



Studies on the disassembly of cowpea chlorotic mottle virus
by Na Li

A thesis submitted in partial fulfillment of the requirement for the degree of Master of Science in Plant Sciences

Montana State University

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Abstract:

The mechanism of the spherical virus disassembly has been under investigation for understanding the early events during virus infection, and eventually for helping to design reagents to block the process for releasing genetic information into host cells, thus preventing viral infection. The virus has to be stable enough to protect the genetic materials inside the virion, yet dynamic enough to release the nucleic acids to establish infection. Cowpea chlorotic mottle virus provides genetic and biochemical advantages for this purpose. The role of virus swelling for disassembly was studied based on characterization of a salt stable mutant of CCMV in vivo and in vitro. The salt stable mutant is as infectious as the wild type, swells like the wild type but shows negative signal for translation, thus swelling is not required for CCMV disassembly. The N-terminus of the coat protein on the virion is not ordered under X-ray crystallography, it is proposed to be involved in the virus disassembly. Biochemical and immunological analysis of the N-terminus shows that the N-terminus is dynamic, undergoes structural transition to the exterior of the virion presumably to form a channel on the five-fold axis during disassembly. This work will lead more biochemical studies in detail on CCMV disassembly process.

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A thesis submitted in partial fulfillment
of the requirement for the degree

of

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in

Plant Sciences

MONTANA STATE UNIVERSITY
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ABSTRACT

The mechanism of the spherical virus disassembly has been under investigation for understanding the early events during virus infection, and eventually for helping to design reagents to block the process for releasing genetic information into host cells, thus preventing viral infection. The virus has to be stable enough to protect the genetic materials inside the virion, yet dynamic enough to release the nucleic acids to establish infection. Cowpea chlorotic mottle virus provides genetic and biochemical advantages for this purpose. The role of virus swelling for disassembly was studied based on characterization of a salt stable mutant of CCMV *in vivo* and *in vitro*. The salt stable mutant is as infectious as the wild type, swells like the wild type but shows negative signal for translation, thus swelling is not required for CCMV disassembly. The N-terminus of the coat protein on the virion is not ordered under X-ray crystallography, it is proposed to be involved in the virus disassembly. Biochemical and immunological analysis of the N-terminus shows that the N-terminus is dynamic, undergoes structural transition to the exterior of the virion presumably to form a channel on the five-fold axis during disassembly. This work will lead more biochemical studies in detail on CCMV disassembly process.

CHAPTER 1

INTRODUCTION

During the infection cycle of a virus, the virus has to be stable enough to protect the genetic information located inside of the virion, yet dynamic enough to disassemble in order to deliver the viral RNA inside the cell to get access to the host translational machinery. Understanding the mechanisms of viral disassembly would help to discover the chemical basis for genetic material release, and eventually help to design reagents to block the process for releasing genetic information into host cells, thus preventing viral infection.

Cowpea chlorotic mottle virus, CCMV, is a small spherical virus with T=3 icosahedral quasi-symmetry. CCMV belongs to the Bromoviridae family (alpha-virus like superfamily) of the plant viruses, along with broad bean mottle virus (BBMV) and brome mosaic virus (BMV). CCMV and BMV have been model systems for studying the spherical virus assembly and disassembly mechanisms (Bancroft *et al.*, 1974, 1975, 1976; Fox *et al.*, 1996; Zhao *et al.*, 1994; Albert *et al.*, 1997). CCMV was the first spherical virus that had been shown to be reassembled *in vitro* from its protein and RNA components (Bancroft *et al.*, 1967). In laboratory studies, *E.coli* and yeast systems for heterologous expression of CCMV particles have been developed (Zhao *et al.*, 1994; Young laboratory, unpublished data). CCMV infects members of the leguminosae family, such as cowpea. CCMV can accumulate to high levels in host plants; normally from one kilogram of infected plant tissue yield one gram virus. The CCMV virus is stable under a

wide range of salt and pH conditions. Infectious clones are available for *in vitro* studies (Ahlquist *et al.*, 1984; Allison, *et al.*, 1988). This provides useful tools to examine CCMV mutants *in vivo*, such as assembled empty virus particles and engineered CCMV coat proteins. Altogether, the availability of genetic and biochemical studies have made CCMV a model system for studying virus structure, assembly and disassembly.

Genome Organization

There are four single stranded positive sense RNAs in the CCMV genome (Fig. 1). RNA 1 and RNA 2 encode for RNA-dependent-RNA replicases, which along with certain host factors, perform viral RNA replication and transcription. RNA 3 is dicistronic, which encodes a 32 kD movement protein and a 20 kD coat protein. The coat protein is expressed from RNA 4 via a sub-genomic promoter (sgp) on RNA 3. RNA 1 and 2 are encapsidated into separate particles, RNA 3 and RNA 4 are copackaged together into a third particle, with 1:1 molar ratio of RNA 1 and 2 particles (Loesch *et al.*, 1980). All three particles are required to establish successful infection in host cells.

CCMV Structure

The structure of the wild type CCMV has been solved to 3.2 Å resolution (Fig. 2; Speir *et al.*, 1995). The structure of a salt stable mutant of CCMV has been also solved to 2.7 Å resolution (Dr. Qu, the Scripps Research Institute, unpublished data). The high resolution of the salt stable structure provides detailed information on the chemical basis for the salt stable phenotype. The CCMV virus particle is composed of 180 identical coat

protein subunits, arranged on a T=3 icosahedral lattice. There are 20 hexameric and 12 pentameric capsomers in the virion. X-ray crystallography structure of the CCMV coat protein has revealed a striking feature. It has an eight-stranded, anti-parallel β -barrel core, with C-terminus and N-terminus arms that extend in different directions from the core structure (Fig. 3). The N-terminus of the coat protein is not ordered in the X-ray

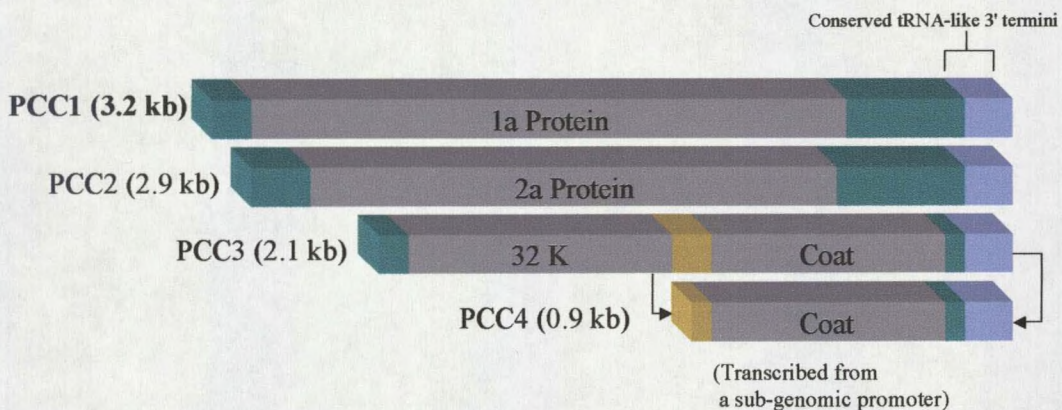


Fig. 1. Genome organization of CCMV (adapted from Arnold, M.S thesis).

The 5' and 3' end untranslated regions are shown in green, and the 3' t-RNA like region is shown in blue. Yellow shows the subgenomic promoter (sgp) on RNA3 and RNA4.

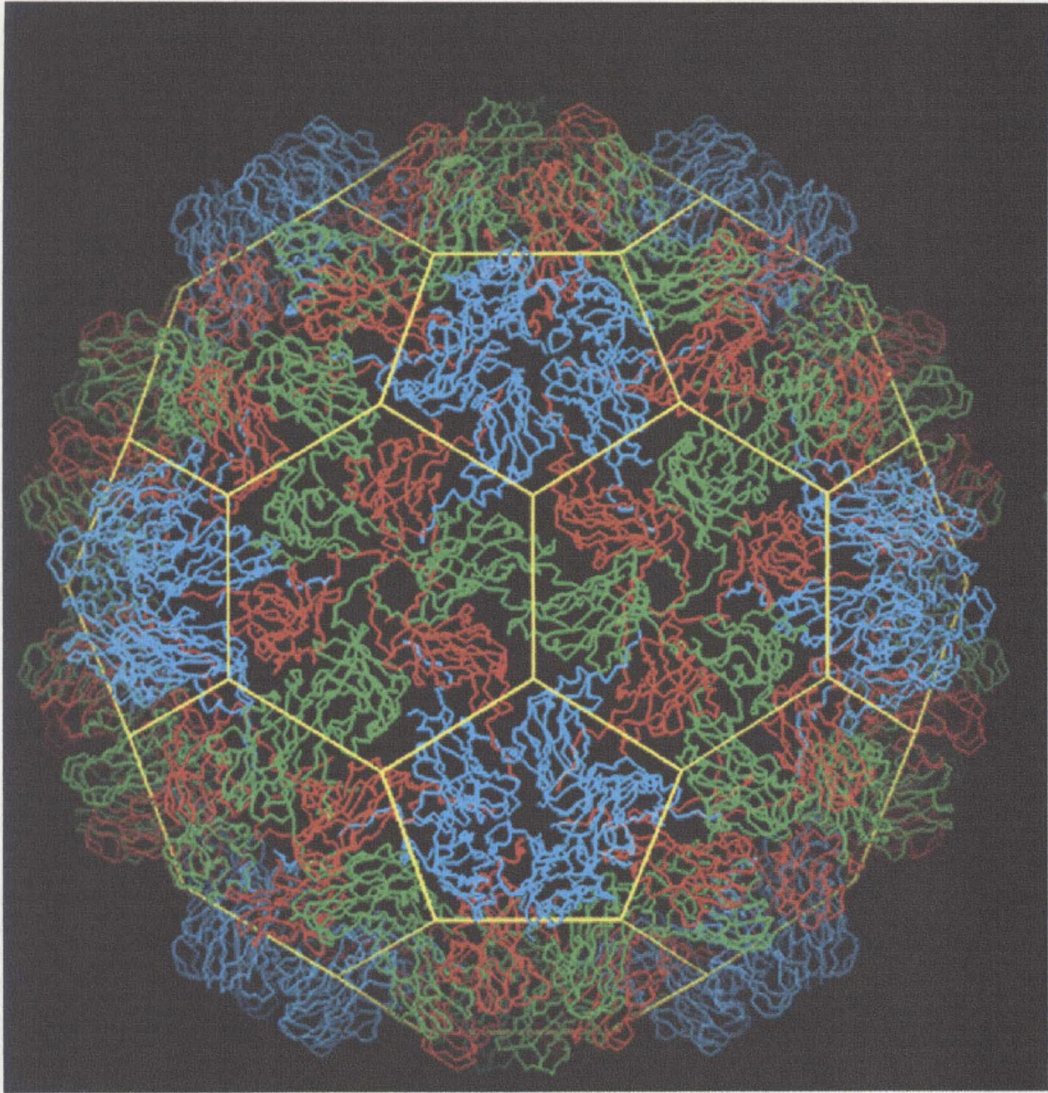


Fig. 2. X-ray crystallography of CCMV virion (adapted from Speir et al., 1995). The virus particle is composed of 180 identical coat protein subunits with a T=3 quasi-symmetry. There are 20 hexameric capsomers and 12 pentameric capsomers on the virion.

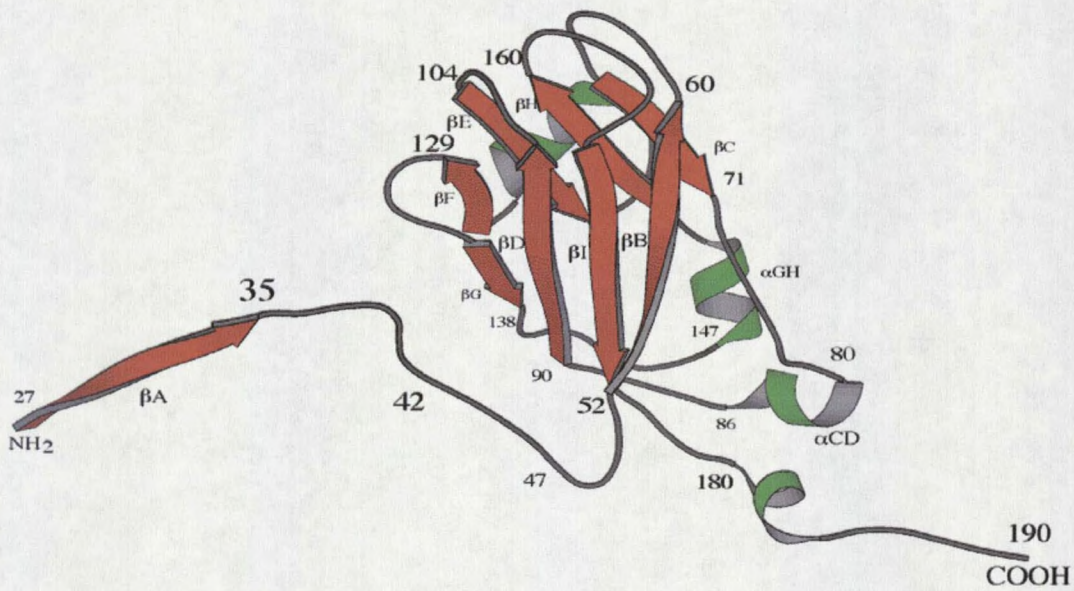


Fig. 3. Structure of the CCMV coat protein (adapted from Speir et al., 1995).

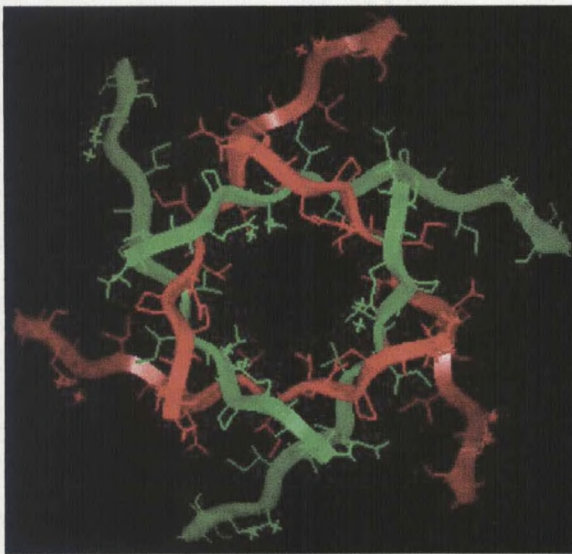


Fig. 4. Hexameric capsomer

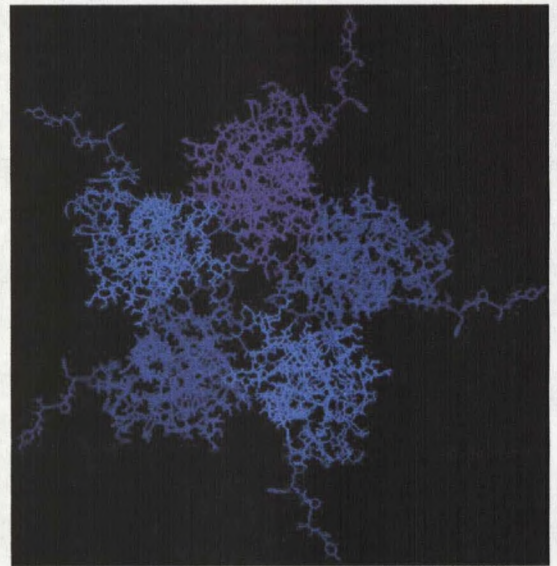


Fig. 5. Pentameric capsomer

crystallography structure (first 27 residues on the hexameric capsomers; first 44 residues on the pentameric capsomers.) (Fig 4, 5). The first 9 amino acids of the coat protein are dispensable for virus assembly, and are believed to be involved with host specificity (Sacher *et al.*, 1989; Zhao *et al.*, 1994; Rao *et al.*, 1995). However, deletion of residues 3-37 at the N-terminal end abolishes the capability for RNA-containing virus particle formation, but can assemble empty particles *in vivo* (Willits, Young lab, unpublished data). There are 9 positively charged lysine and arginine residues in the first 25 amino acids, which make up a conserved arginine-rich motif that is implicated in interactions with viral RNA inside the virion (Wikoff *et al.*, 1997). Substitution of the basic residues with glutamic acid residues results in empty particle formation (Willits, Young lab, unpublished data). The C-termini of two adjacent coat protein subunits undergo reciprocal exchange at the two-fold axis in non-covalent dimer formation. It has been indicated that the noncovalent dimer formation is critical for virus assembly. Deletion of the C-terminal end abolishes the ability to form virion.

Infection

The mechanism of CCMV virus attachment and entry into host cells is poorly understood. In greenhouse studies, mechanical inoculation is used to establish virus infection by rubbing plant leaves with virus or homogenized infected plant tissues. The virus gets into the host cells, and systemic infection is carried through the plasmadesmata and the phloem for cell to cell movement.

Virion Swelling

CCMV particles undergo a structural transition *in vitro* with pH and ionic strength changes (Adolph, 1975a, 1975b). The virus particle is in its compact structure below pH 5.0 with low ionic strength conditions ($I < 0.1$). However, by increasing the pH (> 7.0) under high ionic strength, virions swell from a compact form that sediment on a sucrose gradient of an 88S to a 78S swollen form, which is about a 10% increase in diameter in the linear dimension. The cryo reconstruction study of the CCMV virus has shown that swelling occurs at the pseudo-threefold axis, where a cluster of negatively charged residues (Glu-81, Gln-85 and Glu-148 on one subunit; Gln-149 and Asp-153 on adjacent coat protein subunit) is located (Speir *et al.*, 1995; Fig. 6 and 7). Under high pH, the negatively charged residues are deprotonated and the electrostatic repulsion among them causes swelling. Swelling is reversible by lowering the pH or by addition of divalent cations. At the pseudo-threefold axes the virion is believed to bind 180 Ca^{++} . Further increasing the pH, as well as the ionic strength, will cause the virus particle to disassemble into its free coat protein and RNA components.

There is a salt stable mutant of CCMV that was isolated by random mutation method (Bancroft *et al.*, 1973). The mutant does not dissociate at high salt and high pH conditions, whereas the wild type virus totally disassembles. The mutant was mapped to one amino acid change from a lysine to an arginine residue in the coat protein. The salt stable was regenerated *in vivo* and *in vitro* by genetic means (Fox *et al.*, 1995). It was demonstrated that a single base change from A to G at position 1484 in RNA3 causes the

salt stable phenotype. The structure of the salt stable virus has been recently solved to 2.7 Å resolution (Qu, the Scripps Research Institute, unpublished data). The high-resolution structure demonstrates that there is a new salt bridge formation between the E34 and the substituted R42 on another adjacent coat protein subunit at the threefold axis on the hexameric capsomers. It is proposed that the putative salt bridges formation resulted in the salt stable phenotype (Speir *et al.*, 1995).

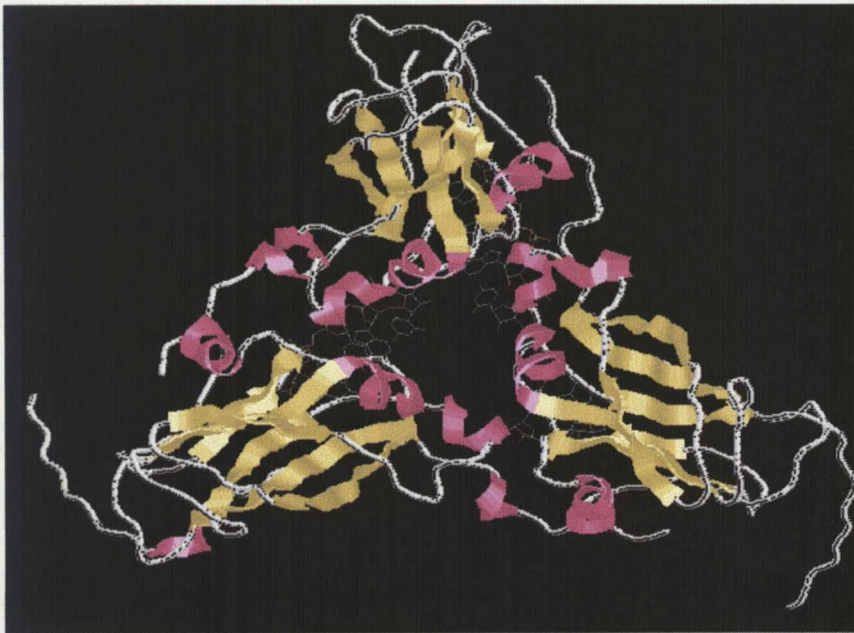


Fig. 6. Structure at the pseudo-three fold axis (adapted from Speir, Ph.D thesis).

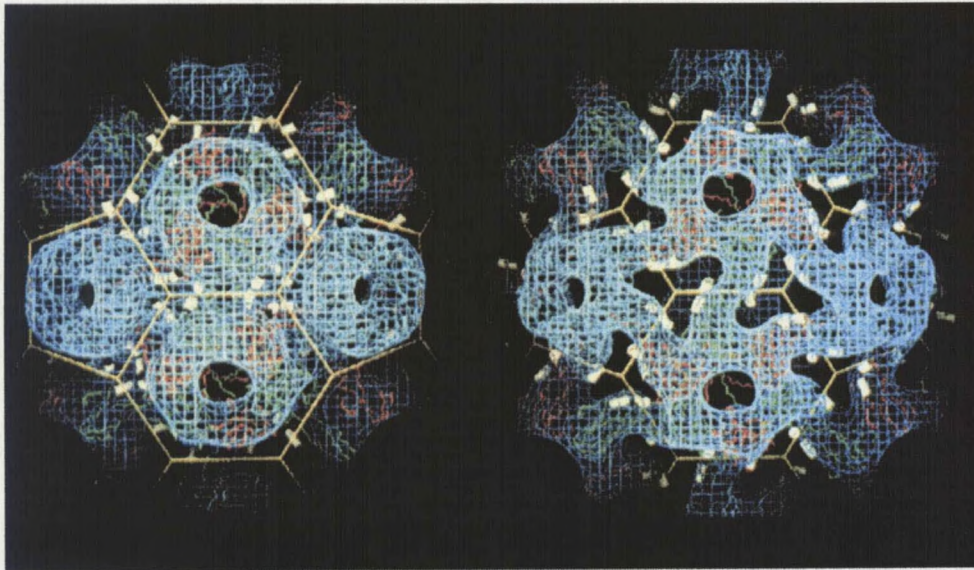


Fig. 7. Electron density map of a native CCMV particle vs. the swollen form.
(adapted from Speir, Ph.D thesis).

CCMV Assembly

CCMV coat protein can spontaneously assemble into polymorphic forms *in vitro* under various pH and ionic conditions, such as sheets, rods and T=1, pseudo T=2 and T=3 particles (Zhao *et al.*, 1994). The particle reassembled *in vitro* has the same characteristics as particles formed *in vivo* observed under cryo-reconstruction (Fox *et al.*, 1997). The coat protein exists as a non-covalent dimer in solution. However for CCMV virus assembly pathway *in vitro*, it was previously believed that assembly was initiated by a hexamer of dimers (Speir *et al.*, 1995). Recent studies with polymerization kinetics observed by light scattering however showed that assembly of CCMV empty particles

starts with a pentamer of dimers in solution for the empty virion formation (Zlotnick *et al.*, 2000). This might not be the mechanism for *in vivo* CCMV assembly. After the pentamer of dimer formation, the assembly pathway can go to T=2 particle or T=3 quasi-symmetry by addition of hexamers.

CCMV Disassembly

A cotranslational disassembly model has been proposed for CCMV (Brisco *et al.*, 1986). The model holds that through swelling, the viral RNA was released and translated. Under the same conditions as virion swelling *in vitro*, the ribosomes bind to the viral RNA and pull it out while the viral RNA is translated. An alternate model predicts that swelling is not required for CCMV cotranslational disassembly *in vitro* based on genetic and biochemical studies of CCMV mutants (Albert *et al.*, 1997). It was suggested that the N-termini of the coat protein subunits on the pentameric capsomers undergo a structural transition and can form a five bundled α -helical channel, and through this channel the viral RNA was presented to the host translational machinery (Fig. 8).

For disassembly mechanisms of spherical virus, there has been a model for poliovirus disassembly that proposed that the N-terminus of VP1 forms a channel after cleavage of VP4 from the coat protein.

For rod-shape virus disassembly mechanisms, tobacco mosaic virus (TMV) is the virus that most of the disassembly studies have been performed. It has been proposed that TMV virus disassembly is initiated by the bi-directional release of the coat protein subunits from the viral RNA, and this process maybe mediated by cotranslational and

coreplicative disassembly mechanisms (for review, Shaw, 1999). The uncoating from the 5'-end of viral RNA results in the 5'-end of the viral RNA exposed first and disassembly is initiated. The disassembly process is then completed by removal of the coat protein subunits in the 3'-5' direction with involvement of TMV replicase proteins

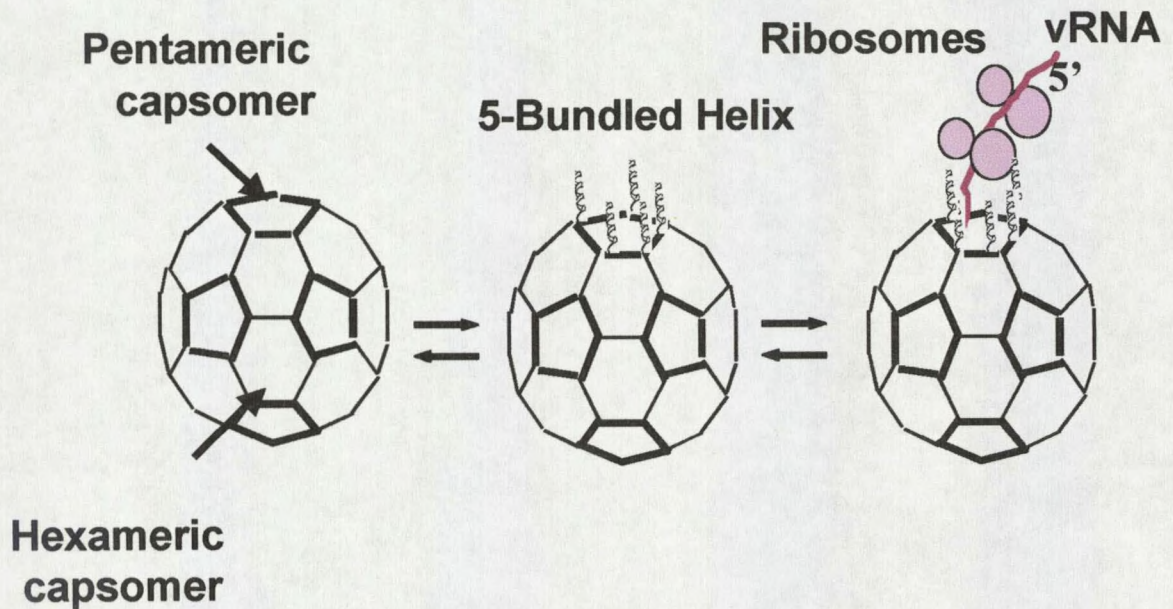


Fig. 8. The N-terminal channel model for CCMV disassembly.

In this model, it was proposed that the N-termini on the pentameric capsomers undergo a structural transition from interior of the virion to the exterior of the virus particle to form a five-bundled α -helix channel. Through this channel, the viral RNA is released to access the host translational machinery.

(Wu *et al.*, 1997; for review, Stubbs, 1999). The possible reason to believe in shedding from 5'-end is because it is very AU-rich region, where RNA-protein interactions are the weakest. Therefore, the 5'-end is exposed first and disassembly is initiated.

The objectives of this thesis was to investigating the role of swelling and the N-terminus dynamics for virus disassembly primarily by studying the characterization of a salt stable mutant, including its *in vivo* infectivity, swelling characteristics, and the chemical basis for the salt stable phenotype. Genetic and biochemical studies have shown that the N-terminus of CCMV coat protein on the virion plays an important role in virus assembly and disassembly. To study CCMV N-terminus dynamics, this thesis also includes biochemical and immunological evidence for the N- terminus mobility by using *in vitro* translation assays, thiol quantitation analysis on mutant viruses and N-terminus specific polyclonal antibodies for tracking the position of the N-terminus in virus particle.

CHAPTER 2

BIOLOGICAL AND GENETIC ANALYSIS OF A SALT STABLE MUTANT OF CCMV AND ITS IMPLICATION FOR DISASSEMBLY

Introduction

Cowpea chlorotic mottle virus (CCMV) belongs to the Bromoviridae family of plant viruses (Alpha-virus like super family). Bromoviruses, such as CCMV and BMV (Brome Mosaic Virus) provide model systems for studying virus assembly and disassembly (Fox *et al.*, 1996; Zhao *et al.*, 1994; Albert *et al.*, 1997). CCMV has been a model system for studying virus assembly and disassembly since the early 1970s (Bancroft *et al.*, 1967, 1968, 1975, 1976). There are four positive sense single stranded RNAs in the CCMV genome, which are encapsidated into three different T=3 icosahedral particles of 28 nm in diameter. RNA 1 and RNA 2, which encode RNA-dependent RNA replicases, are packaged into separate particles. RNA 3 and RNA 4 are co-packaged into a third particle altogether, with a 1: 1: 1 molar ratio with RNA 1 and RNA 2 particles. RNA 3 is di-cistronic, encoding a 32 kD movement protein as well as a 20 kD coat protein. The coat protein is expressed from RNA 4 via a subgenomic promoter (sgp) on RNA 3. All three virus particles are required to establish successful infection in host plants.

The CCMV virus particle is composed of 180 identical coat protein subunits in a T=3 icosahedral lattice. Coat proteins are arranged in 12 pentameric capsomers and 20 hexameric capsomers in the virion. X-ray crystallography of the coat protein has revealed striking features (Speir *et al.*, 1995). It has an antiparallel eight-stranded β -barrel core in it, with its N-terminal and C-terminal arms extended to different directions from the core

structure. The N-terminus is not ordered under the X-ray crystallography (the first 27 amino acids on the hexameric capsomers; and 1-44 residues on the pentameric capsomers). On the threefold axis, the residues from 27 to 35 form a β -hexamer structure, but such a structure is not observed on the fivefold axis. The C-termini of the coat proteins intertwine with each other at the two-fold axis, and this axis was predicted to be where the coat protein non-covalent dimer forms. Protein-protein interactions, such as hydrophobic interactions, hydrophilic interactions, and salt bridges stabilize the virion. Protein-RNA interactions contribute to hold the virion together as well.

The CCMV virions undergo a structural transition *in vitro* in different pH and salt conditions (Adolph, 1975a, 1975b). CCMV virus particles are stable at pH 5.0 with low ionic strength ($I < 0.1$). Increasing the pH to 7.5 while maintaining low ionic strength causes the virus particle to swell from an 88S native virion to a 78S swollen form. Swelling occurs at the pseudo-threefold axis where a cluster of negatively charged residues is located. At high pH, the electrostatic repulsion among them causes swelling at these axes and opens 20 Å pores at these axis. Swelling is reversible by addition of cations, such as Mg^{++} and Ca^{++} , due to neutralization with cation binding. Further increasing the ionic strength ($I > 1.0$) at high pH, causes CCMV virus to dissociate into its coat protein and RNA components.

Studies of CCMV disassembly have suggested that under the same conditions of swelling, the CCMV viral RNA was exposed and consequently, the host ribosome bound with the RNA and pulled the viral RNA out while the RNA was translated. This has been proposed to be cotranslational disassembly for CCMV (Brisco *et al.*, 1986).

Previous studies in the Young laboratory suggested that swelling is not required for CCMV disassembly *in vitro* based on genetic and biochemical studies on a salt stable and a cysteinyl mutant of CCMV (Albert *et al.*, 1997). In this model, it was suggested that the salt stable mutant does not swell *in vitro*, but can be translated, and is as infectious as the wild type virus; the cysteinyl mutant swells like the wild type virus, but does not get translated in the *in vitro* translation system and lost its infectivity under non-reduced condition. Therefore, it was proposed that the N-terminus on the pentameric capsomer on the virus particle forms a five-bundled α -helical channel, and through this channel, the viral RNA was released to the host translational machinery.

CCMV assembly and disassembly mutants provide a valuable source for understanding the chemical basis for virus stability (Bancroft *et al.*, 1969, 1971, 1972, 1973, 1976). One such mutant, the salt stable mutant, was isolated through random mutagenesis in the early 1970's (Bancroft *et al.*, 1971, 1973). This mutant does not disassemble under high salt ($I > 1.0$) and high pH ($pH > 7.5$) conditions, whereas the wild type CCMV virion totally disassociates into its free coat protein and RNA components. Previously in the Young laboratory, it was shown that the salt stable mutant was caused by a single mutation from A to G in the coat protein gene, which resulted in a substitution from a lysine to an arginine residue at position 42 in the coat protein. Therefore, the salt stable (SS) mutant is designated K42R (Fox *et al.*, 1996). The *E. coli* expressed coat protein containing the K42R substitution can spontaneously form virus particles *in vitro* as can the wild type coat protein. The empty virions show the same characteristics as the salt stable native particles (Fox *et al.*, 1996).

My goal was to look at how the salt stability affects both the infectivity of the salt stable mutant and its swelling characteristics. It was predicted from the structure of the salt stable virus that there should be a salt bridge formation between the E34 on one coat protein subunit and the R42 on another adjacent subunit at the threefold axis on the hexameric capsomers. It was proposed that this extra salt bridge contributed to the distinct phenotype in the salt stable virion. The high-resolution structure of the salt stable mutant has been solved recently to 2.7 Å resolution (Qu, Scripps Research Institute, personal communication), which along with biological and genetic analysis has allowed us to expand our understanding of the chemical basis for the salt stable phenotype and its biological consequences *in vivo*.

Materials and Methods

In vitro RNA Transcription and Plant Inoculation of the Salt Stable Mutant

The salt stable mutant was generated by oligonucleotide site-directed mutagenesis in pCC3, which is the complementary DNA (cDNA) for RNA3 of CCMV (Allison *et al.*, 1988), with primer 22 (5'-GGTCCAAGCTTTAATAGCCCTGCCTTGGCC-3') and primer K42R (+) (5'-GGCCAAGGCAGGGCTATTAAAGCTTGGACC-3') as described by the Stratagene protocol (Quick change, Stratagene, San Diego, CA). Primers contain an A to G mutation at position 1484 in pCC3. This mutation causes a lysine to arginine substitution at position 42 in the amino acid sequence of the coat protein. Screening of mutant plasmids was performed by *Bam*H I restriction endonuclease cleavage of mutant

plasmids after transformation. The *Bam*H I site was introduced in the primers as underlined.

RNAs were synthesized *in vitro* using the T7 mMessage mMachine kit as described by the supplier (Ambion, Austin, TX) from 2 µg of *Xba* I linearized complementary DNAs (cDNAs) after they were treated with phenol:chloroform:isoamylalcohol (1:25:24) solution and ethanol precipitation. The RNAs were capped by incorporating p-1-5'-7-methyl-guanosine-p-3-5' guanosine triphosphate (M^7 pppG) into the transcription reaction. CCMV wild type RNAs 1, 2 and 3 were transcribed using *Xba* I digested pCCTP1, pCCTP2 and pCCTP4, which contain cDNAs of RNA 1, RNA 2 and RNA 3 respectively. To analyze transcription products, the RNAs were visualized on denaturing-agarose gel (1.2% agarose/6.6% formalaldehyde) by loading 2 µl out of a standard 20 µl transcription reaction (RNA gel). RNAs were quantified by absorbance at 260 nm (A_{260}) using an extinction coefficient of 33 for CCMV RNA ($E_{260}^{1\text{mg/ml}} = 33$). A mixture of RNA 1, 2 and 3 (or mutant RNA 3') in a 1:1:2 molar ratio was used to inoculate cowpea plants (*Vigna unguiculata* (L.) var. California Blackeye). Inoculations were performed by applying 5 µg of total RNAs on a single 7-10 day old leaf of each plant and rubbing with gloves. The infection was allowed to proceed for 10-14 days at 20 °C with a 14-hour light period.

Virus Detection

Replication and presence of virus were tested with CCMV polyclonal antibody by

an enzyme-linked immunosorbent assay (ELISA). Microplates (Corning Incorporations, Corning, NY) were coated with 100 μ l of 1mg/ml polyclonal antibody specific for CCMV at a 1:5000 dilution in coating buffer (8.0 g/L Na_2CO_3 , 2.93 g/L NaHCO_3 , 0.2 g/l NaN_3 , brought to 1 liter with dH_2O , pH 9.6), and incubated at 4 °C overnight. Plates were washed three times with PBS-Tween (8.0 g/L NaCl, 0.2 g/L KH_2PO_4 , 2.9 g/L $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 0.2 g/L KCL, brought to 1 liter with dH_2O , pH 7.4, 0.5% Tween-20).

A total of 100mg of plant tissue was ground in 500 μ l antigen buffer (10 mM potassium phosphate buffer, pH 6.0, 2% polyvinylpyrrolidone-PVP 4000) with a mortar and pestle. Homogenized plant tissues were spun at 14,000 rpm for 10 minutes in a desktop centrifuge. A total of 100 μ l of the supernatant was loaded into wells, along with a wild type virus standard containing 100ng, 10ng, 1ng, 0.1ng, 0.01ng and 0.001ng in 100 μ l antigen buffer. The plates were incubated overnight at 4 °C, and then washed with PBS-Tween. A total of 100 μ l of alkaline phosphatase (AP)-conjugated CCMV polyclonal antibody was added to each well and incubated at 37 °C for 2 hours. The plates were then washed with PBS-Tween before 100 μ l of 1 mg/ml *p*-nitrophenol phosphate substrate (Sigma, St.Louis, MO) was added in substrate buffer (9.7 % diethanolamine, 1 mM sodium azide, pH 9.8). The colorimetric reaction was allowed to develop from 10 minutes to 1 hour; the absorbance at 405 (A_{405}) was measured and recorded with a Molecular Devices kinetic microplate reader (Molecular Devices, Sunnyvale, CA).

Viral Genome Integrity Testing

To analyze the mutation in the virus genome, a reverse transcription-polymerase chain reaction (RT-PCR) was performed on inoculated plants. Total plant RNA was extracted from 100 mg plant tissue with an RNeasy Plant Mini Kit as described by the manufacturer (Qiagen, Valencia, CA). Primers Ps2c (5'-CCGTTGCGGGCTTCCGACGGCCTCG-3', 1101-1127) and P21 (5'-GTAACGGTCGACAGCGGGC-3', 1934-1953), which flank the coat protein gene on pCC3, were used in the RT-PCR system (Promega, Madison, WI) to amplify the full-length open reading frame of the coat protein gene (Fig. 9). The RT-PCR products were digested with the engineered *Bam*H I (5 µl out of 50 µl RT-PCR product) in a total digestion mixture of 20 µl. RT-PCR products were sequenced with Big-Dye sequencing kit in an Applied Biosystems 7600 thermocycler system (Applied Biosystems, Perkin-Elmer, Foster City, CA) and sequences were analyzed for mutations.

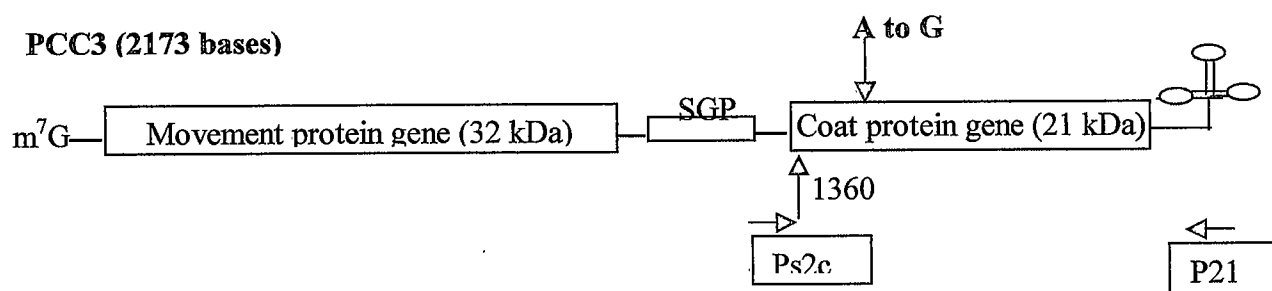


Fig. 9. This graph represents pCC3 of RNA3 and the position of primers for RT-PCR.

Ps2c and P21 were used for amplifying the full open reading frame for coat protein gene. The small bar represents subgenomic promoter (SGP). The arrow on the top indicates the A to G mutation in the salt stable mutant.

Virus Purification and Quantification

The plant tissues were harvested and the viruses were purified as following.

Infected plant leaves were frozen at -20°C and stored. The plant tissues were frozen immediately with liquid nitrogen, and were ground with a Waring blender for 3 minutes at high speed in homogenization buffer (0.2 M sodium acetate, 0.2 M acetic acid, 0.01 M ascorbic acid, 0.01 M disodium EDTA, pH 4.8). The homogenized plant tissues were filtered through two layers of cheesecloth, and were placed on ice for one hour before centrifugation at 20,000g for 20 minutes. The virus in the semi-clear supernatant was precipitated with 10% (w/v) polyethylene glycol (PEG)-8000 at 4°C overnight with slow stirring. The virus was pelleted by centrifugation at 20,000g for 20 minutes, and the pellet was subsequently resuspended in virus buffer (0.1 M sodium acetate, 1 mM sodium azide, 1 mM sodium EDTA, pH 4.8, 0.22 μM filtered), with a 1/10 volume of the homogenization buffer. Undissolved materials were removed by centrifugation at 20,000 g for 20 minutes. The virus was precipitated a second time with 15 % (w/v) PEG-8000 in solution overnight at 4°C , and then was pelleted by centrifugation at 20,000g and resuspended in virus buffer with 5% the volume of the homogenization buffer.

Undissolved materials were removed by centrifugation at 20,000 g for 20 minutes. A total of two ml of supernatant was put on a 10 ml of 38% (w/v) CsCl gradient for ultra centrifugation at 38,000 rpm for 18-20 hours at 4°C (Beckman, SW41, Fullerton, CA).

The virus band was removed with a glass pipette, and then dialyzed against a total of 600 ml virus buffer three times to get rid of CsCl.

The purified virus was quantified both by absorbance at A_{260} with an extinction coefficient of 5.87 (Gene Quant RNA/DNA Calculator, Pharmacia Biotech, Cambridge, England) and a direct protein quantification using a BCA protein assay kit (Pierce, Rockford, IL) with BSA (bovine serum albumin) as a standard. The virus purity was determined by the ratio of A_{260} to A_{280} (1.6-1.7 for pure CCMV virus). The virus with low yield was further concentrated with CentriconTM 100.

Local Lesion Assay

A local lesion assay was performed with a *Chenopodium quinoa* plant, which is one of the permissive local lesion hosts for CCMV. Virus was quantified and purity was determined as described above in this chapter. A standard curve was first constructed with known amount of wild type virus. A total of 10 μ l of a series of diluted virus at 0.04, 0.02, 0.01, 0.005 mg/ml was inoculated onto duplicated half-leaves of physiologically similar leaves from different plants to eliminate the difference between leaves. The log of the lesion number was plotted against the minus log (-Log) of virus concentration to construct a standard curve for a particular experiment. The following experiments were performed within the linear range according to the standard curve, both with the salt stable and the wild type virus at the same concentration (0.02 and 0.01 mg/ml). Inoculated plants were incubated at 25 °C with a 24-hour light period, and lesions were allowed to develop for four to seven days. Leaves were harvested once lesions were visible. Local lesions from

each sample were counted under a light microscope and numbers were recorded and compared for wild type and the salt stable viruses.

Sucrose Gradient Assay

A total of 200 µg of CsCl purified wild type and the salt stable viruses in 200 µl of virus buffer (0.1 M sodium acetate, 1 mM sodium EDTA, 1 mM sodium azide, pH 4.8, 0.02 µm filtered) were dialyzed against either the swollen buffer (0.1M Tris-HCl, 1 mM sodium EDTA, 0.1M NaCl, pH 7.5), or the compact buffer (10 mM CaCl₂, 0.1M Tris-HCl, 0.1M NaCl, pH 7.5), at 4°C overnight to keep viruses swollen or not swollen. Both 5% (w/v) and 25% (w/v) sucrose solutions were made in each buffer. The gradients were made by mixing equal volume of 6 ml of 5% and 25% sucrose solution with a gradient master (Biocomp, Canada) as described by the manufactures protocol. Samples in a 200 µl volume were loaded on the top layer of the 12-ml gradient, which was then spun at 37,000 rpm for 2 hours at 4 °C in a SW41 Ti rotor (Beckman, Fullerton, CA).

After centrifugation, the gradients were fractionated with an HPLC system (Bio-Rad, Hercules, CA) at a speed of 3 ml/minute. Absorbance at A₂₆₀ and A₂₈₀ was monitored by a UV spectra-photometer (Bio-Rad, Hercules, CA). The peak positions were recorded and compared.

Second position E34 mutants

From structural studies, it was proposed that amino acid residue E34 could form a new salt bridge with the substituted R42 from an adjacent coat protein subunit in the salt stable mutant (Speir *et al.*, 1995). To test the potential role of the salt bridge, I wanted to interrupt the salt bridge by changing E34 to a similar negatively charged residue, D; a neutral residue, A; and a positively charged residue, R.

Mutations were generated by nucleotide site-directed mutagenesis as described by Stratagene protocol (Stratagene, San Diego, CA). Primers with engineered restriction sites on pCC3 are shown in Table 1:

E34A (+): CCTGTTATTGTAGCACCGATCGCTTCAGGCC (*PvuI*)

E34A (-): GGCCTGAAGCGATCGGTGCTACAATAACAGG (*PvuI*)

E34D (+): CCTGTTATTGTGGATCCATCGCTTCAGGCC (*Bam*HI)

E34D (-): GGCCTGAAGCGATGGGATCCACAATAACAGG (*Bam*HI)

E34R (+): CCTGTTATTGTAAGGCCTATCGCTTCAGGCC (*Stu*I)

E34R (-): GGCCTGAAGCGATAGGCCTTACAATAACAGG (*Stu*I)

Table 1. Primers for site-directed mutagenesis.

Underlined bases represent restriction endonuclease sites introduced in primers for each mutation. Capped mutant RNA 3 was transcribed from *Xba* I linearized perfect-kit purified plasmids (Qiagen, Valencia, CA) E34A1, E34A6, E34D1, E34D2, E34R4, and E34R7, each mutant with a duplicated clone. Wild type RNAs 1, 2, and 3 were

synthesized and capped as described above using a T7 mMessage mMachine kit (Ambion, Austin, TX). RNAs were visualized on a denaturing agarose gel (1.2% agarose/6.6% formaldehyde) after transcription. Plants were inoculated by applying 5 µg total RNAs on a single 7-10 day old leaf of each plant and rubbing with gloves. The infection was allowed to proceed for 10-14 days at 20 °C with a 14-hour light period. Primary plant tissues were tested by ELISA for the presence of virus as described above. Mutations were confirmed by RT-PCR with Ps2c and P21 from plant tissues followed by sequencing and enzyme digestion as described above. Viruses were purified as described above (Zhao *et al.*, 1995). Viruses were then quantified both by absorbance at A_{260} (using an extinction coefficient of 5.87 ($E_{260}^{1\text{mg/ml}} = 5.87$)) and protein analysis using a BCA protein assay kit (Pierce, Rockford, IL).

To test whether a mutant can be passaged from plant to plant, plant leaves inoculated with mutant transcribed RNA were ground in inoculation buffer (100 mM sodium phosphate buffer, 10 mM MgCl_2 , pH 6.0) and then inoculated to cowpea plant. Plants were grown at 20 °C with a 14-hour light period in the green house to allow virus accumulation. Subsequently, plant tissue was tested by ELISA using a CCMV polyclonal antibody seven to ten days post infection, harvested and stored at -20 °C. Viruses were purified as described above (Zhao *et al.*, 1994).

The purified wild type and mutant viruses were tested for salt stability by dialyzing against a disassembly buffer (1.0 M CaCl_2 , 20 mM Tris buffer, 1 mM EDTA, pH 7.5) at 4 °C overnight. The wild type virus dissociates in disassembly buffer, but the salt stable mutant stays intact. The ratio of A_{260}/A_{280} before and after dialysis was used for measuring virion stability.

Results

Local Lesion Assay

The salt stable mutant replicated at the same level as the wild type in primary inoculated leaves and passaged plants, which indicated that the salt stable virus replicated systemically as the wild type. RT-PCR showed the correct pattern for restriction endonuclease digestion. Sequencing of the RT-PCR product also confirmed that only one base was changed in the salt stable mutant.

The local lesion assay, like the plaque assay for bacterial phage and animal viruses, is a method to measure the infectivity of a plant virus. It requires all three CCMV virus particles that contain RNA 1, RNA 2 and RNA 3/4 to establish a successful infection. To compare the infectivity of wild type and the salt stable intact viruses, a standard curve was first constructed with a series of diluted wild type viruses (Fig. 10). The reason that a greater amount of virus does not produce higher numbers of lesions is due to viral precipitation; therefore, the lesion number is not directly correlated with the amount of virus that was inoculated on the plant.

The following experiments were performed within the linear range of the standard test. The numbers of lesions caused by same amount of wild type and the salt stable virus, showed no statistical difference (with an average of 47 and 51 at 0.02 mg/ml; and an average of 8 and 7 at 0.01 mg/ml for wild type and the salt stable respectively) (Fig. 11). This indicated that the infectivity of both viruses is the same *in vivo* although the salt

stable virus is high salt and high pH resistant. As predicted before, from purification of the viruses from the systemic host, cowpeas, the salt stable virus will accumulate at the

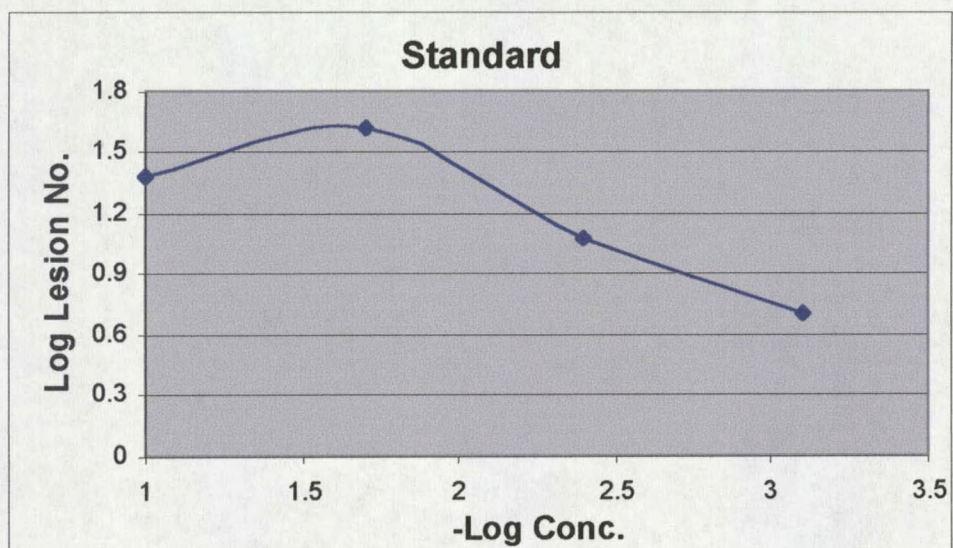


Fig. 10. Wild type virus standard for local lesion assay.

The log of the lesion numbers was plotted against $-\text{Log}$ of the virus concentration. The following experiments were performed within the linear range.

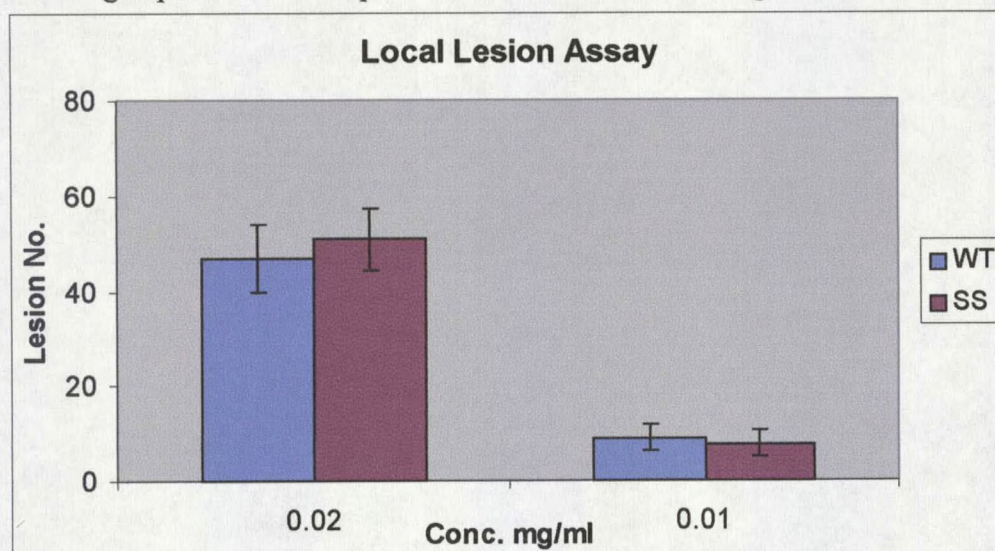


Fig. 11. Local lesion numbers for wild type and salt stable viruses.

10 μl of 0.02 mg/ml and 0.01 mg/ml viruses were inoculated on chenopodia leaves. Lesion was allowed to develop for 4-7 days, and lesion numbers were counted under a light microscope. The bars represent standard deviation from triplicate leaves.

same level as the wild type, which suggests that the salt stable mutant is as infectious as the wild type *in vivo*.

Sucrose Gradient Assay

A sucrose gradient separates molecules based on their sizes and shapes, which corresponds to a sedimentation coefficient, or an S value. As shown in Fig.12, in swollen buffer, both the wild type and the salt stable swell; therefore, the peak shifted upwards in the gradient. From previous data in the laboratory, the swollen form of CCMV has a 78 S value, but the native WT has an 88S value. Comparing the peak position of wild type virus with the salt stable mutant in the swollen form, there is no difference between them (Fig. 12 and 13, upper peaks in red). The swelling buffer contains 1 mM EDTA at pH 7.5; however, the compact buffer contains 10 mM CaCl_2 . This demonstrated that swelling can be reversed by the addition of cations in solution, possibly due to the neutralization of negatively charged residues by ion binding at the pseudo-threefold axis thus neutralizing electrostatic repulsion among them. The results indicate that the salt stable mutant has the same swelling characteristic as the wild type virus.

It was previously suggested that the salt stable virus does not swell under high salt and high pH conditions (Albert *et al*, 1997). Previous experiments used 10-40% sucrose with 1.0 M NaCl, in a 50 mM sodium-phosphate buffer, pH 6.5, 1 mM EDTA, which might not be as sensitive as 5-25% sucrose at pH 7.5. However, the data presented showed that the salt stable virion swells like the wild type *in vitro*. The structure of the salt stable virus at the pseudo-threefold axis agrees with the results

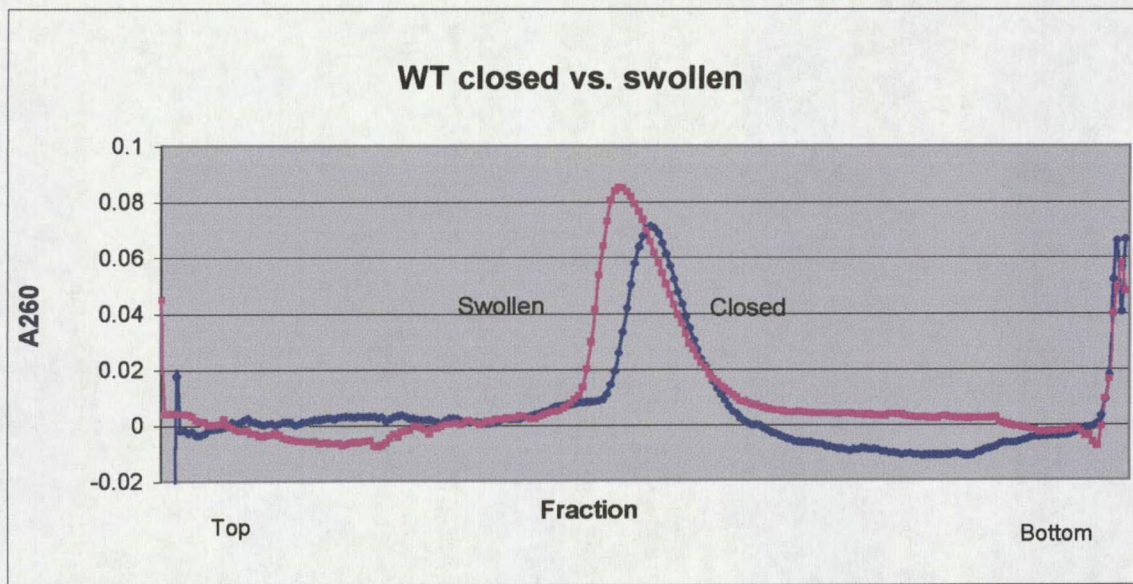


Fig. 12. Swollen vs. closed forms of wild type virus.

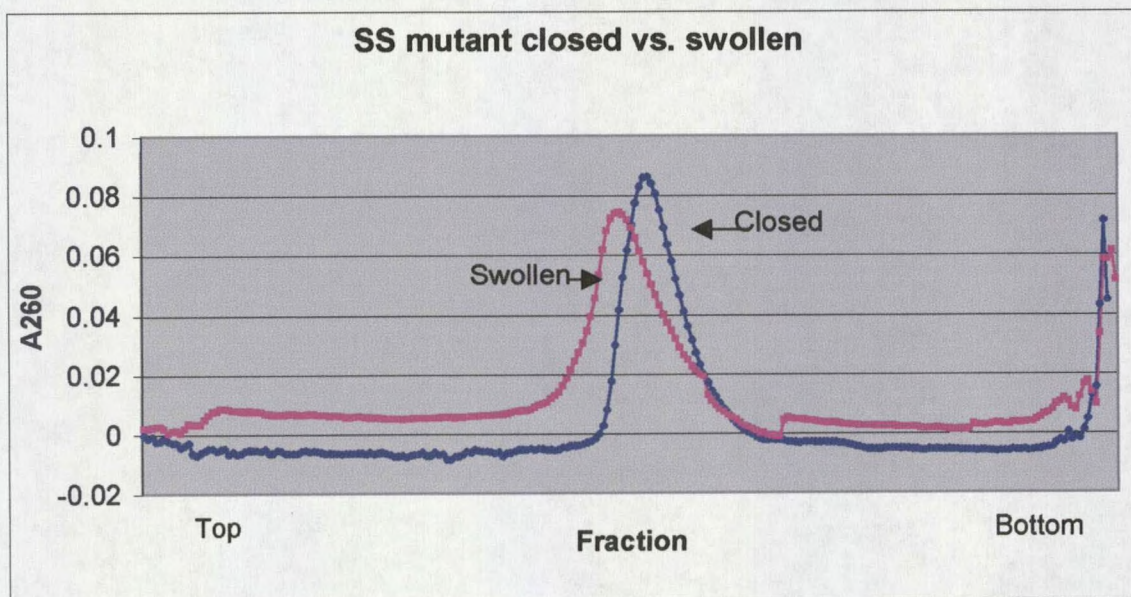


Fig. 13. Swollen vs. closed forms of the salt stable (SS) viruses.

The swelling buffer (1.0 M NaCl, 20 mM Tris-buffer, 1 mM EDTA, pH 7.5) and the compact buffer (1.0 M NaCl, 20 mM Tris-buffer, 10 mM CaCl_2) were used for this assay. The swollen form (red) shifted upward compared to the closed form (blue) in the gradient, from an 88 S virus particle shifted to a 78 S swollen form, which is about 10% increase in diameter in the linearized dimension.

presented here since there are only subtle changes at these axes, and the interactions among the cluster of negatively charged amino acids at high pH is still the same as that in the wild type virus.

Second Position E34 Mutants in the Salt Stable Virus

To test the hypothesis of salt bridge formation, E34 was substituted by three residues: D, A, and R. RNAs inoculated onto primary leaves resulted in positive ELISA reactions 10 days after inoculation. As shown in Fig. 14, E34A, E34D, and E34R replicated at the same level as the wild type control (with an ELISA value of 0.9) (Fig. 14). Healthy plant tissue showed a background signal on ELISA with a value of 0.28. The mutated RNA product was confirmed by RT-PCR and restriction endonuclease digestion. Subsequently sequencing of RT-PCR products from each mutant confirmed the mutation at E34; no other base changes occurred in the open reading frame of the coat protein.

As shown in Fig. 15, E34A and E34D showed ELISA positive results both in the primary and secondary leaves, at the same level as the wild type control (with an ELISA value of 1.2). However, with E34R inoculated plants, the presence of coat protein was detected in primary leaves but not in the inoculated secondary leaves, which had a background ELISA value of 0.25. This indicated that the transcribed RNAs were infectious *in vivo*, but unable to move systemically. The E34R virus might be deficient in assembly or movement; therefore, it could not be passaged from leaf to leaf since there

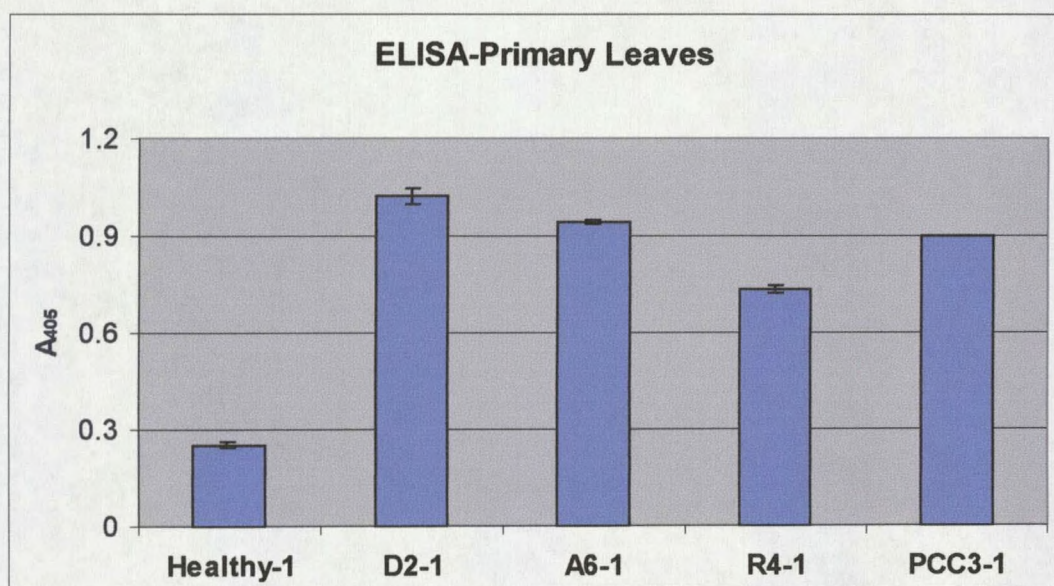


Fig. 14. ELISA results of the primary leaves of E34 mutants.

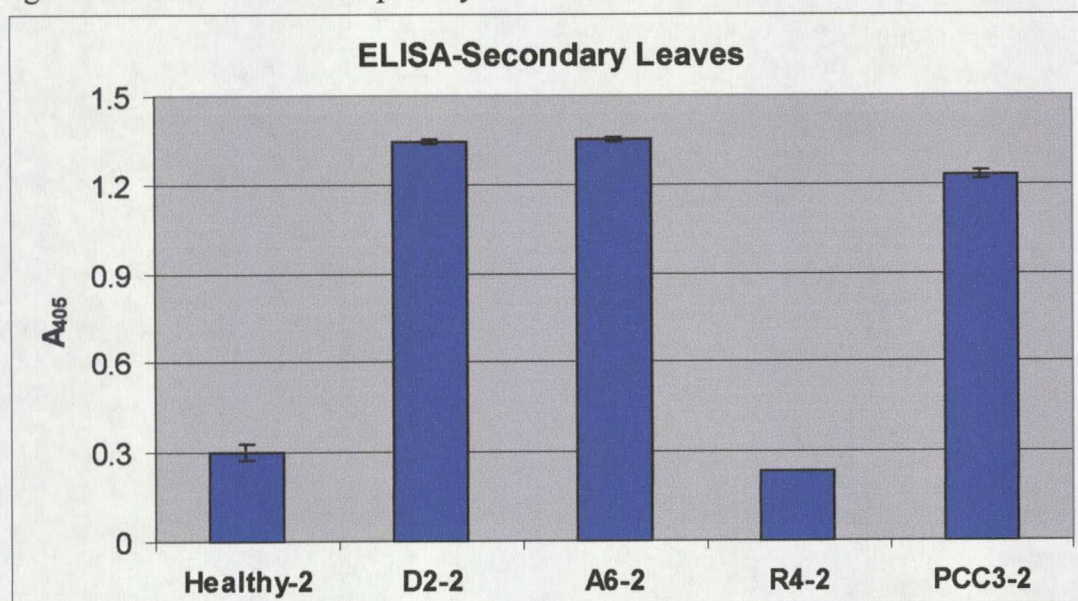


Fig. 15. ELISA results of the secondary leaves of E34 mutants.

Healthy-1: Primary leaves of healthy plant Healthy-2: secondary leaves of healthy plants

D2-1: E34D2 primary leaves D2-2: E34D2-2 secondary leaves

A6-1: E34A6 primary leaves A6-2: E34A6 secondary leaves

R4-1: E34R4 primary leaves R4-2: E34R4 secondary leaves

PCC3-1: CCMV wild type primary leaves PCC3-2: CCMV wild type secondary leaves

The bars represent standard deviation of triplicate experiments.

was no packaged viral RNA for establishment of infection. Effort to purify the E34R virus from plants was unsuccessful along with the wild type as a positive control.

To test virion stability, mutant viruses were dialyzed against the disassembly buffer along with the same amount of the wild type and salt stable viruses. The ratio of A_{260}/A_{280} before and after dialysis is shown in Table 2. :

	Before dialysis (Ratio)	After dialysis (Ratio)
WT-PCC3	1.705	0.951
Salt Stable	1.694	1.612
E34A6	1.691	0.945
E34D2	1.690	0.876

Table. 2. A_{260}/A_{280} ratios of the viruses before and after dialysis against the disassembly buffer.

Normal full CCMV virus particle has an A_{260}/A_{280} ratio of 1.6-1.7. After virus disassembly, the ratio drops to around or below 1.0 for the free coat protein. Therefore, the A_{260}/A_{280} ratio can be an indication for disassembled and full virus particle for CCMV.

For the E34A6 mutant, before dialysis against disassembly buffer, the virion looked like WT under the EM. After dialysis, the ratio dropped from 1.691 to 0.945, which indicated that the E34A virus dissociated into free coat proteins and viral RNA. Therefore, it showed that the E34A mutant reverted to wild type phenotype in disassembly buffer.

E34D2 behaved like the E34A1 mutant that also showed reversion to the wild type phenotype in disassembly buffer, with ratio from 1.69 before dialysis to 0.876 afterwards. These results indicated that E34 and R42 salt bridge is essential for maintaining the salt stable phenotype.

The drastic substitution at E34 to R totally abolished the ability to assemble into full virus particle and move systemically *in vivo*. Therefore, efforts to purify the mutant virus from infected plant tissues along with the wild type control was unsuccessful, even though high levels of protein synthesis were detected in the primary inoculated leaves.

Discussion

The goal of this research was to understand the chemical basis for the salt stable mutant and how enforced protein-protein interactions affect CCMV virion stability and virus disassembly. Here I show that the salt stable mutant is as infectious as the wild type *in vivo*, swells under low salt and high pH conditions; and that an extra salt bridge formation, however, contributes to the distinct phenotype. Swelling occurs at the pseudo-threefold axis opening a 20 Å pore. The chemical basis for swelling is that under high pH, the cluster of negatively charged amino acids (Glu-81, Gln-85 and Glu-148 on one subunit; Gln-149 and Asp-153 on adjacent coat protein subunit) are deprotonated, and the electro-static repulsion among them causes repelling, thus causing swelling at the threefold axis. It was speculated that E34 could form a salt bridge with mutated R42 from an adjacent subunit (Fig. 16).

The second mutation study on E34 showed that this salt bridge is essential in the salt stable mutant for maintaining this salt stable phenotype. Mutation to the same negative charge residue but one carbon shorter on the side chain, and change to a neutral residue both reverse the phenotype to wild type. The E34R mutant does not form virus particles *in vivo*; this might indicate that E34 also interacts with viral RNAs. Change of E34 to a positively charged amino acid abolishes the protein-RNA interaction. Thus, this may totally interrupt protein-RNA interaction in formation of full virus particles, since ELISA result showed that there is coat protein synthesis in host plants.



Fig. 16. Computer modeling at the six-fold axis of the salt stable virus.

Arg 42 is shown in white, Glu 34 is shown in green from an adjacent coat protein subunit. vRNA is shown in blue, yellow and red (adapted from Speir *et al.*, 1995).

The putative extra salt bridge formation between E34 and the mutated R42 indicates that it is the enforced protein-protein interaction that holds the salt stable virus together under high salt and high pH conditions, whereas, wild type virus totally dissociates into its coat protein and free viral RNA components. The structure of the salt stable mutant shows that K42R and E34 from an adjacent coat protein subunit can form the salt bridge and the interactions are enhanced at the threefold axis by a factor of 120 times in virus particles. Therefore, the interaction holds the coat protein altogether under high salt and high pH conditions. Computer modeling with A, E and D residues at position E34 indicated that mutation interrupts not only salt bridge formation, but also protein-RNA interaction in the case of E34R. It will be interesting to study whether the E34R protein can form empty particle *in vitro*. If the E34R coat protein maintains the capability to form empty particles *in vitro*, this might suggest that E34R mutation only interrupt protein-RNA interaction but not protein-protein interaction to form empty particles.

The same salt bridge was studied in tobacco mosaic virus (TMV) (Lu *et al.*, 1996). It showed that an engineered salt bridge in the TMV coat proteins inhibited wild type virus disassembly both *in vivo* and *in vitro*, and caused unnaturally longer virion formation of TMV by inhibition of virus disassembly. Therefore, salt bridges play an important role in virus assembly and disassembly pathways. Taken together, these showed that the salt bridge is essential for maintaining the salt stable phenotype for CCMV.

The salt stable virus is as infectious as the wild type *in vivo*, swells like the wild type virus, but it is very stable under high salt and high pH conditions *in vitro*. This indicates that disassembly might not be caused by way of changing in salt and pH condition *in vivo*. We have proposed a model for CCMV cotranslational disassembly for

which the N-terminus on the pentameric capsomers can form an α -helical channel; and through this channel, the viral RNA gets access to the host translational machinery and decoded.

CHAPTER 3

BIOCHEMICAL ANALYSIS OF THE SALT STABLE MUTANT AND
ITS N-TERMINUS MOBILITYIntroduction

Cowpea chlorotic mottle virus (CCMV) has been a model system for studying the mechanisms of spherical virus assembly and disassembly since the early 1970's (Bancroft *et al.*, 1969, 1971, 1972, 1974, 1975). CCMV is a member of the Bromoviridae family of plant viruses, with a positive sense single stranded RNA genome and T=3 quasi-symmetry structure. For establishing infection, the virus has to deliver its genetic information into the host cells to release its genetic materials for transcription and replication. In the case of CCMV, it was believed that swelling was required for CCMV cotranslational disassembly *in vitro* (Brisco *et al.*, 1986). The cotranslational disassembly model held that through swelling at the pseudo-threefold axis, viral RNA was presented to the host translational machinery. However, the model was called into doubt when a disassembly deficient mutant of CCMV, with a cysteine substitution at R26 (R26C), does not dissociate under high salt and high pH conditions (Bancroft *et al.*, 1967). This mutant still swells like the wild type, but is noninfectious *in vivo* under non-reduced conditions. Therefore, swelling is not correlated with CCMV cotranslational disassembly (Albert *et al.*, 1997). There has been an alternate model for CCMV cotranslational disassembly, in which it was proposed that the N-terminus of the coat protein plays an important role in the viral disassembly by formation of a channel on the five-fold axis of the virus particle.

Through this five-bundled α -helical channel, viral RNAs were able to get accessed to host ribosomes and were translated.

The N-terminus of the CCMV coat protein appears not to be ordered under the x-ray crystallography (first 26 amino acids on the hexameric capsomers, first 44 amino acids on the pentameric capsomers) (Speir *et al.*, 1995). Genetic studies on the N-terminus have shown that the first 8 amino acids are dispensable for virus assembly and infection (Sacher *et al.*, 1989; Zhao *et al.*, 1995), but it may be involved in determining host specificity (Rao *et al.*, 1995). Deletion of the first 25 amino acids eliminates the ability for virus coat proteins to form full RNA-containing particles, but not empty particles *in vitro* (Vriend *et al.*, 1981). Among the first 25 amino acids, the N-terminus contains nine positively charged residues of lysine and arginine. These residues have been shown to interact with viral RNA on the interior of the virus particle (Verdium *et al.*, 1984; Vriend, 1983; Vriend *et al.*, 1986). Recent studies on the N-terminus has shown that deletion of amino acids 3-36 on the N-terminus (N-34) could form empty virus particles *in vitro*, but not full particles (Willits, Young laboratory, unpublished data). Substitution of all the nine positively charged amino acids with negatively charged glutamic acid (Sub-E) maintained the capability for empty particle formation *in vitro* (Young laboratory, unpublished data). This indicates that the interaction of the N-terminus with RNA is important for virion formation. Taken together, this demonstrates that the N-terminus plays an important role in virus assembly and disassembly.

My goal was to study CCMV disassembly. Two basic approaches were taken: (1) a cell free translation assay programmed with intact virus particles, and (2) introduction of a chemically reactive functional group to track the position of the N-terminus of the

coat protein on the virus particle. The goal of developing both of these assays was to obtain direct evidence for the disassembly assay based on the formation of a five bundled helix channel.

Cell free translation assays have been used to study virus disassembly for TMV (tobacco mosaic virus) (Wu *et al.*, 1997; for review, Shaw, 1999); BMV (brome mosaic virus), poliovirus and CCMV (Brisco *et al.*, 1986; Albert *et al.*, 1997). Viral RNAs were translated into their products, and by incorporating ^{35}S -labeled methionine in protein synthesis; protein products can be detected by auto radiography. The goal of this project was to study the correlation of the salt stability of the salt stable mutant and the translation of its RNA by using a cell free translation assay.

There have been many reagents used to detect thiol groups on proteins. Ellman's reagent and a more sensitive thiol-quantitation kit are commonly used ones. An Ellman's reagent (5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB) is reduced by free thiol groups and produces a colorimetric reaction that can be detected at 405 nm. Another thiol and sulfide quantitation kit is an ultra sensitive colorimetric assay for quantitating both protein and non-protein thiols. Briefly, a disulfide-inhibited derivative of papain is reduced by thiols and the active enzyme is stoichiometrically released. The activity of the enzyme is then measured by the chromogenic papain substrate, N-benzoyl-L-arginine, p-nitroanilide (L-BAPNA). Although thiols and inorganic sulfides can be quantitated using the traditional Ellman's reagent (5, 5'-dithiobis-(2-nitrobenzoic acid), DTNB), the amplification of the enzyme step in the thiol-quantitation increases the sensitivity about 100-fold greater than that in Ellman's reagent reaction. To study the mobility of the N-terminus on the mutant viruses, both were applied to detect the free thiol groups.

Material and methods

In vitro Translation of Viral RNA and Intact Viruses

The *in vitro* translation was carried out with a cell free Flexi rabbit reticulocyte system (Promega, Madison, WI). Briefly, this translation system contains all components for translation, such as ribosomes, translation initiation and elongation factors, and ATP as the energy source. Exogenous RNA or RNA-containing virus was added into the system, and RNA was translated into its protein products. With incorporation of ^{35}S -Methionines, the proteins can be visualized by auto radiography.

Before being translated, the virus concentration was quantified by A_{260} with an extinction coefficient of 5.87 (for purified CCMV virus). The purity of the virus was determined before translation by absorbance at 260 nm (A_{260}) and 280 nm (A_{280}), and their ratio (A_{260}/A_{280}) was found to be 1.6-1.7 for the CCMV virion. The viral RNA extraction was performed by treating 200 μg virus in extraction buffer (1% SDS, tris-glycine buffer, pH 7.5, 1 μl proteinase K), and then extracted three times with phenol-chloroform. The RNA was subsequently precipitated with ethanol and dissolved in DEPC-treated double distilled water (diethyl pyrocarbonate treated ddH₂O). RNA was then quantified by the absorbance at 260 nm (A_{260}) with an extinction coefficient of 33 ($E_{1\text{mg/ml}} = 33$). The RNA was also visualized on a denaturing-agarose gel (1.2% agarose / 6.6% formaldehyde) before being translated.

A linearized standard curve for both viral RNA and the virus was constructed according to previous studies in the Young laboratory (Albert, *et al.*, 1996). The translation was performed with a Flexi translation kit as described by the provider (Promega, Madison, WI). For a 12.5 μ l translation mix, it contained 8.25 μ l Flexi, 0.25 μ l RNase inhibitor, 0.25 μ l amino acid mixture minus methionine (1.0 mM), 0.35 μ l KCl (0.5M), 0.5 μ l 35 S-methionine (1,200Ci/mmol, Amersham, Buckinghamshire, England), and 1.4 μ l nuclease-free water. A total of 1.0 μ l of the wild type and the salt stable viruses at 125 μ g/ml were each added to the reactions.

For viral RNA, 1.0 μ l of 25 ng/ μ l extracted RNA was added to the reaction based on the fact that about 20% of the mass of CCMV is made of the viral RNA. To test the effect of the salt stable virion on the translation reaction, a total of 125 μ g of the salt stable virus was added to the wild type RNA and wild type virus reaction. To further test the affect of Mg^{++} , a 4-mM final concentration of $MgCl_2$ solution was adjusted for the translation. The reaction was incubated at 30 $^{\circ}$ C for 90 minutes after which an aliquot of the reaction was mixed with an equal volume of SDS-loading buffer (2% SDS, tris-glycine buffer, pH 7.5, 0.5% β -mercaptoethanol). The samples were then boiled for 10 minutes, and run out on a 12.5% SDS-PAGE gel (Phast gel, Pharmacia Biotech, England). The total 35 S-labeled proteins on the dried gel were exposed to a Phosphor-imager Plate II overnight. The plate was scanned with a molecular dynamic software and quantified with an Image Quantitation program (Molecular Dynamics, Menlo Park, CA.) by the incorporation of the coat protein as well as combined with the movement protein.

Ellman's and Thiol-quantitation of S2C Mutants

Oligonucleotide Site-directed Mutagenesis for S2C Mutants

In order to put a biochemical marker on the N-terminus of the coat protein, the second amino acid, serine, was substituted by a cysteine residue (S2C). Both an Ellman's reagent and a more sensitive thiol-quantitation kit were used for quantifying the cysteine.

The S2C was engineered in both the wild type and the salt stable background, which was designated as S2C-WT and S2C-SS respectively. To eliminate the background for colorimetric reactions, both the C59 and the C108 cysteine residues on the wild type coat protein were subsequently substituted with serine residues using S2C-WT and S2C-SS as templates.

The S2C mutants were generated by oligonucleotide site-directed mutagenesis (Kunkel method, Kunkel, *et al.*, 1987) with primer S2C in both the wild type and the salt stable background as shown in table 3. The primers contain an A to G mutation at position 1364 on pCC3, which is the CCMV cDNA of RNA 3. An engineered *Bsr*GI restriction endonuclease site was introduced into the primers for screening purpose as underlined.

The C59 and C108 cysteine residues were substituted with serines with primers Cys-59 and Cys-108 as shown in Table 1. Engineered restriction endonuclease sites are underlined in primers for screening as shown below:

S2C: 5'-CCCTGTTCCGACTGTACACATGATAAATTACTA-3' (1348-1381, *Bsr* G I)

Cys-59: 5'-GGCAGCCGCGGAAGAGGTCC-3' (1523-1546, *Sac* II)

Cys-108: 5'-GGAGGCTGCAGCAGTAGTCGTCTGCGTCTCTGTAAACAGAGGA

TTTCAC-3' (1681-1726, *Pst* I)

Table 3. Primers for site-directed mutagenesis.

The *in vitro* RNA transcription and plant inoculation were performed as previously described (Chapter 2). ELISA, RT-PCR, sequencing and virus purification were carried out as previously described in Chapter 2.

Once the virus was purified on a CsCl gradient, viral quantitation was determined by absorbance at 260 nm (A_{260}) with an extinction coefficient of 5.87 for purified CCMV virus. The purity of the virus was measured by the ratio of A_{260}/A_{280} (1.6-1.7 for CCMV virus).

Ellman's Reagent Reaction

Ellman's reagent (5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB) was first used for thiol quantitation on viruses and free coat proteins. Briefly, once a free thiol group reduces the Ellman's reagent, the Ellman's reagent produces a colorimetric reaction that can be detected at 405 nm. A series of diluted wild type and the salt stable mutant viruses in a total of 100 μ l buffer were added into the reaction along with free coat protein as a

positive control. The buffer alone was used as a negative control. The absorbance at 405 nm (A_{405}) was measured with a microplate reader at 10 minutes and overnight after incubation (Molecular Devices, Sunnyvale, CA).

Thiol-quantitation of the S2C-C59S-WT and the S2C-C59S-SS mutant and wild type viruses

A Thiol and sulfide quantitation kit (T-6060, Molecular Probes, Eugene, OR) was used for thiol quantitation of viruses. This kit is an ultra sensitive colorimetric assay for quantitating both protein and non-protein thiols. Briefly, a disulfide-inhibited derivative of papain is reduced by thiols and the active enzyme is stoichiometrically released. The activity of the enzyme is then measured by the chromogenic papain substrate, N-benzoyl-L-arginine, p-nitroanilide (L-BAPNA). Although thiols and inorganic sulfides can be quantitated using the traditional Ellman's reagent (5, 5'-dithiobis-(2-nitrobenzoic acid), DTNB), the amplification of the enzyme step in the thiol-quantitation increases the sensitivity about 100-fold greater than that in Ellman's reagent reaction.

The Ellman's reagent is first used for accurately determining the actual thiol concentration of L-cysteine in a standard solution. Furthermore, cystamine, which is a disulfide, undergoes an exchange reaction with poorly accessible thiols on proteins, yielding 2-mercaptothylamine (cysteamine), which can release active papain. Therefore, thiol-quantitation can also detect poorly accessible thiols.

To construct a standard curve for thiol-quantitation, the Ellman's reagent reaction was first used for a series of diluted L-cysteine (Molecular Probes, Eugene, OR). A 100

mM L-cysteine stock solution was made by dissolving cysteine in Buffer A (5 mM NaOAc, 50 mM NaCl, 0.5 mM EDTA, pH 4.7, degassed at ~30 Torr for 30 minutes, then < 1 Torr for another 30 minutes prior to use). The cysteine stock was then diluted in Buffer A at 1:1000 to give a 0.1 mM final concentration. The standard curve for cysteine was constructed by diluting the 0.1 mM stock solution in buffer A to give 0.1, 0.08, 0.06, 0.04, and 0.02 mM solutions. A total of 138 μ l Buffer B (40 mM sodium phosphate buffer, 2 mM EDTA, pH 7.6, degassed as previously described) was added subsequently to each well on a 96-well microplate followed by addition of 80 μ l standard cysteine in buffer A and addition of 2 μ l of Ellman's reagent (100 mM DTNB in dimethyl sulfoxide (DMSO)). The plate was immediately mixed well and subsequently incubated for one to two seconds at room temperature. The absorbance at 405 nm (A_{405}) was then measured by a microplate reader (Molecular Devices, Sunnyvale, CA) blanked with water. The actual thiol concentration value in the reaction was calculated by a formula for the Ellman's reagent as below:

$$\text{mM of thiol} = A_{405} \times 220\mu\text{l} \times 1000 / 13600 \text{ cm}^{-1}\text{M}^{-1} / 80\mu\text{l}$$

where 13600 is the molar extinction coefficient ($\text{cm}^{-1}\text{M}^{-1}$) of the 5-thiol-2-nitrobenzoate generated from the Ellman's reaction with free L-cysteine. The calculated thiol concentration was then used for generating a standard curve in the following papain-based assay. The standard curve was plotted with A_{405} (subtracting control absorbance value minus L-cysteine) against the actual thiol concentrations.

After the standard curve was constructed, viruses were first quantified as previously described in Chapter 2. Each virus sample at 9 mg/ml was diluted at 1:1, 1:2, or 1:3 in Buffer A. The sample was then quantified with the thiol-quantitation kit along

with the standard cysteine calculated above. A total of 100 μ l of 0.6 mg/ml Papain diluted at 1:1 in buffer B (40 mM sodium phosphate buffer, 2 mM EDTA, pH 7.6, degassed as previously described) was then added to each well. A 4 mM solution of cystamine was diluted at 1:10 in buffer A, and 3 μ l of diluted cystamine was subsequently added to each well along with addition of 3 μ l of each sample at different dilutions. The plate was then incubated for one hour at room temperature before 100 μ l of 4.9 mM L-BAPNA solution was added and well mixed with a multi-channel pipit. The plate was then placed at room temperature for one hour before the A_{405} was measured by a microplate reader (Molecular Devices, Sunnyvale, CA) with water as a blank.

The thiol quantitation standard curve was constructed by plotting A_{405} (subtracting background without L-cysteine in the reaction) against the actual thiol concentrations that were calibrated from the Ellman's reagent reaction. The thiol content of each experimental sample was determined according to the standard curve. The actual thiol content of each virus was calculated with two cysteine residues in each protein subunit in the virion, and then the thiol contents of each sample were compared.

Results

In vitro Translation of Viral RNA and Intact Viruses

In vitro translation system is a powerful tool to detect translated proteins because it is very sensitive and can be quantitated. As shown in Fig. 17, there are four protein products from the CCMV viral RNA translation reaction as well as the virus translation:

RNA-dependent-RNA replicase proteins, 1a and 2a proteins (104 kD and 94 kD) for RNA replication; a 32 kD movement protein, and a 20 kD coat protein which is expressed via a subgenomic promoter on RNA3 (Fig. 1, Chapter 1). The intensity of the protein bands corresponds to the methionine content in the protein. The movement protein contains 8 methionines, and there are two methionine residues on the coat protein. It was suggested that viral RNA of both wild type and the salt stable were translated at the same level (Albert *et al*, 1997); however, the salt stable mutant virus showed no bands contrasting with wild type virus with all four protein products (Fig. 17).

By mixing the salt stable virus with the wild type virion in the translation reaction, the integration of the coat protein was 76.25 and 76 with and without the salt stable virus respectively; furthermore, with the movement and the coat protein combined for integration; it showed 123.6 and 124 with and without the addition of the salt stable virus to the translation. For the viral RNA translation, without the salt stable virus in the reaction, the integrations were 118.4 and 225.3 with the labeled coat protein and the coat protein combined with the movement protein respectively; with the salt stable virus, the integrations were 118.2 and 225.3 for both quantification with the coat protein and the coat protein along with the movement protein (Fig. 18). This therefore indicated that there was no effect of the salt stable virus on the translation reaction.

Furthermore by addition of 4 mM $MgCl_2$ solution into the translation mixture did not show any effect on the translation reaction, thus indicating that the salt stable virus, which is at closed form as the wild type virus, in contrast to the wild type virus showed a negative signal in the translation assay.

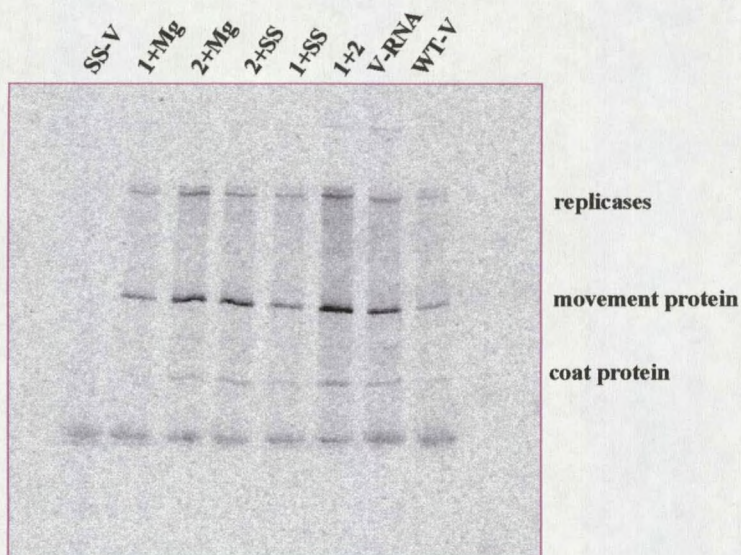


Fig. 17. ^{35}S -labeled SDS-PAGE gel of the *in vitro* translation of viruses and the viral RNA.

WT-V: 125 ng WT virus in 1 ul of virus buffer

V-RNA: 25 ng of WT RNAs in 1 ul DEPC-treated water

1+2: 125 ng of the wild type virus and 25 ng of viral RNAs in the translation

1+SS: 125 ng of WT virus plus 125 ng SS virus

2+Mg: 25 ng WT RNAs plus 2 ul of 4 mM MgCl_2

1+Mg: 125 ng of WT virus plus 2 ul of 4 mM MgCl_2

SS-V: 125 ng of SS virus in virus buffer

The four bands on the gel represent the CCMV RNA-dependent RNA replicases (104, 94 kD), the movement protein (32 kD) and the coat protein (20 kD).

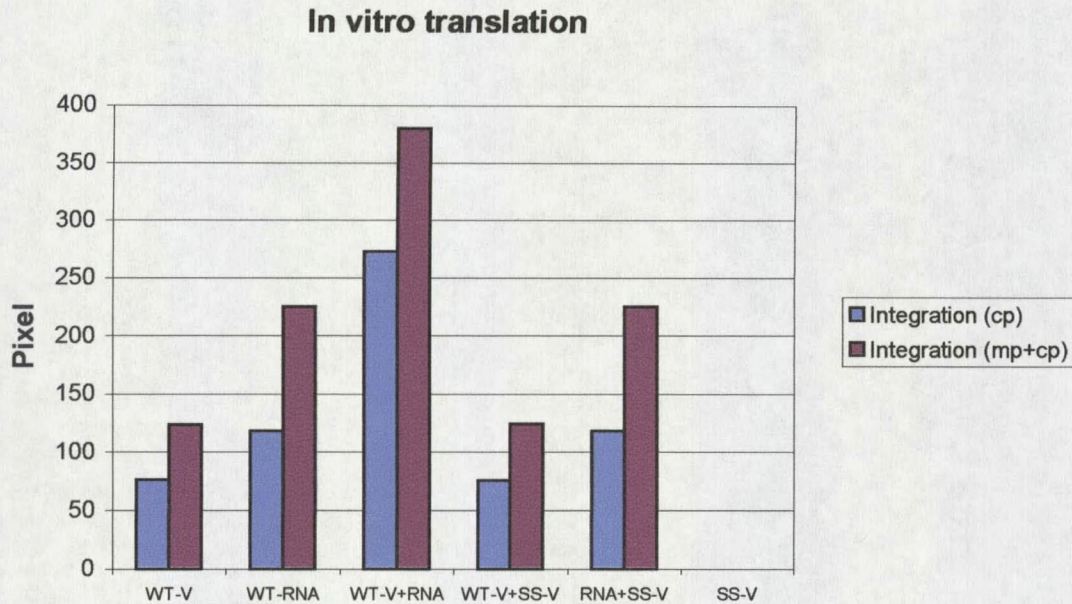


Fig. 18. Quantitation of the labeled coat protein and quantitation of the labeled movement protein plus the coat protein.

The salt stable virus does not get translated into any protein products compared with the wild type virus. The salt stable virus does not affect the translation by mixing the same amount if salt stable virus with wild type virus or wild type RNA.

Ellman's Reagent Reaction

In the Ellman's reagent reaction, the free coat protein from the disassembled virus was used as a positive control and showed a positive signal after 10 minutes of incubation with the Ellman's reagent. The wild type virus showed about 70% of the free coat protein signal after a 10 minutes reaction with the Ellman's reagent. The S2C-SS showed the same background signal as the buffer after 10 minutes of incubation (Fig. 19). With a 24-

hour incubation for the Ellman's reagent reaction as shown in Fig. 20, the wild type virus eventually reached around 95% of the value of the free coat protein; the S2C-SS had the same background signal as the buffer. These results indicate that the wild type virus might dissociate in the solution although the solution was at pH 7.5 with low ionic strength. The salt stable background S2C mutant did not disassemble and, therefore, the thiol groups were not accessible for the colorimetric reaction. It was speculated that the substituted C2 was modified *in vivo*; as a consequence, the free thiol was not available for the reaction.

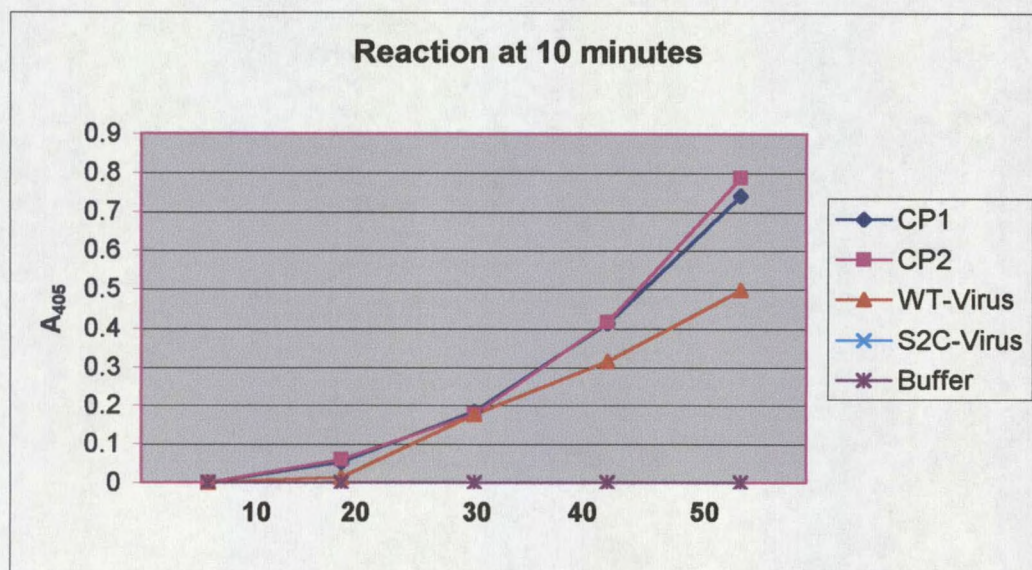


Fig. 19. Ellman's reagent reaction with viruses at 10 minutes

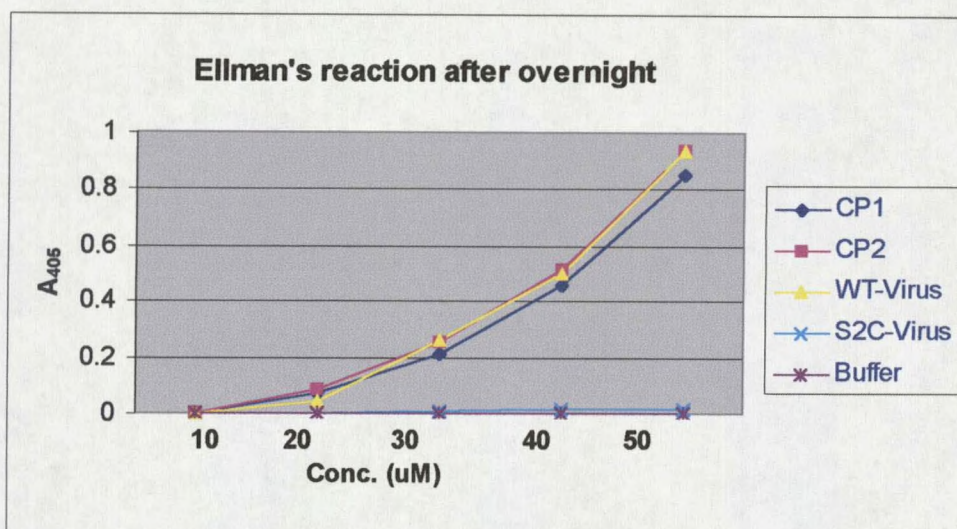


Fig. 20. Ellman's reagent reaction with viruses after overnight incubation.

CP1: wild type coat protein

CP2: duplicated wild type coat protein

WT-Virus: wild type virus

S2C-Virus: S2C mutant at salt stable background with C59S

Buffer: virus buffer

Thiol-quantitation

For the more sensitive thiol-quantitation, the Ellman's reagent reaction was first used for construction of a standard curve with a series of a standard cysteine concentration. The actual thiol concentration calibrated with the Ellman's reagent formula was then used as a standard for consequent thiol-quantitations.

Fig. 21 shows the Ellman's reagent reaction against the standard cysteines, with an R^2 of 0.9999. Subsequently the actual thiol concentration was then plotted against

absorbance at 405 nm in the following thiol quantitation reaction with an R^2 of 0.9788 (Fig. 22).

From mutagenesis and sequencing data, cysteine 108 was not included despite the fact that the engineered restriction endonuclease site was there. This might be because the enzyme site is not at the same position as the mutation. Therefore, the final mutant was S2C-C59S in both the wild type and the salt stable background for the thiol quantitation.

The wild type virus showed colorimetric reaction, with absorbance of 0.667, 0.618, and 0.322 for no dilution, 1:2 and 1:3 dilutions respectively. For the reduced S2C-C59S-SS mutant, it showed values of 0.667, 0.415 and 0.274 at no dilution, 1:2 and 1:3 dilutions. For the non-reduced mutant virus, it showed a higher than background level at no dilution; with a value of 0.281 compared with the salt stable control value of 0.17. However, at 1:2 and 1:3 dilution of the virus, the signals were the same as the background (0.21 and 0.17 for the virus; 0.16 and 0.14 for the salt stable virus control at 1:2 and 1:3 dilutions respectively) (Fig. 23 and 24). The actual thiol concentration in the reaction was about 20% of the calculated thiol content from the virus added to the reaction.

This might be due to modification of the second cysteine although the genetic data from RT-PCR and sequencing both showed that S2C is there. The first methionine on the coat protein is cut off *in vivo* in the wild type virus and the second serine becomes the first residue and is acetylated in the wild type coat protein. Therefore, in the MASS data, the free coat protein is 141 more than the calculated molecular weight. Since in the S2C mutants, cysteine becomes the first amino acid residue, MASS data on S2C mutants

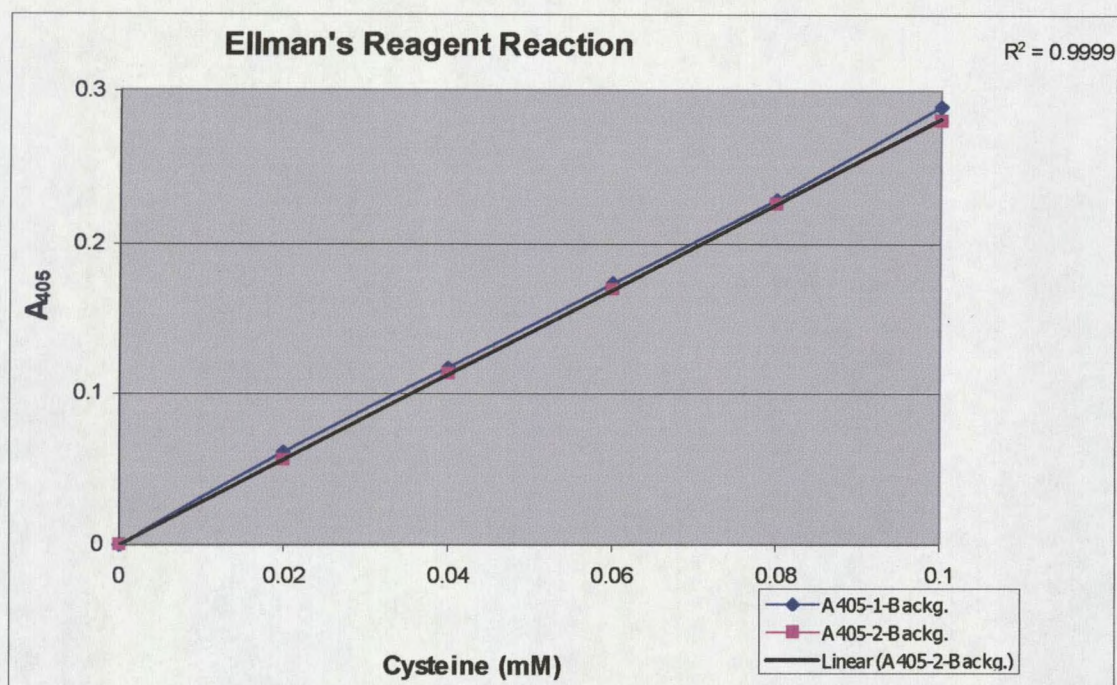


Fig. 21. Ellman's reagent reaction and a standard for Thiol-quantitation.

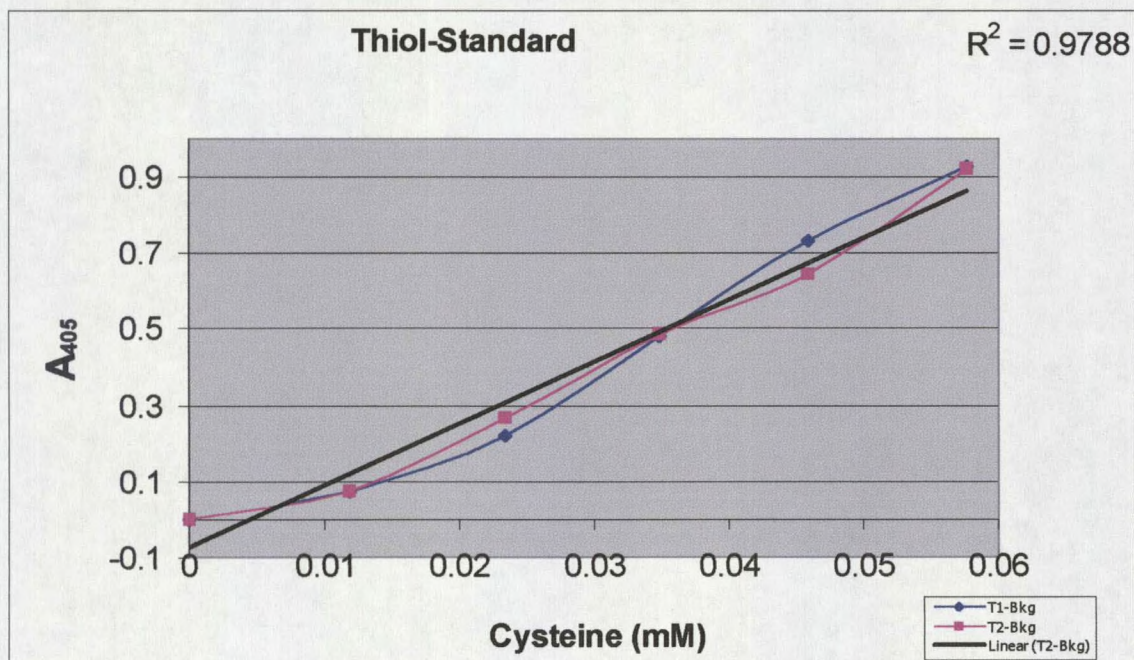


Fig. 22. Standard curve for thiol quantitation.

The actual thiol content was calibrated from Ellman's reagent, with an R^2 of 0.9788.

showed that the second cysteine might be modified somehow *in vivo*. As a consequence, the free sulfhydryl groups are blocked and are not accessible to the thiol-quantitation reaction. The free coat protein and the standard free cysteine work fine as controls for the thiol quantitation as shown in Fig. 24 and 25. Furthermore, the thiol quantitation was carried out in pH 7.6 and low ionic strength conditions as in the *in vitro* translation system, therefore, the virion might be in the swollen form. It was also speculated that the wild type virus might dissociate in the solution.

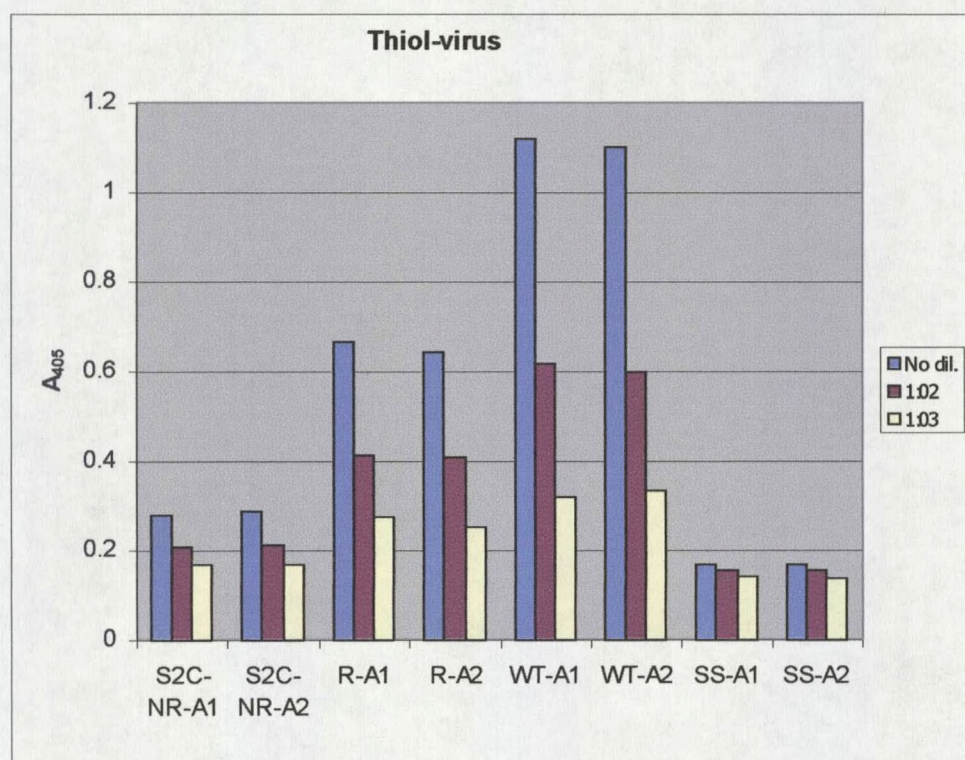


Fig. 23. Thiol-quantitation on viruses.

S2C-NR: S2C-C59S-WT non-reduced virus S2C-R: S2C-C59S-WT reduced virus
 WT: wild type virus SS: salt stable virus

After reducing with DTT, S2C-C59S-WT showed a higher reaction absorbance than non-reduced virus, but it is lower than that of wild type virus in the reaction, although they both contain two cysteines in their coat protein. The salt stable virus was negative as was the S2C-C59S-WT non-reduced virus. Viruses were at 9 mg/ml concentration, and were diluted in 1:2 and 1:3 in virus buffer for colorimetric reaction.

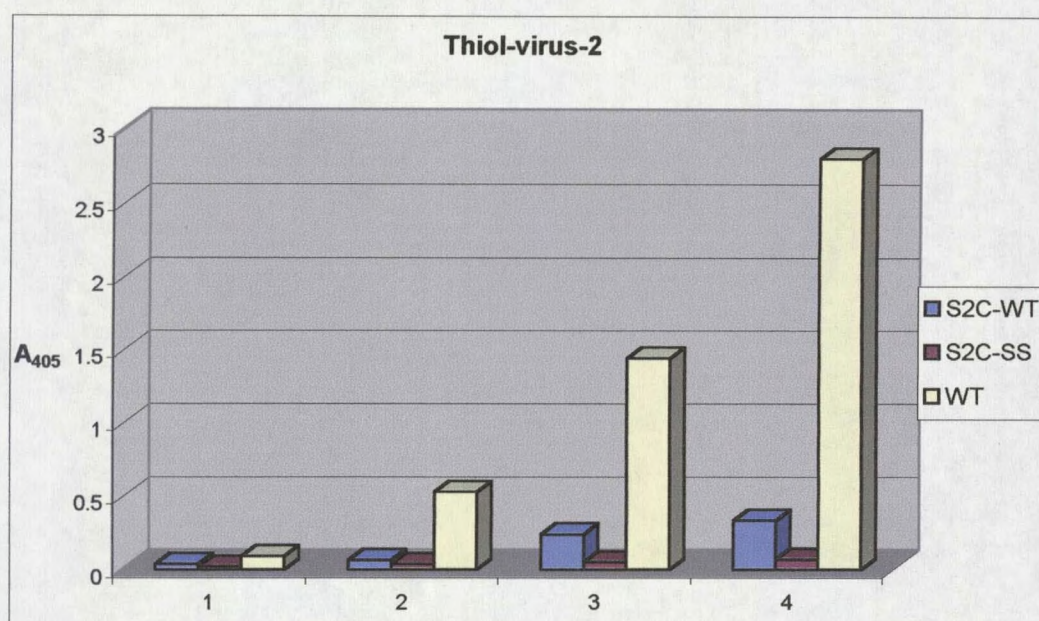


Fig. 24. Thiol-quantitation on viruses.

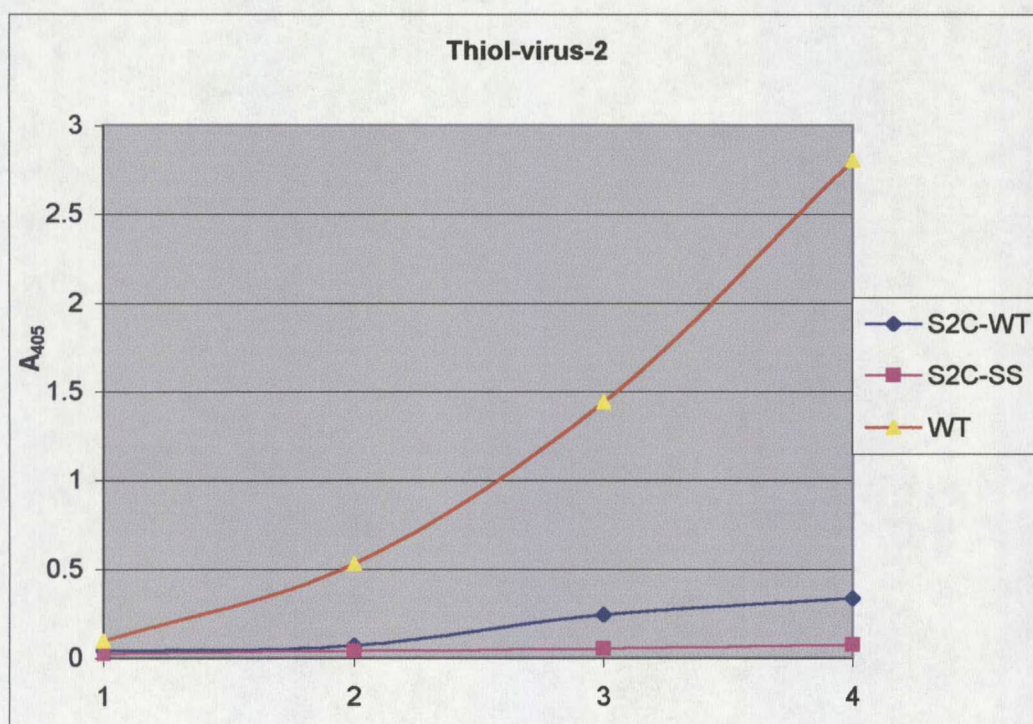


Fig. 25. Thiol-quantitation on viruses.

Thiol quantitation of the wild type, the S2C-C59S-WT and the S2C-C59S-SS viruses.

1: 1:10 dilution

2: 1:5 dilution

3: 1:2 dilution

4: no dilution of virus at 9 mg/ml.

Discussion

The cell free translation assays have been used for tracking virus disassembly, especially for studying TMV disassembly mechanisms. It is very powerful and can be quantified. The Flexi system contains HEPES buffer at pH 7.6 in a low salt condition (Promega, Madison, WI). It was believed that the virus swells under such conditions. However, the result indicates that swelling is not required for *in vitro* translation for CCMV since the salt stable showed no signal in the Flexi system. It was therefore speculated that *in vitro* translation measures viral disintegration; as a consequence, viral RNAs get access to the translational machinery. Although previous data in the lab showed that salt stable virus could be translated at the same level as the wild type (Albert *et al*, 1997), which might be due to the fact that the salt stable mutant used in the experiment was mixed up with the wild type virus, and therefore, the data were not reliable. The results indicated that the *in vitro* translation system does not mimic the *in vivo* conditions for CCMV cotranslational disassembly despite that fact that the same system has been used for the TMV cotranslational disassembly (Lu *et al.*, 1995).

The thiol-quantitation results showed no colorimetric reaction with the salt stable virus or the salt stable background S2C-C59S virus. This might be due to modification of the second cysteine although the genetic data from RT-PCR and sequencing both showed that S2C is there. MASS spectrometry data on S2C mutants showed that there is a molecular weight change of the coat protein, which indicated that the second cysteine might be modified somehow *in vivo*.

More supportive data came from collaboration with the Scripps Research Institute (Qu, unpublished data) with studies of the surface accessibility of virus particles by limited proteolysis with matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MASS). MALDI is a very powerful and sensitive method, it can tell a mass difference of one hydrogen. Briefly, wild type and the salt stable virions were dialyzed against swollen and compact buffer (as described in Chapter 2), then were digested with trypsin in a time course and put on the MALDI-MASS at different time point. The results showed that even after 24 hours digestion with trypsin, the salt stable mutant would not give small pieces of digested peptides in both forms. On the contrary, for wild type virus digestion, the N-termini were first products after 10 minutes of incubation with trypsin, although it seemed that in the compact form, more N-terminus was digested than in the swollen form. These results indicated that the N-terminus was dynamic in the wild type virus and transiently exposed on the virion (Qu, unpublished data, Fig. 26 and 27). These data all agreed with the translation data and showed that salt stable virus did not expose thiols on the virion, and did not get digested by trypsin. This suggested that the N-terminus of the salt stable was orientated inside of the virion instead of exposed as that in wild type virus.

For further studies on the thiol quantitation, it might be more useful to substitute the third or the fourth amino acid with cysteine residue, or engineer a series of cysteine residues on the N-terminus of the coat protein to get more signals for the thiol quantitation, therefore, making it easier to detect the thiols on the N-terminus of the coat

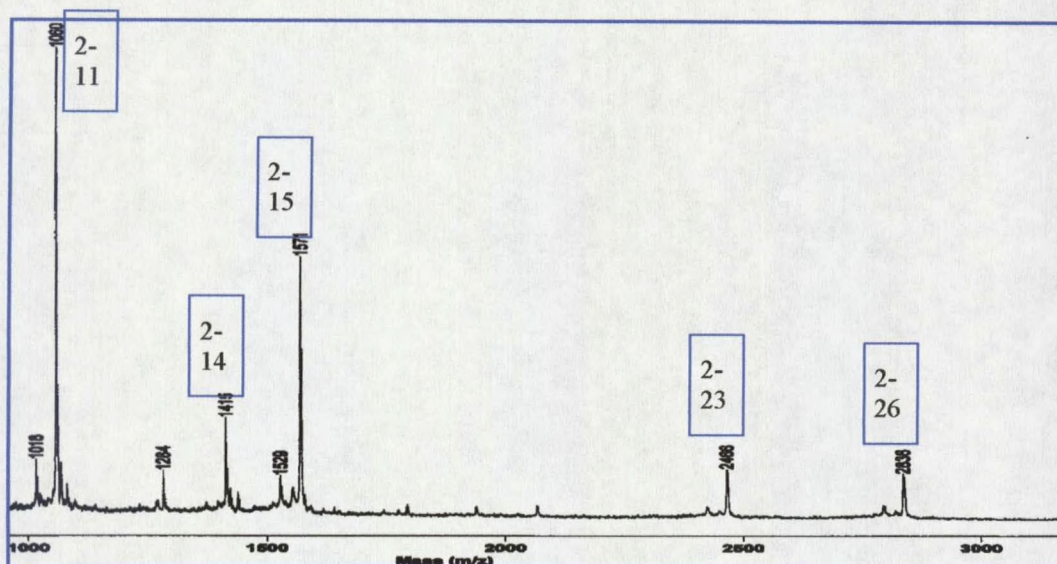


Fig. 26. MALDI-MASS spectrometry of wild type virus with trypsin digestion at 10 minutes.

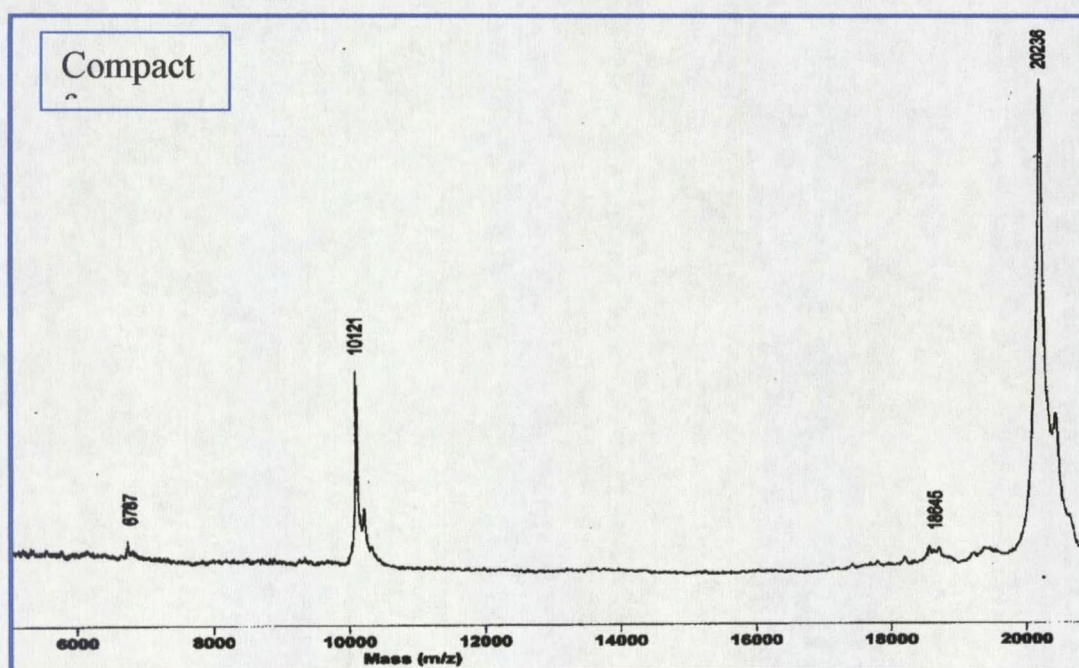


Fig. 27. MALDI-MASS spectrometry of the salt stable virus after 24 hours of incubation with trypsin.

protein. It also might be helpful to screen for disassembly deficient mutants by local lesion and protoplast analysis. In this way, it will provide more information for understanding the mechanism of CCMV disassembly.

The other factor for disassembly would be host components. Screening for interaction between the salt stable virus and the host components, then put the purified material into the *in vitro* translation system to monitor for protein products from the salt stable mutant. By doing this, it would shed some light on the mechanism of virus disassembly. There have been reports on isolating host factors for RNA-specific RNA replication for BMV, and the same techniques might be applied for isolating host components for CCMV disassembly.

CHAPTER 4

IMMUNOLOGICAL ANALYSIS OF THE CCMV N-TERMINUS AND VIRUS
DYNAMICSIntroduction

The paradox of virus disassembly is that the virion must be stable enough to protect its genetic information located inside the virus particle, while at the same time, it has to be dynamic enough to release its genetic material into host cells to establish infection. It was believed that swelling is required for CCMV cotranslational disassembly *in vitro* (Brisco *et al.*, 1986). In this model, the swelling of the virion at the pseudo-threefold axis allowed viral RNAs to be presented to the host translational machinery. However, an alternative model was proposed in the Young laboratory. In this model, it was proposed that the five N-termini of the five coat protein subunits in the pentameric capsomer undergo a structural transition to form a five-bundled α -helical channel, through which the viral RNA gains access to the host translational machinery (Albert *et al.*, 1997). This model suggests that there is a dynamic structural transition of the N-terminus of the coat protein from the inside to the outside of the viruses. Here I present evidence for this model using immunological methods.

The experimental approach taken was to generate monoclonal antibodies to CCMV or polyclonal antibodies to the N-terminus of the coat protein, which can either only detect the whole virion or only detect the free coat protein or the N-terminus.

Monoclonal antibodies are powerful tools for mapping the antigen-antibody interaction, preventing diseases *in vivo*, and labeling the antigen-antibody complex.

The CCMV virion has T=3 quasi-symmetric structure; the N-terminus of the CCMV coat protein is not ordered by X-ray crystallography. The first 26 amino acids are invisible on the hexameric capsomer and the first 51 residues are not ordered on the pentameric capsomer (Speir *et al.*, 1995).

Studies have shown that monoclonal antibody against the N-terminus bound on the exterior on the virion, which indicated that the N-terminus of the coat protein was exposed to the exterior of the virus particle. It was proposed that the N-terminus of CCMV coat protein could form a α -helix channel upon interaction with RNA (Van der Spoel *et al.*, 1996). Therefore, it is predicted that the N-terminus is dynamic and interacts with viral RNA inside the virion. To study the dynamic nature of CCMV and to track down the position of the N-terminus on the virion, I wanted to generate N-terminus specific polyclonal antibodies with a synthetic N-terminus peptide.

Material and Methods

Generation and Characterization of Monoclonal Antibodies of CCMV

Immunization of Mice

Balb/c mice were immunized with 50 μ g of either a mixture of wild type and the salt stable viruses or free coat proteins, and were boosted two months later with 50 μ g of the same antigens. The initial immunization was given subcutaneously in Freund's

complete adjuvant (FCA). The final boosts were performed intravenously in saline solution.

Cell Fusion

Three days after the boost, the mouse was sacrificed and the spleen was removed. Subsequently SP2/0 myeloma cells (ATCC) and ground spleen cells from the mouse were spun at 1000 rpm for 10 minutes, and then washed twice with serum free RPMI 1640 (Gibco, Gathersburg, MD), and then were resuspended in 5 ml of RPMI media. Cells were counted using a hemocytometer (Improved Neubauer-Clay Adams) with 4% trypan blue stain (Gibco Laboratories, Grand Island, NY).

A total of 2×10^8 spleen cells (in 5 ml) were added to 6×10^6 SP2/0 (in 5 ml). Cells were then spun at 1000 rpm for 10 minutes. The combined cell pellet was then resuspended and agitated one minute at 37 °C in 0.8 ml of 50% PEG (polyethylene glycol) /10% DMSO (dimethyl sulfoxide) (Pontecorvo, 1976). Subsequently, a total of 25 ml of serum free RPMI media was added slowly over a six-minute period to dilute off the PEG. The cells were then spun at 1000 rpm for 5 minutes, and were gently resuspended in 50 ml of HAT media (5×10^{-3} M hypoxanthine, 2×10^{-5} aminopterin, and 8×10^{-4} M thymidine hydrated in RPMI 1640. Littlefield, 1964; Ephrussi *et al.*, 1969). A total of two 24-well plates were prepared, and 1-ml total volume of the resuspended cell was put into each well. The cells were maintained at 37 °C in a CO₂ incubator.

Creation of Hybridomas

Neither of the parent cells can survive under the selection of the HAT media, but the properly fused ones can. The fused cells, which were potentially immortal and antibody producing, were maintained in complete RPMI 1640 containing 10% fetal calf serum (FCS) (Gibco Laboratories, Grand Island, NY).

Selection of Monoclonal Antibodies

Supernatants of hybridoma cells were tested 10 to 14 days after fusion to determine if any of the cells were secreting anti-CCMV or anti-coat protein antibodies. An ELISA (enzyme-linked immunosorbent assay) was used to measure the reactivity of the antibodies to intact wild type and the salt stable viruses, or free coat proteins. Microplates (Corning Incorporation, Corning, NY) were coated with 100 μ l of 10 ng/ μ l viruses or the coat protein, incubated at 4 °C overnight. The plates were then blocked with 100 μ l of 5% BSA dissolved in PBS buffer (8.0 g/L NaCl, 0.2 g/L KH_2PO_4 , 2.9 g/L $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 0.2 g/L KCL, brought to 1 liter with dH_2O , pH 7.4; 5% bovine serum albumin) overnight at 4 °C. Then 100 μ l of the supernatant was added to each well and incubated at 37 °C for 2 hours or 4°C overnight. The plates were then washed and incubated with a goat-anti-mouse-IgG (H+L) antibody conjugated with alkaline phosphatase (Zymed, San Francisco, CA) for 2 hours at 37 °C, followed by washing and addition of a colorimetric substrate, *p*-nitro phenyl phosphate tablets (Sigma, St. Louis, MO) dissolved in the substrate buffer (9.7% diethanolamine, 1 mM sodium azide, pH 9.8). The A_{405} (absorbance at 405 nm) was determined using a microplate reader (EL-320). The negative control contained all reagents except for the tested antibody. For

identification of a potentially specific antibody for CCMV virus or for the free coat protein, the wells, which showed the highest or the lowest ratios of the ELISA value on either the virus or the coat protein-coated plates were selected.

Limiting Dilution Cloning

Immediately following the cell fusion, once positive cells were identified, cells in the well were subsequently cloned by limiting dilution (Nowinski *et al.*, 1979) at 10 cells/ml, 3 cells/ml, or 1 cell/ml. The diluted cells were placed in 96-well microplates at 0.2 ml/well in order to establish a single cell clone releasing monoclonal antibody. Single clones were screened by ELISA for anti-virus or anti-coat protein antibody production. The positive cells were expanded to 2 ml in complete RPMI, allowed to grow, and then tested for cross-reactivity using ELISA assays against the antigens.

If positive cells came from plates with more than 10 wells having cell growth, the cells could not be guaranteed to be cloned. These cells were recloned at 10, 3, and 1 cell(s)/ml in complete RPMI. And the positive cells were expanded in 2 ml RPMI and tested by ELISA against antigens again.

Freezing Procedure

To preserve the cell lines, all cells were frozen at -70°C until positive clones were found. To freeze cell lines, a total of 10 ml of cell culture was placed in a centrifuge tube and spun at 1000 rpm for 5 minutes. The supernatant was then removed and 1 ml of the freezing media (45% FCS, 45% RPMI, and 10% DMSO (dimethyl sulfoxide) was added.

A total of six aliquots of each cell line were frozen at -70°C for three days and then moved to liquid nitrogen for long-term storage.

To thaw out the cell lines, cells were placed in a 37°C water bath until thawed, and then were spun at 1000 rpm for 5 minutes. The supernatant solution was removed and the cells were resuspended with 2 ml of complete RPMI. Cells were then grown in 2 ml-well plates at 37°C in a CO_2 incubator.

Testing of Ig class

The class of the monoclonal antibody was determined using ELISA assays with anti-mouse-IgG or anti-mouse-IgM antibodies. The virus and the coat proteins were diluted in the coating buffer (8.0 g/L Na_2CO_3 , 2.93 g/L NaHCO_3 , 0.2 g/l NaN_3 , brought to 1 liter with dH_2O , pH 9.6) in 96-well microplates. A total of 100 μl supernatant was added to each well and incubated at 4°C overnight. Plates were washed and subsequently incubated with an alkaline phosphatase (AP)-linked goat-anti-mouse IgG; or an AP-linked goat-anti-mouse IgM antibody (Zymed, San Francisco, CA) for 2 hours at 37°C , followed by washing and addition of a colorimetric substrate dissolved into the substrate buffer (9.7% diethanolamine, 1.0 mM sodium azide, pH 9.8). The A_{405} (absorbance at 405 nm) was determined using a microplate reader (EL-320).

Purification of monoclonal antibodies

Selected single cloned positive cell lines were expanded to 100 ml in cell culture flasks and allowed to grow for 7 to 10 days in a CO_2 incubator at 37°C . The antibody from

a total of 1 liter of the supernatant was collected for each clone. For IgG antibody, supernatant was passed through a Protein-G affinity column (Pierce, Rockford, IL) at 4 °C. The column was washed with 200 ml PBS buffer before elution with 0.2 M glycine-HCl, pH 2.5. The eluted antibody was then neutralized with 1/5 volume of 1 M Tris-buffer (pH 8.0) while the A_{280} (absorbance at 280 nm for protein) was monitored with a HPLC fractionator system (Bio-Rad Laboratories, Hercules, CA). Subsequently, the neutralized antibody was dialyzed three times against a total of two liters of PBS buffer.

Purification of the IgM antibody was performed by precipitation with ammonium sulfate in the supernatant. A final of 40% (w/v) ammonium sulfate was added to the supernatant and then stirred slowly at 4 °C over night. The antibody was then precipitated by centrifugation at 8000 rpm for 30 minutes in a GSA rotor, and the pellet was resuspended in a total of 15 ml sterile PBS buffer.

Finally, the antibodies were concentrated with CentriconTM 100 to a minimum volume. The concentration of the antibodies was determined with a BCA protein assay kit (Pierce, Rockford, IL). Antibodies were stored at -20 °C in PBS buffer containing 1.0 mM sodium azide.

Characterization of Monoclonal Antibodies

Monoclonal antibodies were characterized by ELISA assays described as above.

Generation and Characterization of Polyclonal Antibodies Directed Against N-terminal 24

Amino Acids of the CCMV Coat Protein

Synthesis of N-terminal Peptide

The N-terminal 24 amino acids with an addition of a cysteine residue at C-terminus (NH₂-MSTVGTGKLTRAQRRRAARKNKRNC-COOH) of the CCMV coat protein was commercially synthesized (United-Biochemical Research, Inc., Seattle, WA) at >70% HPLC purity.

Conjugation of the Synthetic Peptide with mKLH

A total of 0.5 mg of the peptide was dissolved in 10 µl of DMSO (dimethyl sulfoxide), and mixed immediately with 50 µl of reconstituted (activated in double distilled water) mKLH (~0.5 mg, mariculture KLH, Pierce, Rockford, IL). The mixture was subsequently incubated at room temperature for two hours to allow the reaction to proceed. The reaction volume was then brought up to 1.0 ml with sterile PBS buffer after incubation, and was refrigerated overnight at 4 °C to allow the conjugation reaction to reach equilibrium.

The conjugated sample was concentrated with a Centricon™ 100 (30,000 Dalton molecular weight cut off (MWCO)) to 100 µl by centrifuging at 4000 rpm for 15 minutes three times in a desktop centrifuge in order to remove the unconjugated free peptide. The conjugated peptide was diluted to 500 µl with sterile PBS, and was then tested for protein concentrations with a BCA protein assay kit (Pierce, Rockford, IL) before immunization.

Immunization of Rabbits

The conjugated KLH-peptide was mixed with an equal volume of Freund's complete adjuvant (Pierce, Rockford, IL) by sonication on ice four times, with a time period of 30 seconds each time. The sonicated mixture was then placed into a 2.0 cc Leur-Lock glass syringe for immunization. The initial injection was performed with 100 µg (200 µl of emulsion) of the immunogen into six to ten subcutaneous sites on the rabbit's back by staff at the Animal Resource Center, MSU. The first booster was at day 28 with 50 µg sonicated KLH-peptide in the incomplete Freund's adjuvant. After 35-42 days, the first serum was collected; and then the second bleeding was performed and serum was collected three weeks after the first one.

Serum Testing

The serum from each rabbit was tested with a pre-immune bleed by an ELISA against the N-terminal peptide and CCMV virus particles for antibody production. The plates were coated with 100 µl of 10 µg/ml antigens in coating buffer (8.0 g/L Na₂CO₃, 2.93 g/L NaHCO₃, 0.2 g/l NaN₃, brought to 1 liter with dH₂O, pH 9.6); first with the synthetic peptide, then with CCMV wild type, the salt stable, S2C-SS and N-34 viruses (lacking the 3-37 residues at the N-terminus of the coat protein). The serum was diluted 1:300, 1:1000 and 1:3000 with PBS buffer, and then 100 µl of each diluted serum was put to each well. The plates were incubated at 4 °C overnight before being washed three times with PBS-Tween, followed by addition of 100 µl of an alkaline phosphatase (AP)-

linked goat-anti-rabbit secondary antibody diluted at 1:2000 with PBS to the wells. The plates were then incubated at 37 °C for 2 hours and washed three times with PBS-Tween. The substrate *p*-nitro phenol phosphate substrate (Sigma, St.Louis, MO) dissolved in substrate buffer (9.7% diethanolamine, 1.0 mM sodium azide, pH 9.8) was then added to each well and the colorimetric reaction was allowed to develop for 10-30 minutes. The A_{405} was determined with a microplate reader (Molecular Dynamics, Valencia, CA).

Purification of the Polyclonal Antibody

Once the ELISA results showed positive reactions, a total of 70 ml of serum was collected after the second bleed from rabbit #4756, and the IgG antibody was then purified by an affinity column. The serum was diluted at 1:2 with a sterile PBS buffer before it was passed through a protein-G affinity column (Pierce, Rockford, IL) for IgG binding. The bound antibody was then eluted with 0.2 M glycine-HCl (pH 2.5) while the A_{280} was monitored with a HPLC fractionator (Bio-Rad Laboratories, Hercules, CA). The eluted antibody was subsequently neutralized with 1/5 volume of 1.0 M Tris-buffer (pH 8.0). The collected antibody was dialyzed three times against a 2 liters of sterile PBS buffer before it was tested for specificity and cross-reactivity on ELISA, dot-blot and Western-blot with CCMV viruses and free coat proteins.

ELISA Analysis using N-terminal Specific Polyclonal Serum

ELISA was performed with purified IgG as described above. The antibody was diluted 1:1000 in the coating buffer (8.0 g/L Na_2CO_3 , 2.93 g/L NaHCO_3 , 0.2 g/l NaN_3 ,

brought to 1 liter with dH₂O, pH 9.6). A total of 100 µg wild type and the salt stable viruses in 100 µl were used to coat the plates. A N-34 mutant was used as a negative control since it is a mutant with deletion of amino acids 3-37 at the N-terminus of the coat protein. An alkaline phosphatase (AP)-linked CCMV polyclonal antibody was then used as the secondary antibody at a 1:2000 dilution with PBS. The plates were washed with PBS-Tween three times before 100 µl of 1 mg/ml substrate of *p*-nitro phenol phosphate substrate (Sigma, St.Louis, MO) in the substrate buffer (9.7 % diethanolamine, 1 mM sodium azide, pH 9.8) was added to each well. The colorimetric reaction was then allowed to proceed for 30 minutes, and the A₄₀₅ was recorded with a microplate reader (Molecular Dynamics, Valencia, CA)

Dot-blot and Western-Blot Assays using N-terminal Specific Polyclonal Serum

For dot-blots, total of 1, 0.2, 0.04 and 0.008 ng viruses or the coat protein were first spotted in 2 µl volume on a piece of nitro cellulose membrane. The viruses were the wild type, the salt stable, the N-34 mutant and the Sub-E mutant (Sub-E mutant contains substitutions of all nine positively charged amino acid residues to E in the N-terminal 25 amino acids); and the free coat proteins were from disassembled wild type and the N-34 viruses. The membrane was then blocked with 5% BSA (bovine serum albumin) in PBS buffer before the antibody at 1:5000 dilution was added to 10 ml PBS. The membrane was subsequently incubated at room temperature for 2 hours, washed with PBS-Tween (PBS containing 0.5% Tween-20) three times before a horseradish peroxidase (HRP)-linked goat-anti-rabbit IgG secondary antibody was added at a 1:2000 dilution in the

PBS-Tween. The plates were then incubated at room temperature for 2 hours and washed with PBS-Tween three times before an Opti-4CN colorimetric detection kit (Bio-Rad Laboratories, Hercules, CA) was used for detection. The colorimetric reaction was allowed to proceed for 30 minutes. Finally a total of 10 ml double distilled water was added to stop the reaction.

A Western-blot was performed to assay IgG reactivity against denatured coat proteins of the wild type virus, the cysteinyl virus (a substitution at R26 on the coat protein, Fox *et al.*, 1997), the N-34 mutant and the KLH-conjugated peptide. A total of 0.5 μ l at 0.6 mg/ml of each protein boiled in the SDS-loading buffer (tris-glycine buffer, pH 7.5) was loaded onto a Phast gel system (PhastSystem, Pharmacia Biotech, Uppsala, Sweden), and then transferred to a piece of nitro cellulose membrane according to the transfer program as described by the Phast gel transferring system.

The membrane was first blocked with 10 ml 5% BSA in the PBS buffer, and then washed three times with PBS-Tween. The membrane was incubated with the N-terminal polyclonal antibody diluted at 1:5000 in a 10 ml PBS-Tween for 2 hours at room temperature. A secondary horseradish peroxidase (HRP)-linked goat-anti-rabbit IgG antibody diluted at 1:2000 was used for enhanced chemiluminescent detection (Amersham, Buckinghamshire, England). A piece of X-ray film was used to detect chemiluminescence with 10-30 minutes of exposure.

Results

Monoclonal Antibody Generation, Purification and Characterization

ELISA Assay of the Mouse Serum

The serum from each mouse showed positive reactions in ELISA assays (Fig. 28 and Fig. 29). Mouse #1 showed a lower ELISA value of 0.4 compared with the other three with higher than 1.6 ELISA values on the virus coated plate. All the ELISA values on the coat protein coated plates were higher than 2.9, which indicated that the reaction was saturated. These results suggest that antibodies cross react with both intact virus and the free coat protein. And mouse #1 might be secreting coat protein specific antibodies as shown with the ELISA value on the virus coated plate.

To screen for virus or coat protein specific antibodies, wells with the highest ELISA value on either virus coated or the coat protein coated plate were selected. However, after limiting dilution and antibody production, the cells showed the same cross-reactivity level for both antigens.

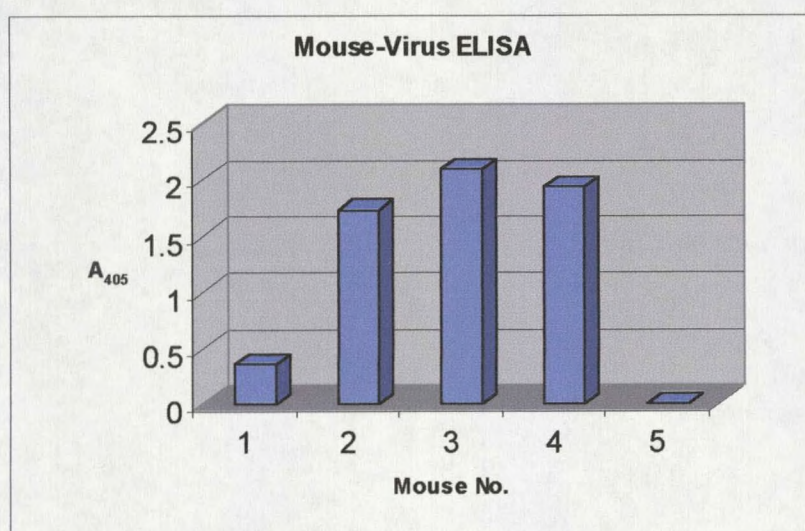


Fig. 28. ELISA with CCMV wild type virus

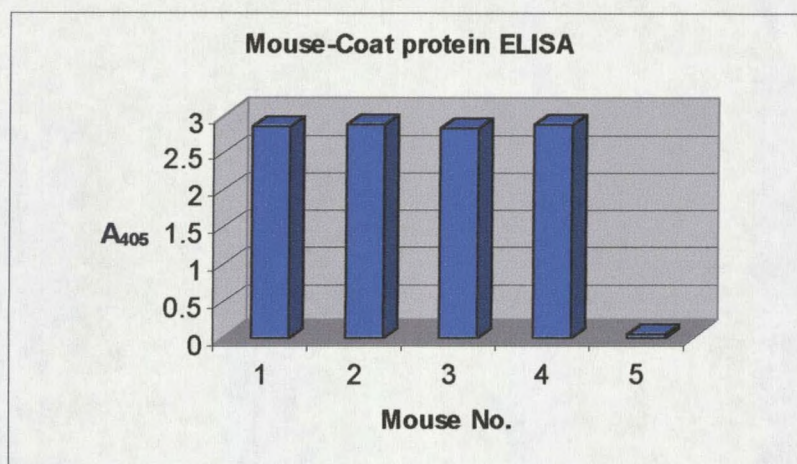


Fig. 29. ELISA with the free coat protein

ELISA results of mouse serum on virus and free coat protein coated plates. 100 μ l of 10 μ g/ml of virus or coat protein was used to coat plates in coating buffer. Mouse serum was diluted at 1:300, 1:1000 and 1:3000 in PBS. A secondary AP-linked rabbit-anti-mouse antibody was used for detection.

Mouse 1 and 2: mice immunized with the coat protein

Mouse 3 and 4: mice immunized with the whole virions containing the wild type and the salt stable viruses

Mouse 5: pre-immune bleed

Antibody Classification

The mouse 2 was selected for hybridoma production. After the fusion, screening procedure and the limiting dilution, among all positive clones, #1, #18 and #20 (labeled by position on the 96-well microplates) were selected for cell line expansion and antibody purification. The antibodies were subsequently tested for Ig class. As shown in Fig. 30, #1 and #20 showed average ELISA values of 0.89 and 0.91 with the anti-IgG antibody; with the anti-IgM antibody, both showed background signals (0.07 and 0.02 for #1 and #20 respectively). In contrast, #18 showed an average ELISA value of 1.27 with anti-IgM antibody reaction; with anti-IgG antibody reaction, it showed an ELISA value

of 0.13 as the background signal. These results indicate that antibodies #1 and #20 are IgG class antibodies, while #18 is an IgM class antibody.

A BCA protein assay kit was used to determine the concentration of the antibody with a serial diluted BSA as a standard. The concentrations were 2.3 mg/ml, 2 mg/ml

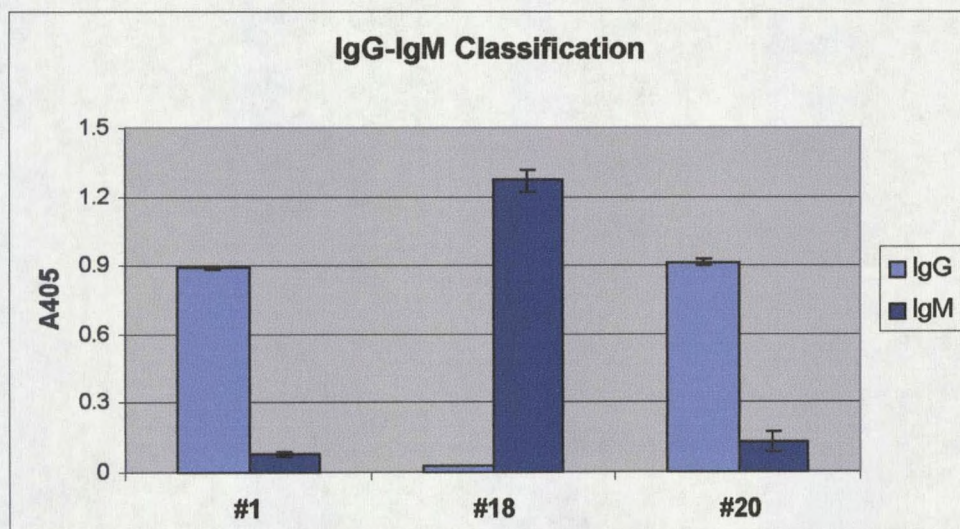


Fig. 30. ELISA with goat-anti-mouse IgG or IgM antibody. Purple and blue bars represent anti-IgG and anti-IgM antibodies as secondary antibodies.

and 1 mg/ml for antibody #1, #20 and #18 respectively. Antibodies were then stored at -20°C in PBS containing 1.0 mM sodium azide.

ELISA of Purified Monoclonal Antibodies

Antibodies were tested for reactivity with wild type virus and coat protein by ELISA assays. Monoclonal antibodies could not distinguish between virion and free coat.

Generation and Characterization of Polyclonal Antibody against the N-terminus of CCMV Coat Protein

After the conjugation reaction and concentration, the conjugated KLH-peptide resulted at a final concentration of 0.6 mg/ml. This is slightly higher than the theoretical 0.5 mg/ml yield, which might be due to the efficacy of the conjugation reaction.

Test of Serum with ELISA Assays

The serum from each rabbit was tested by an ELISA assay for cross-reactivity with the synthetic peptide. The serum of rabbit #4756 showed positive ELISA values with an average of 0.67, 0.66 and 0.43 at 1:300, 1:1000 and 1:3000 dilutions respectively. The pre-immune bleed control showed background ELISA values of 0.03 (Fig. 31). The serum from rabbit #4757 showed 0.75, 0.74 and 0.56 ELISA values with dilutions at 1:300, 1:1000 and 1:3000 respectively. The negative control with BSA showed background ELISA values of 0.03 (Fig. 32).

ELISA of rabbit #4756 serum demonstrated positive cross reactivity with wild type, the salt stable virus and the S2C mutant (S2C-SS mutant, Chapter 3) with average values of 0.68 and 0.43 at 1:1000 and 1:3000 dilutions respectively. With the N-34 virion, a mutant lacking the N-terminal 34 residues, it showed a background ELISA value of 0.008. From the negative control, the ELISA value was 0.005 (Fig. 33), thus indicating that the polyclonal serum is N-terminus specific.

The ELISA was saturated at 1:300 dilution of the serum, which did not show difference between dilutions at 1:300 and 1:1000 with PBS buffer.

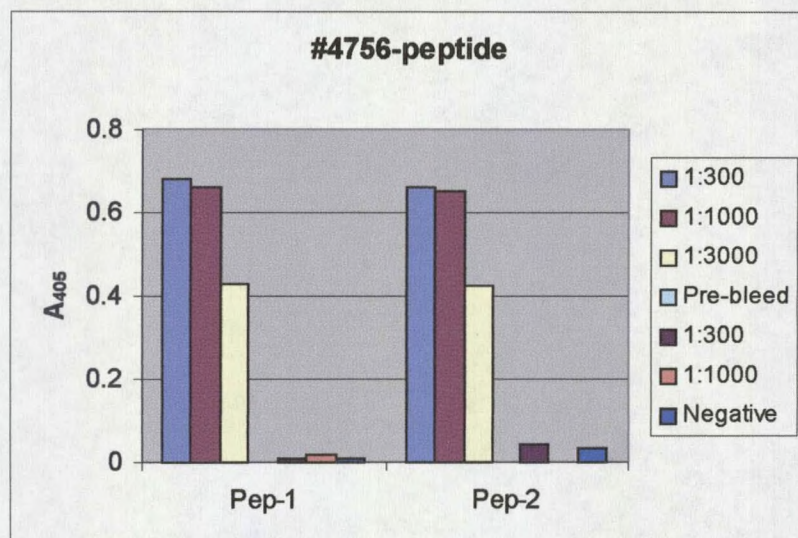


Fig. 31. ELISA of rabbit serum #4756 with the N-terminal peptide

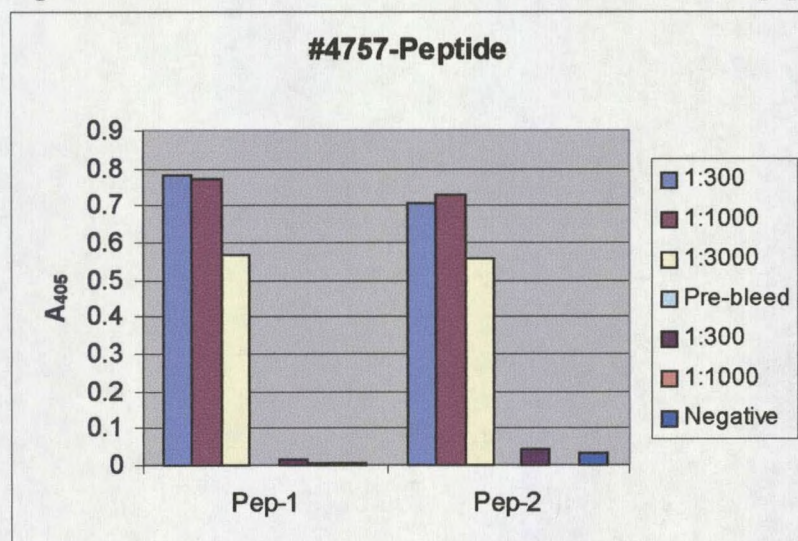


Fig. 32. ELISA of rabbit serum #4757 with the N-terminal peptide

Results of an ELISA of rabbit serum with the N-terminal peptide of the CCMV coat protein.

The serum from both rabbits shows positive cross-reaction with the synthetic peptide. A pre-immune bleed shows a background signal in a serial dilution. BSA serves as the negative control, and shows the background signal.

ELISA with Purified IgG

After the protein-G affinity column purification, a total of 15 ml of 1.2 mg/ml of the polyclonal IgG was obtained. The purified IgG was then tested by an ELISA for cross reactivity and specificity with the intact wild type virus, the salt stable virus, the S2C-salt stable virus, the N-34 virus and free wild type coat proteins. As shown in Fig. 34, showed average ELISA values of 0.25, 0.51, 0.59 and 0.72 for wild type, the salt stable, the S2C-SS mutant and coat protein of the wild type virus respectively; from the E-34 wells, it showed an average ELISA value of 0.08 as the background signal. The results indicated that the polyclonal antibody cross reacted with wild type, the salt stable and the S2C-salt stable viruses; with a higher cross-reactivity for the salt stable background mutants, which might be experimental variations. The background colorimetric reaction was shown with N-34 mutant virus at a value of 0.03. The wild type coat protein served as a positive control with an average ELISA value of 0.72.

Dot-blot and Western of IgG

A dot-blot assay was then used to test the cross-reactivity of IgG with intact virions and coat proteins on a piece of nitro-cellulose membrane. A series of diluted intact viruses and free coat proteins were applied for the experiment. The polyclonal antibody cross-reacted with the wild type, the salt stable, and the S2C-SS virions, but

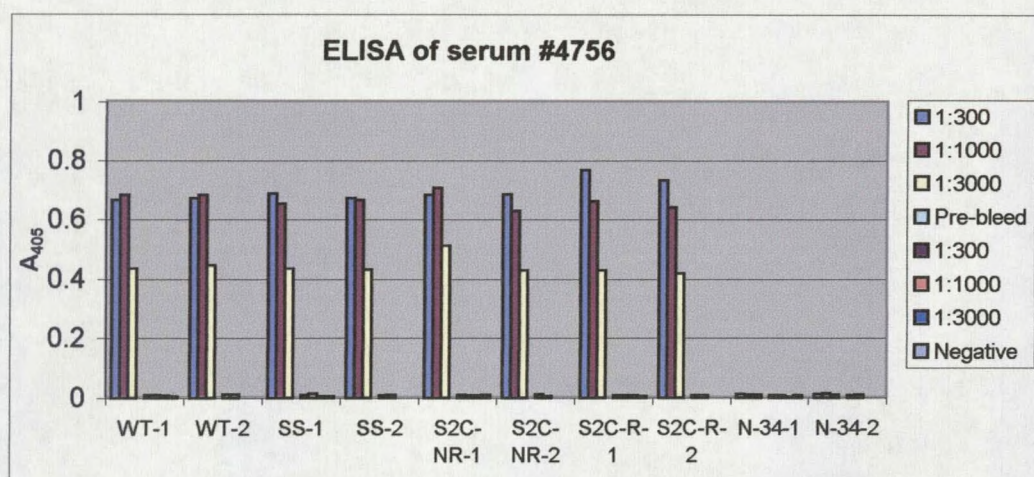


Fig. 33. ELISA of the serum with CCMV viruses.

WT: wild type virus

SS: salt stable virus

S2C-NR: non reduced S2C mutant

S2C-R: reduced S2C mutant

N-34: N-terminal 3-34 amino acid deletion mutant

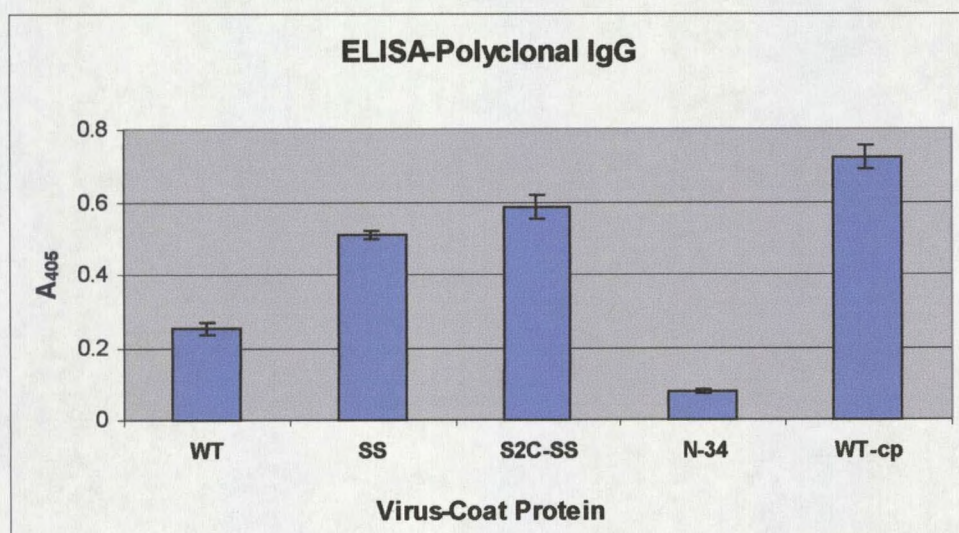


Fig. 34. ELISA of purified IgG with CCMV viruses and the coat protein

WT: wild type virus

SS: the salt stable virus

S2C-SS: a second cysteine substitution in a salt stable background

N-34: the N-terminal 3-37 amino acids deletion mutant

WT-cp: the wild type coat protein purified from disassembled virus

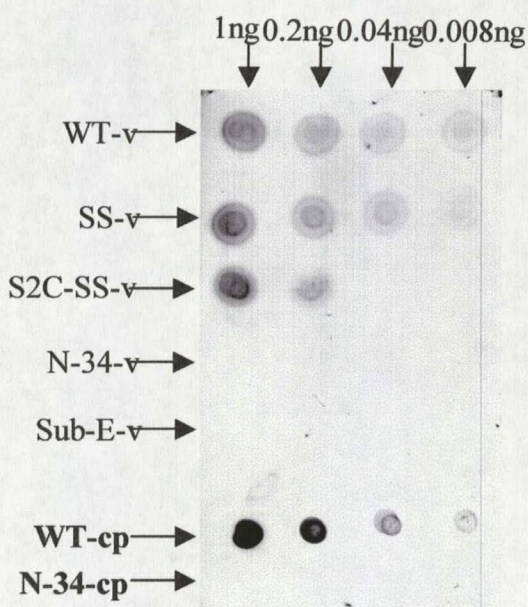


Fig. 35. Dot-blot of polyclonal IgG with viruses and coat proteins.

Total of 1.0, 0.2, 0.04, 0.008 ng of viruses or coat proteins in 2 μ l of buffer was placed on the piece of nitro cellulose membrane. The IgG was diluted 1:5000 with PBS.

v: virus

cp: coat protein

not with the N-34 and the Sub-E mutant viruses. For coat protein samples, the polyclonal antibody crossreacted with wild type coat protein, but not with the N-34 free coat protein (Fig. 35). These results indicate that the polyclonal serum is N-terminus specific and the N-terminus on the virus is susceptible with the antibody binding.

The free CCMV coat proteins exists as non-covalent dimers in solution. The mutant R26C coat proteins form polymers and more dimers due to a disulfide bond

formation at the N-terminus of the coat protein. Western-blot results showed that the polyclonal IgG cross-reacted with wild type coat protein monomers and dimers; with R26C monomer, dimers and polymers. The polyclonal IgG also cross reacts with the mcKLH-conjugated peptide, but not with the N-34 free coat protein (lane N-34) (Fig. 36).

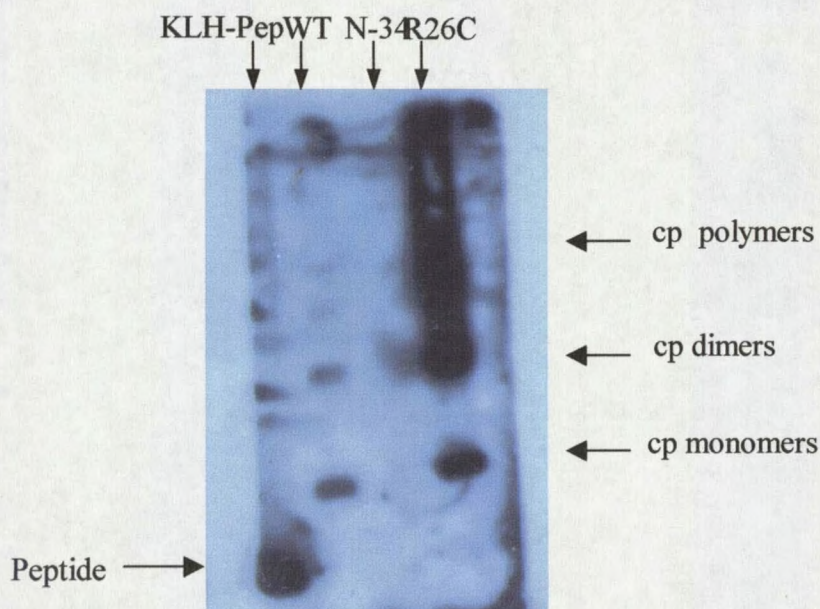


Fig. 36. Western-blot of purified polyclonal IgG with free coat proteins.

1:5000 diluted IgG cross-reacts with the KLH-Conjugated peptide, the wild type coat protein and the R26C coat protein, but not with the N-34 free coat protein. The lower band on the gel represents coat protein monomers (20 kD), the middle band represents protein dimers (40 kD), and the upper band represents coat protein polymers. For the KLH-peptide lane, the lower band represents the free synthetic peptide, and the upper band represents the conjugated peptide.

Discussion

The objective of this research was to generate and use immunological tools to probe for the CCMV structural transitions. Antibodies provide powerful tools for tracking down the position of peptides on the macromolecules, such as the CCMV structural protein, and even the whole CCMV virion. Studies with a monoclonal antibody against N-terminus of CCMV coat protein have shown that the antibody binds to the surface of the virus particle. This implicated that the N-terminus of the coat protein is on the exterior of the virus particle. The data presented in this chapter also agrees with this conclusion. ELISA, Dot-blot and Western-blot assays with the N-terminal polyclonal antibody using the N-34 and the Sub-E mutant viruses and their coat proteins as negative controls suggested that the polyclonal N-terminal antibody is N-terminus specific. The wild type and the salt stable virion showed positive cross reactivity at the same level, which indicated that the N-terminus on the virion is accessible to the exterior of the virus particle. However, this appears to contradict with the MALDI-MASS spectrometry data (Qu, unpublished data). In the MASS spectrometry experiments, it was demonstrated that in the salt stable virion, the N-terminus of the coat protein is not accessible to tryptic digestion even 24 hours after incubation (Qu, chapter 3, unpublished data). In contrast, the N-terminus peptides were the first products digested by tryptic digestions of the intact wild type virus. This suggests that the N-terminus on the salt stable virus remains inside of the virion, instead of exposing itself to the outside as it does in the wild type virus.

One interpretation of these contradictory data is that since the antigen-antibody binding is irreversible, once the antibody binds to the exposed N-terminus on the virion,

the N-terminus does not go back to the inside because the antibody "locks" it on the exterior of the virus particle. Therefore, the structural transition equilibrium is driven to the "out" confirmation for the N-terminus in the presence of the antibody. As a consequence, ELISA does not show difference between the wild type and the salt stable virus in their capability of binding the antibody. In contrast, in the MASS spectrometry experiments, it is conceivable that the rapid transition of the N-terminus between the virion's interior and exterior is too fast for interactions with trypsin, but slow enough for antibody binding.

Further by passing the virus and N-terminus polyclonal antibody through a column showed that the salt stable virus was co-eluted with the antibody (Willits, unpublished data). Therefore, the interaction between the N-terminus of the coat protein on the salt stable virion and the polyclonal antibody is stable even though the MALDI-MASS spectrometry experiment, the N-terminus of the coat protein of the salt stable mutant was not digested by trypsin.

Another explanation for the MASS spectrometry results is that, like that in the *in vitro* translation, the MASS spectrometry experiment is detecting disassembled wild type coat protein digested by trypsin, whereas the salt stable virion stays intact.

The monoclonal antibodies were tested for N-terminus specificity by an ELISA. From the selected three antibodies, one of the three purified antibodies, #1 showed positive cross reaction with the N-terminus peptide although with lower affinity compared to the CCMV polyclonal serum, which implicated that the N-terminus is antigenic and can stimulate immune response (Fig. 37). It might be helpful in the future studies to choose synthetic peptide of the hidden part of the coat protein in the virion for

obtaining coat protein specific antibodies, such as the C-terminal parts, where the dimers form.

The purified #1, #20 and #18 antibodies did not show different affinities for viruses and coat proteins in ELISA assays. This might be due to the fact that the hidden motifs in the virus are hard to be exposed. But these monoclonal would be helpful for other projects in the laboratory since they are specific for CCMV virus and the coat protein.

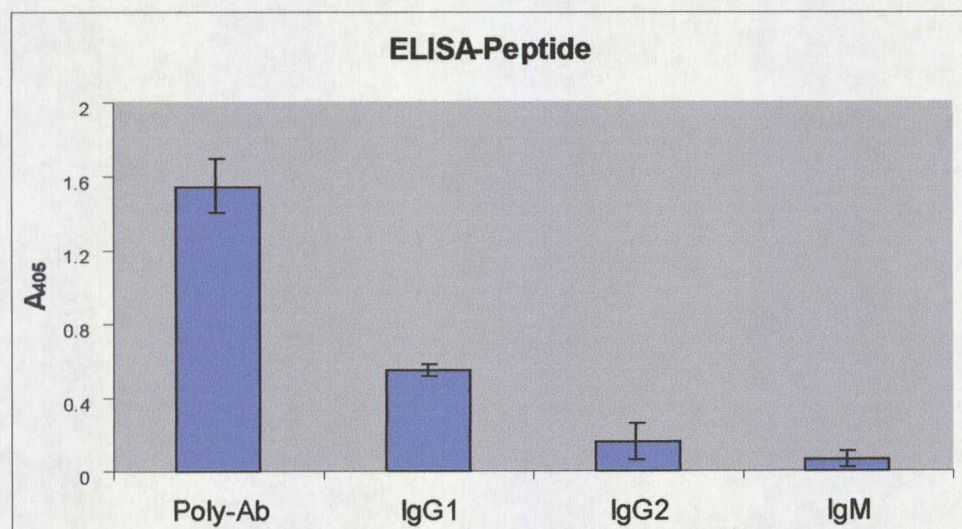


Fig. 37. ELISA of monoclonal antibodies for N-terminus specificity

Poly-Ab: polyclonal antibody of CCMV

IgG1: monoclonal antibody # 1

IgG2: monoclonal antibody # 19

IgM: monoclonal antibody # 18

CCMV polyclonal antibody cross reacts with N-terminus peptide. IgG # 1 shows positive cross reactivity with N-terminal peptide, however, with a low ELISA value of 0.55.

For future N-terminal mobility study, one further step would be to label the virus with N-terminus polyclonal antibody, and then observe labeled virion under a cryo-reconstruction EM. In this way, it would show the image of the antibody bound on the surface of the virion, and provide even close insight on the interaction, therefore helping to understand the role of the N-terminus in virus disassembly.

CHAPTER 5

CONCLUSION

This thesis covers studies on CCMV virus disassembly based on detailed characterization of a salt stable mutant of CCMV, and biochemical and immunological analysis of the N-terminus dynamics of the viral coat protein on virus.

The salt stable virus was regenerated by site directed mutagenesis. The mutant showed the same infectivity and swelling characteristics as wild type virus, but stayed intact under high pH and high salt conditions. Structural studies suggested that the new salt bridge formation between the E34 residue on one coat protein subunit and the R42 on an adjacent subunit at the threefold axis enhanced the protein-protein interaction in the virion and contributed to the distinct salt stable phenotype. As predicted before the salt bridge could be observed in the x-ray crystallography structure of the salt stable virus. I have further demonstrated that this new salt bridge is essential for maintaining the salt stable phenotype for the virus by interrupting the salt bridge by substituting the E34 with different amino acids, alanine (E34A), glutamic acid (E34D) and arginine (E34R). The new salt bridge could be observed in the x-ray crystallography of the virus.

I have demonstrated that the salt stable virus showed another phenotype distinct from wild type CCMV in the Flexi cell free translation assay. In contrast to the wild type virus translation that gave four protein products, the salt stable virus showed no translation products in the translation reaction. Furthermore, the salt stable virus did not affect the translation assay indicated by addition of the same amount of the salt stable virus to the viral RNA and wild type reactions and did not show difference. These results indicated that swelling is not correlated with CCMV cotranslational disassembly *in vitro*.

Furthermore disassembly deficient mutants such as R26C non-reduced form and the calcium-binding deficient mutants (Young laboratory, unpublished data) that do not disassociate under high salt and high pH conditions did not get translated in the Flexi system. But the *in vivo* infectivity for the mutants is the same as wild type. Therefore we suspected that the translation assay measures disassociated viral RNA for the wild type, in contrast the salt stable virus stays intact. The translation system did not mimic the disassembly conditions of CCMV *in vivo* despite the fact that it has been widely used for studying TMV virus disassembly mechanisms.

For the other spherical plant viruses, such as cucumber mosaic virus (CMV), the swelling characteristic does not exist. The structure has been solved recently at 2.9 Å resolution for CMV. From the high-resolution structure studies, it has demonstrated that the cluster of negatively charged residues at the pseudo-threefold axis is not present in the CCMV virus, which agrees with the hypothesis that the CMV virion does not undergo swelling *in vitro*. This indicates that the CMV disassembly is by a mechanism other than swelling.

The N-terminus of the coat protein of CCMV plays important roles in virus assembly and disassembly. The first 27 amino acids on the hexameric capsomers and the first 44 amino acid residues on the pentameric capsomers are not ordered. Another alternative disassembly model was proposed in which the N-termini on the pentameric capsomers undergo a structural transition to form a five-bundled α -helix channel, and through this channel the viral RNA was released to the host translational machinery and translated.

The Ellman's reaction and the thiol quantitation were utilized to analyze the N-terminus mobility in intact viruses. The data indicated that in the salt stable background viruses (S2C-C59S-SS) and the salt stable mutant, the thiol groups present on the second position cysteines showed negative signal, in contrast wild type virus showed positive in the same assays. Therefore as in the *in vitro* translation assay, the thiol quantitation probably measured the disassembled coat proteins for wild type virus. Another possible interpretation is that the substituted second position cysteine residue was modified *in vivo* since the MALDI-MASS spectrometry showed a change of the molecular weight of the mutant virus coat protein. As a consequence the free thiol group is blocked and cannot get access to the reaction.

The N-terminus polyclonal antibodies generated with a synthetic N-terminal peptide of 25 amino acid residues however showed a contradictory point for the N-terminus dynamics of the salt stable mutant. My results suggest that the polyclonal antibody can recognize the N-termini of the coat proteins on the viruses at the same level as the wild type. The negative controls of a N-34 mutant (lacking the N-terminal 3-37 residues of the coat protein) and a Sub-E (the N-terminus substitution with glutamic acids) showed negative signal for the antibody binding assays, which demonstrated that the polyclonal antibody was N-terminus specific. These data indicated that probably due to the fact that the antibody binding to the N-terminus in the viruses shifts the N-terminus mobility equilibrium to the final "out" phase and therefore both the wild type and the salt stable viruses showed the same cross reactivity with the polyclonal antibody. Furthermore, an FPLC size-exclusion chromatography assay demonstrates that the salt stable virus was co-eluted with the N-terminal specific antibody, which indicates that the

N-terminus of the coat protein in the salt stable virus and the antibody interactions are stable.

Future steps for the N-terminus mobility project would be to label viruses with the N-terminus specific antibody and observe the labeled virions under cryo reconstruction electronic microscopy to gain details of the N-terminus with the bound antibody. Inhibition studies with the N-terminus specific antibody *in vivo* for infectivity assay would help to illustrate the role of the N-terminus by inoculating viruses along with the antibody to the local lesion hosts.

Studies presented here have not excluded the N-terminus channel formation for CCMV disassembly, but have demonstrated that swelling is not correlated with CCMV cotranslational disassembly *in vitro*. The antibody and MALDI MASS spectrometry assays demonstrate CCMV virus undergoes structural transition on the N-terminus of the coat protein although in this thesis there is no direct evidence to tie this structural transition with virus translation despite the N-terminus channel formation model. But the dynamics of the N-terminus plays important role in virus disassembly.

Altogether, the studies in this thesis have not excluded the possibility of swelling in the CCMV cotranslational disassembly. It will be helpful to screen for disassembly deficient mutants, and by comparison to the wild type it will illustrate the mechanisms of virus disassembly. Furthermore, the virus dynamics studies using the N-terminus specific antibodies will facilitate the understanding of the role of the N-terminus of the coat protein for virus disassembly.

REFERENCES

- Adolph, K. W. (1975a). Conformation features of cowpea chlorotic mottle virus RNA and the stability of the virus. *J. Gen. Virol.* **28**, 137-145.
- Adolph, K. W. (1975b). Structural transition of cowpea chlorotic mottle virus. *J. Gen. Virol.* **28**, 147-154.
- Albert, F. G., Fox, J.M., and Young, M. J. (1997). Swelling is not required for cotranslational disassembly of cowpea chlorotic mottle virus in vitro. *J. Virol.* **71**, 4862-4865.
- Allison, R., Janda, M., and Alquist, P. (1988). Infectious in vitro transcripts from cowpea chlorotic mottle virus cDNA clones and exchange of individual RNA components with brome mosaic virus. *J. Virol.* **62**, 3581-3588.
- Alquist, P. (1992) Bromovirus RNA replication and transcription. *Curr. Opin. Gen. And Dev.* **2**(1), 71-76.
- Arthur, L. O., and Massey, R. J. (1981). "A workbook on Hybridomas and Monoclonal Antibody Technology." Hood College, Frederick, Maryland.
- Bancroft, J. B., and Hiebert, E. (1967). Formation of infectious nucleoprotein from protein and nucleic acid isolated from a small spherical virus. *Virology* **32**, 354-356.
- Bancroft, J. B., and Horne, R. W. (1977). Bromovirus (brome mosaic virus) group. In "The Atlas of Insect and Plant Virus." (K. Maramorosch, Ed.), Vol. **8**, pp. 287-302. Academic Press, New York.
- Bancroft, J. B., Bracker, C. E., and Wagner, G. W. (1969). Structures derived from cowpea chlorotic mottle and brome mosaic virus protein. *Virology* **38**, 324-335.
- Bancroft, J. B., McDonald, J. G., and Rees, M. W. (1976). A mutant of cowpea chlorotic mottle virus with a perturbed assembly mechanism. *Virology* **75**, 293-305.
- Bancroft, J. B., McLean, G. D., Rees, M. W., and Short, M. N. (1971). The effect of an arginyl to a cysteinyl replacement on the uncoating of a spherical virus. *Virology* **45**, 707-715.
- Bancroft, J. B., Rees, M. W., Dawson J. R. O., McLean, G. D., and Short, M. N. (1972). Some properties of a temperature-sensitive mutant of cowpea chlorotic mottle virus. *J. Gen. Virol.* **16**, 69-81.

- Bancroft, J. B., Rees, M. W., Johnson, M. W., Dawson J. R. O. (1973). A salt-stable mutant of cowpea chlorotic mottle virus. *J. Gen. Virol.* **21**, 507-513.
- Bancroft, J. B., Wagner, G. W., and Bracker, C. E. (1968). The self-assembly of a nucleic-acid free pseudo-top component for a small spherical virus. *Virology.* **36**, 146-149.
- Brisco, M., Hull, R., and Wilson, T. M. A. (1986). Swelling of isometric and of bacilliform plant virus nucleocapsids is required for virus-specific protein synthesis in vitro. *Virology* **148**, 210-217.
- Ephrussi, B., and Weiss, M. C. (1969). Hybrid somatic cells. *Sci. Am.* **220**, 26-35.
- Fox, J. M., Albert F. G., Speir, J. A., and Young M. J. (1997). Characterization of a disassembly deficient mutant of cowpea chlorotic mottle virus. *Virology* **227**, 229-233.
- Fox, J. M., Zhao, X., Speir, J.A., and Young M. J. (1996). Analysis of a salt stable mutant of cowpea chlorotic mottle virus. *Virology.* **222**(1), 115-122.
- Harlow, E., and Lane, D. (1988). *Antibodies: A laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 56-100.
- Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987). Rapid and efficient site-specific mutagenesis without phenotypic selection. *Meth. In Ezym.* **154**, 367-382.
- Lane, L. C. (1981). Bromovirus. In "Handbook of plant virus infection and comparative diagnosis" (E. Kurstak, Ed.). Elsevier/ North-Holland, Amsterdam.
- Littlefield, J. W. (1964). Selection of hybrids from mating of fibroblasts in vitro and their presumed recombinants. *Science* **145**, 709-710.
- Loesch-Fries, L. S., and Hall, T. C. (1980). Synthesis, accumulation and encapsidation of individual brome mosaic virus RNA components in barley protoplasts. *J. Gen. Virol.* **47**, 323-332.
- Lu, B., Stubbs, G., and Culver, J. N. (1996). Carboxylate interactions involved in the disassembly of tobacco mosaic tobamovirus. *Virology* **225**(1): 11-20.
- Male, D., Champion, B., and Cooke, A. (1987). *Advanced Immunology.* London: J. B. Lippincott Co., Gower Medical Publishing 8.1-8.8.
- Malik, V. S., and Lillehoj, E. P. (1994). *Antibody Techniques.* Academic Press. pp71-102.
- Nowinski, R. C., Lostrom, M. E., Tam, M. R., Stone, M. R., and Burnette, W. N. (1979). The isolation of hybrid cell lines producing monoclonal antibodies against the p15(E) protein of murine leukemia virus. *Virology* **93**, 111-126.

Pontecorvo, G. (1976). Production of indefinitely multiplying mammalian somatic cell hybrids by polyethylene glycol (PEG) treatment. *Somatic Cell Genet.* **1**, 397.

Rao, A. L. N., and Grantham, G. L. (1995). A spontaneous mutation in the movement protein gene of brome mosaic virus modulates symptom phenotype in *Nicotiana benthamiana*. *J. Virol.* **69** (4), 2689-2691.

Sacher, R., and Alquist, P. (1989). Effects of deletion in the N-terminal basic arm of brome mosaic virus coat protein on RNA packaging and systemic infection. *J. Virol.* **63** (11), 4545-4552.

Sell, B. (1987). *Immunology, Immunopathology and Immunity*. Elsevier, N.Y. 69-78.

Shaw, J. G. (1999). Tobacco mosaic virus and the study of early events in virus infections. *Philos Trans R Soc Lond B Biol Sci* **354** (1383): 603-611.

Speir, J. A., Munshi, S., Wang, G., Baker, T. S., and Johnson, J. E. (1995). Structure of the native and swollen forms of cowpea chlorotic mottle virus determined by X-ray crystallography and cryo-electron microscopy. *Structure* **3**, 63-78.

Stubbs, G. (1999). Tobacco mosaic virus particle structure and the initiation of disassembly. *Philos Trans R Soc Lond B Biol Sci* **354** (1383): 551-557.

Van der Spoel, D., Feenstra, K. A., Hemminga, M. A., and Berendsen, H. J. (1996). Molecular modeling of the RNA binding N-terminal part of cowpea chlorotic mottle virus coat protein in solution with phosphate ions. *Biophys. J.* **71**, 2920-2931.

Verduin, B. J., Prescott, B., and Thomas, G. J., Jr. (1984). RNA-protein interactions and secondary structure of cowpea chlorotic mottle virus for in vitro assembly. *Biochemistry* **23**: 4301-4318.

Vriend, G. (1983). Molecular interactions during the assembly of cowpea chlorotic mottle virus studied by magnetic resonance. Ph.D Thesis, van de Landbouwhogeschool to Wageningen.

Vriend, G., Hemminga, M. A., Verduin, B. J. M., DeWit, J. L., and Schaafsma, T. J. (1981). Segmental mobility involved in protein-RNA interaction in cowpea chlorotic mottle virus. *FEBS Lett.* **134**, 167-171.

Vriend, G., Verduin, B. J., and Hemminga, M. A. (1986). Role of the N-terminal part of the coat protein in the assembly of cowpea chlorotic mottle virus. A 500 MHz proton nuclear magnetic resonance study and structural calculations. *J. Mol. Biol.* **191**: 453-460.

Wu, X., and Shaw, J. G. (1997). Evidence that a viral replicase protein is involved in the disassembly of tobacco mosaic virus particle in vivo. *Virology* **239** (2): 426-434.

Zhao, X., Fox, J. M., Olson, N. H., Baker, T. S., and Young, M. J. (1994). In vitro assembly of cowpea chlorotic mottle virus from coat protein expressed in *Escherichia coli* and in vitro-transcribed viral cDNA. *Virology* **207**, 486-494.

Zlotnick A., Aldrich R., Johnson J. M., and Young M. J. (2000). Mechanism of capsid assembly for an icosahedral plant virus. *Virology* **277**, 450-456.

APPENDIX A

ABBREVIATIONS

BSA: bovine serum albumin
CCMV: cowpea chlorotic mottle virus
°C: Celsius
cDNA: complementary DNA
CO₂: carbon dioxide
cp: coat protein
DMSO: dimethyl sulfoxide
DNA: deoxyribonucleic acid
ELISA: enzyme-linked immnosorbent assay
FCS: fecal calf serum
HAT: hypoxanthine aminopterin thymidine
HGPRT: hypoxanthane guanine phosphoribosyl transferase
Ig: immunoglobulin
mcKLH: maricultured keyhole limpet hemocyanin
ml: milliliter
mg: milligram
PBS: phosphate buffered saline
rpm: revolutions pre minute
RPMI 1640: a standard cell culture medium
SP2/0: myeloma cell lines used in fusion
SS: salt stable virus
µg: microgram
µl: micro liter
v: virus
WT: wild type virus

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