoculated into *N. benthamaina* for 5 passages. The leaf extract of the infected plants was inoculated into 5 new plants every 28 days. Mild symptoms were consistently observed on all inoculated plants throughout almost five months passaging period. The systemic leaf tissues for each passage were collected and subjected to RT-PCR. Figure 4.11 showed that mutants MRTAL and MGVDT were detectable in all five passages, thus confirming the stability of the mutants.

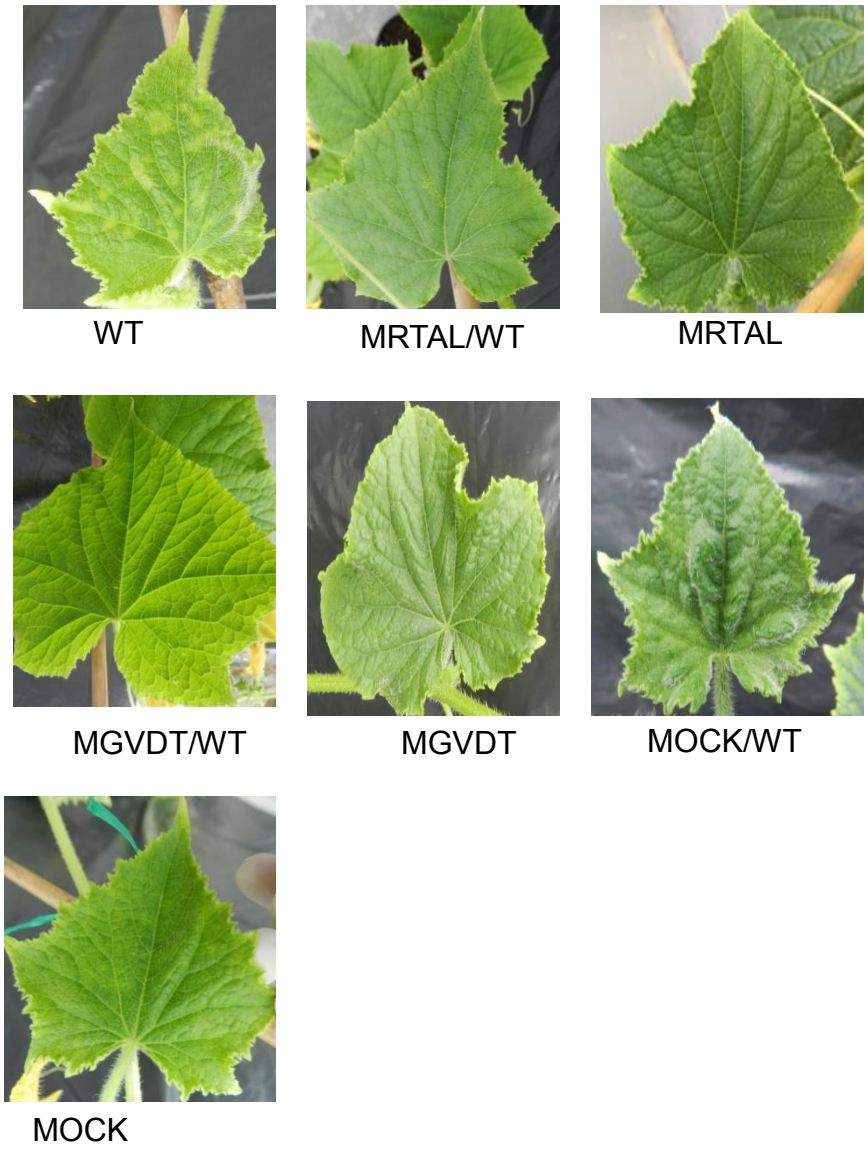

Figure 4.8 Mutants MRTAL and MGVDT are the robust vaccine that remains the same cross-protection capability in cucumber. Mock, a buffer-inoculated plant or negative control; MGVDT, a plant inoculated with the lab attenuated mild strain MGVDT only; MGVDT/WT, a plant initially inoculated with the lab attenuated mild strain MGVDT then challenged with the wild type severe CGMMV. WT, a plant inoculated with the wild type severe CGMMV; Mock/WT, a plant first inoculated buffer only then challenged with the wild type severe CGMMV. Pictures were taken 20 days after secondary inoculation.

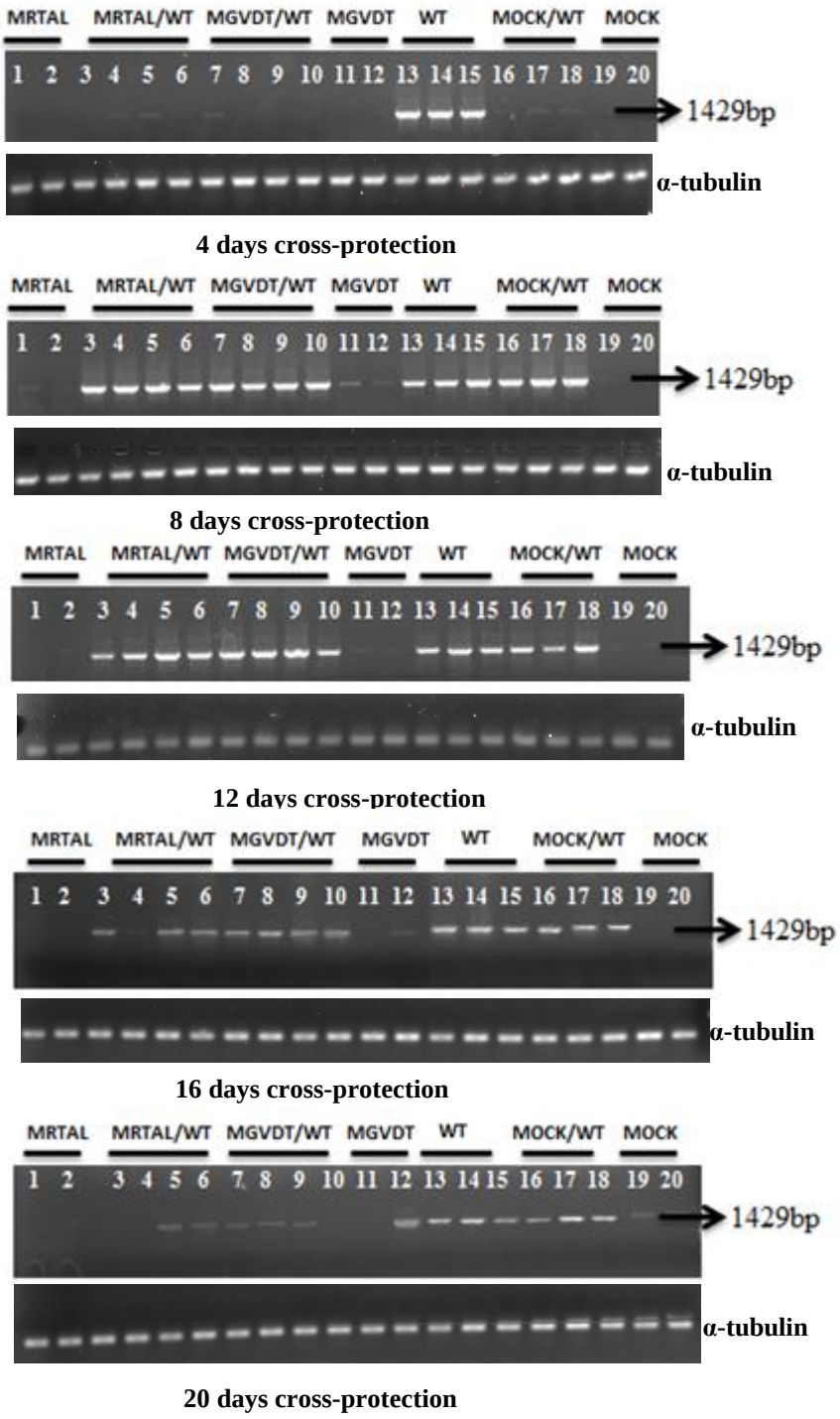

Figure 4.9 Mutants MRTAL and MGVDT inhibit the replication of the wild type CGMMV in the secondary-infected cucumber plants. RT-PCR was carried out to monitor the viral RNA accumulation level using a pair of CP special primers by every 4 days.

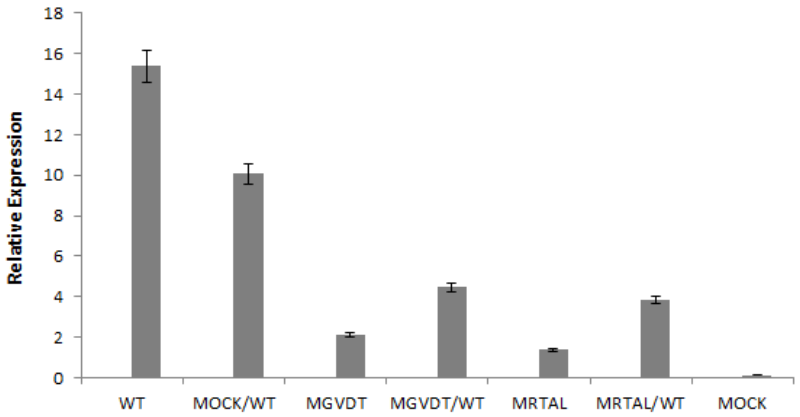

Figure 4.10 Evaluation of the viral RNA accumulation level four weeks after secondary inoculation using qRT-PCR with a pair of primers specific for the CP gene.

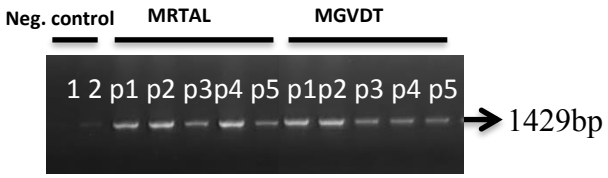

Figure 4.11 Verification of the stability of mutants MRTAL and MGVDT by RT-PCR to amplify a cDNA fragment amplified using specific CP region.

4.4 Discussion

In this study, I developed an infectious clone of CGMMV by insertion of the full-length cDNA of CGMMV into the vector pCB301 with a double 35S promoter and the ribozyme sequence (RZ) (Figure4.1). The transcripts produced from the full-length CGMMV cDNA were infectious in both *N. benthamiana* and cucumber plants based on results from symptom observation, RT-PCR or qRT-PCT and ELISA analyses. The site-directed mutagenesis was applied to change amino acids in the replication region and the intervening region to generate the virus variants to their capacity for cross-protection.

As metioned earlier, an attenuated CGMMV isolate SH33b was successfully developed against CGMMV for greenhouse muskmelon production in Japan (Ali *et al*., 2015). SH33b was shown to accumulate approximately two-thirds of the parental isolate SH in the melon plants (Ali *et al*., 2015). When the genome sequence of SH33b was compared with that of SH, nine nucleotide substitutions were found, five of which lead to aa substitutions including four in the replicase coding region and one in the CP coding region. Apparently these mutations in the replicase coding region are important (Ali *et al*., 2015). Based on these findings, we generated three CGMMV-Ca variants: the single mutant MG (Glu480Gly) containing one aa change in the intervening region, the triple mutant MGVD (Glu480Gly, Ala1124Val, Asn1157Asp) consisting of two additonal substitutions with one in the helicase region and the other one in the RdRp polymerase region, in addition to the mutation in the MG mutant, and the quadruple mutant MGVDT (Glu480Gly, Ala1124Val, Asn1157Asp, and Val1397Thr) resulting from one more substitution introduced into the RdRp region of the mutant MGVD. Moreover, we also generated three more mutants, MRT (Lys546Arg, Val557Thr), MRTA (Lys546Arg, Val557Thr, Val651Als), and MRTAL (Lys546Arg, Val557Thr, Val651Als, Ser762Leu) with aa substitutions in the intervening region. Those three virus variants were generated based on the genome sequence analyses of other attenuated tobamoviruses (TMV-V36, ToMV-L11A, ToMV-K, PMMoV Pa18, PMMoV C1421) published previously (Nishiguchi *et al*., 1985; Lewandowski and Dawson, 1993; Hagiwara *et al*., 2002; Yang *et al*., 2002; Ichiki *et al*., 2005).

We showed that both mutants MRTAL and MGVDT produced mild disease symptoms in both *N. benthamaina* and its natural host cucumber. Additionally, no stunted growth of the plants

infected with those mutants was observed, thus I selected them as potential cross protection candidates. I further showed that the viral variant MGVDT exhibited cross- protection against its parental virus CGMMV-Ca in both *N. benthamiana* and cucumber. In comparison with MGVDT, the quadruple substitutions in the viral variant MRTAL also showed nice cross-protection against CGMMV-Ca. Based on a number of previous studies, the intervening region seems an ideal target for virus attenuation, suggesting that the intervening region plays an essential role in symptom induction, viral replication and pathogenicity. Indeed, the attenuated tobamovirus ToMV strain L11, which has a caudal mutation in the intervening region of 130K protein, showed a reduced ability to suppress RNA silencing and the mutated 130K protein did not prevent the accumulation of siRNA, but did inhibit the formation of RISC (Liu *et al*., 2015), revealing a possible role of RNA silencing involved in the cross protection mechanism. It would be interesting to determine if this mechanism also functions in the mutant MRTAL I generated in this study.

To date, considerable efforts have been made in producing virus-free propagative stocks, in preventing the spread of viral diseases with the vectors, in breeding virus-resistant varieties, and in developing genetically engineered virus-resistant plants. However, there are still a number of barriers to be overcome in the future. In this regard, cross protection could be thought as a promising but underutilized alternative approach for plant viral pathogen management. In this chapter, I have indicated the use of a severe wild type virus (CGMMV) to generate the attenuated virus strains (MRTAL and MGVDT) with cross protection. The results provide a foundation for commercial greenhouse trials to test if the mutants MRTAL and MGVDT can protect cucumber against CGMMV in greenhouses and if the primary infection of these mutants has any significant side effects on other traits.

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Chapter 5. General Discussion

5.1 Conclusion Remarks

The experiments included in this dissertation identified six viruses on three major greenhouse vegetables in Ontario by using next-generation sequencing techniques. Analyses of the contig sequences assembled based on obtained sequencing data revealed a high sequence similarity/identity to the sequences of BPEV, PMMoV, TSV, CGMMV, and PepMV and a relatively low similarity/identify to the sequence of PMoV. We further cloned and determined the complete genome sequences of BPEV, PMoV, and CGMMV which had not been reported previously in Canada. Our results support that small RNA sequencing technique may be used to query thousands of small RNA and miRNA sequences with unprecedented sensitivity and dynamic range (Motameny *et al*., 2010). In comparison with other detection methods, this technology offer an entirely generic, specific and, apparently sensitive approach to identify plant viruses. Given the genetic and structural diversity of the characterized viruses, it is possible that there are viral and subviral pathogens yet to be identified which exhibit no similarity to any of the known viruses detectable using available bioinformatics tools (Cox-Foster *et al*., 2007; Quan *et al*., 2008; Li *et al*., 2012). In this regard, the unique features of virus deep sequencing and assembly of total small RNAs might facilitate the development of bioinformatics tools used for screening particular contigs and virus discovery.

Many studies have been reported that the use of recirculating nutrient solutions in greenhouse hydroponic systems could facilitate the rapid spread of water-transmissible plant pathogens throughout the entire crops. In the Chapter 3, we suggested the CGMMV could be water-transmissble through recirculating hydroponic systems. Our combined results from TEM, RT-PCR, qRT-PCR, and western blot have shown that CGMMV is accumulated in infected plant root. Our data suggest that CGMMV in the roots of the infected plants could be released to, and transmitted through the nutrient solution to infect the root system of the healthy plants. As a result, the healthy plants could be infected by the virus. Then the question becomes how the virus is released from and enters into the root system of the plants. It is possible that the nutrient circulating system might damage the roots of hydroponic plants, and that the possible mode of entry for the virus into the roots tissues would be either from the damaged root or through lateral

roots. To my knowledge, this is the first study to show that transmission mode of CGMMV in experimental hydroponic systems.

Based on preliminary results, CGMMV is a devastating viral pathogen for greenhouse cucumber production in Canada. As discussed in chapter 4, many efforts were made to develop an attenuated variant for protection against CGMMV and to establish effective management strategies so as to control the viral pathogens in greenhouse vegetables and avoid possible catastrophic losses. Six attenuated CGMMV variants were generated based on the full length cDNA CGMMV infectious clone in this study, and the mutants MRTAL and MGVDT produced mild disease symptoms and low viral RNA accumulation levels in both *N. benthamaina* and its natural host cucumber. For cross-protection experiments, MRTAL and MGVDT were examined in both *N. benthamiana* and cucumber plants to demonstrated low viral RNA or coat protein accumulation levels and no symptom expression in systemic leaves. It is suggested that the mutants MRTAL and MGVDT as attenuated CGMMV variants have induced effective cross protection.

Although many efforts have been undertaken to control plant viral pathogens, there are still a number of barriers to be overcome. For example, pesticides used against viral transmission may not efficiently prevent vector transmission and have negative impact on the environment. Conventional breeding of virus-resistance plant may not be possible when the resistance gene is lacking (Ziebell and Carr 2010). Therefore, cross protection as a form of microbial resistance offers great promise in plant viral disease management. If properly utilized, it could be effective for most viruses in many different crop species. To effectively use this resource, it is important to exploit rapid and reliable procedures for generating mildly symptomatic viruses. The method to generate attenuated viruses presented here is a rapid procedure for using wild type viral pathogens and engineering them into attenuated virus variants which can induce effective cross protection. The attenuated virus variants produced in this study are also good candidates (mutants MGVDT and MRTAL) to study host factors that are playing a major role in host responses to infection. However, before the attenuated mutants could be used in crop protection, they should be examined in each of hosts to which they will be applied. Because cross protection

does not rely on harmful materials or chemicals, such as pesticides and fungicides, it is suitable for developing sustainable agriculture in the coming years.

5.2 Future prospects

The purpose of this study is to develop effective strategies to control viral pathogens in greenhouse vegetables and to avoid possible catastrophic losses. Although cross protection has been given serious attention as a control measure for few virus diseases, results show that it may be effective for many others. Careful attention to selection of the best attenuated variants of virus and their introduction into the crop to be protected are essential. Two mutants MRTAL and MGVDT have been developed to serve as attenuated CGMMV variants for cross protection against CGMMV for cucumber production.

Effectiveness of cross protection is not the the sole criterion for its usefulness for diseases control (Channon *et al*., 1978). The control strategy being most economical and environmentally friendly will be adopted. Cross protection may be superseded by the disease resistance because the expenditure of time and effort by the greenhouse grower need to be considered.

CGMMV apparently is water-transmissible in the hydroponic system. This information is essential for greenhouse vegetable growers to decide how to reuse CGMMV-contaminated hydroponic nutrient solutions. Recycling of hydroponic solutions is not only important for cost savings but also for environmental protection. For example, the virus-contaminated nutrient solution may be reused directly for culture of non-host vegetables such as tomato and pepper, but will need to deactiviate the virus for cucuber production. Growers can also take advantage of this property to inoculate cucumber plants with mild strains of CGMMV such as attenuated mutants MRTAL and MGVDT through the recirculating nutrient solutions for cross protection against CGMMV. Of course, the efficiency and effectiveness of this inoculaiton method needs to be tested in the experimental hydroponic system before it is used in the commerical greenhouse.

5.3 Reference

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