National Library of Canada
Bibliothèque nationale du Canada

Canadian Theses Service
Service des thèses canadiennes

Ottawa, Canada
K1A 0N4

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

NL 339 (r. 88/04) c

Canada

CHARACTERIZATION OF THE INTERFERENCE BETWEEN TWO COMOVIRUSES IN CO-INOCULATED *VIGNA UNGUICULATA*

by

Gabriel Bela Kalmar

B.Sc., University of British Columbia, 1983

M.Sc., University of British Columbia, 1986

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in the department

of

Biological Sciences

© Gabriel Bela Kalmar 1989

SIMON FRASER UNIVERSITY

March 1989

All rights reserved. This work may not be reproduced in whole or in part, by photocopy or other means, without permission of the author.

National Library of Canada
Bibliothèque nationale du Canada

Canadian Theses Service
Service des thèses canadiennes

Ottawa, Canada
K1A 0N4

The author has granted an irrevocable non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of his/her thesis by any means and in any form or format, making this thesis available to interested persons.

L'auteur a accordé une licence irrévocable et non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de sa thèse de quelque manière et sous quelque forme que ce soit pour mettre des exemplaires de cette thèse à la disposition des personnes intéressées.

The author retains ownership of the copyright in his/her thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without his/her permission.

L'auteur conserve la propriété du droit d'auteur qui protège sa thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

ISBN 0-315-59371-7

Canadä

APPROVAL

Name: GABRIEL BELA KALMAR

Degree: Doctor of Philosophy

Title of Thesis:

CHARACTERIZATION OF THE INTERFERENCE BETWEEN TWO COMOVIRUSES IN CO-INOCULATED *VIGNA UNGUICULATA*

Examining Committee:

Chairman: Dr. B. Honda, Associate Professor

Dr. K. Eastwell, Visiting Professor, Senior Supervisor

Dr. M. Smith, Professor

Dr. J. Kane, Professor

Dr. L. Kemp, Associate Professor

Dr. R. Martin, Research Scientist, Agr. Cda. Research Station, Vancouver, B.C.

Dr. J. Webster, Professor, Public Examiner

Dr. G. Prody, Associate Professor, Chemistry, W. Washington University, U.S.A., Public Examiner

Dr. R. Stace-Smith, Research Scientist, Agr. Cda. Research Station, Vancouver, B.C., External Examiner

Date Approved 17 April 1989

PARTIAL COPYRIGHT LICENSE

I hereby grant to Simon Fraser University the right to lend my thesis, project or extended essay (the title of which is shown below) to users of the Simon Fraser University Library, and to make partial or single copies only for such users or in response to a request from the library of any other university, or other educational institution, on its own behalf or for one of its users. I further agree that permission for multiple copying of this work for scholarly purposes may be granted by me or the Dean of Graduate Studies. It is understood that copying or publication of this work for financial gain shall not be allowed without my written permission.

Title of Thesis/Project/Extended Essay

Characterization of the interference between two comoviruses in co-inoculated *Vigna unguiculata*

Author: ______________________
(signature)

Gabriel Bela Kalmar
(name)

4/5/89
(date)

ABSTRACT:

The molecular interactions between the two comoviruses cowpea mosaic virus (CPMV) and cowpea severe mosaic virus (CPSMV) co-inoculated in *Vigna unguiculata* cultivars Black and Arlington were examined; both black and Arlington cowpeas were "operationally" immune to CPMV infection. The replication of each viral genome was studied independently by nucleic acid hybridization analysis. The hybridization probes were generated with SP6 and T7 DNA-dependent RNA polymerases from pGEM vectors with CPMV and CPSMV cDNA inserts. The templates were representative sequences cloned from each RNA of the bipartite genomes. The accumulation of viral capsid was determined by enzyme-linked immunosorbent assays (ELISA) using monoclonal antibodies which specifically recognized either CPMV or CPSMV.

In seedlings inoculated with CPMV alone, the RNAs were replicated in the "operationally" immune cowpeas Black and Arlington; however, the accumulation of RNA was delayed considerably when compared to CPMV infection of the susceptible cowpea variety Blackeye-5. In Black and Arlington cowpeas, CPMV RNA was restricted to the primary inoculated leaves, and the accumulation of capsid polypeptides or infectious virus was not detected in the immune cowpeas.

Co-inoculation of CPMV and CPSMV in Black and Arlington seedlings resulted in a delay of the symptoms expressed in response to CPSMV. All co-inoculated plants eventually

developed CPSMV associated symptoms, but 25% of the plants developed secondary leaves uninfected by CPSMV. In the latter plants, CPSMV was localized in distinct necrotic lesions which formed on the primary inoculated leaves. Experiments were performed to determine the specific conditions required for optimal interference. The data suggested that interference of CPSMV replication by CPMV was initiated by a direct interaction between the viruses at the site of co-infection.

Monoclonal antibodies were used to characterize epitopes of both viruses. Internal and external epitopes were identified for both viruses. External epitopes were unique to each virus, whereas the majority of the internal epitopes were common to both viruses. Most of the epitopes were confirmational, (rather than sequential) resulting from amino acids brought into close proximity by the secondary and tertiary structure of the coat protein subunits. The monoclonal antibodies were used to identify antigenic differences between empty capsids and capsids containing viral RNA. These differences suggested that packaging of the viral RNA altered the three-dimensional structure of the capsid.

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to Dr. Kenneth C. Eastwell for his encouragement and advice throughout this work, and also for his critical comments and tolerance in the preparation of this manuscript. His support and dedication to his students throughout this work were invaluable.

The helpful and supportive discussions with Drs. C.L. Kemp, R.R. Martin and M.J. Smith are gratefully acknowledged.

The use of facilities and equipment provided by Dr. B.M. Honda was greatly appreciated.

Appreciation is expressed to Dr. V. Bourne for helpful assistance with preparation of graphics for this manuscript.

Finally, I wish to express my sincerest appreciation to my wife, Gloria, and my family for their support and tolerance of me during the last three years.

TABLE OF CONTENTS

PAGE

Title Page i

Approval Page ii

Abstract iii

Acknowledgements v

Table of Contents vi

List of Tables ix

List of Figures xi

Chapter 1: General Introduction 1

The Comoviruses 1

Chapter 2: Comparison Of Monoclonal Antibodies Against Two Comoviruses 12

Introduction 12

Methods 16
- Virus and Plant Stocks 16
- Separation Of Virus Components 17
- Plant Extracts 18
- Virus Particle Disruption 18
- Ribonuclease Treatment Of Virus 20
- Immuno-blotting 20
- Production Of Rabbit Immunoglobulins 21
- Production Of Hybridoma Cell Lines 21
- Ascites Fluid Collection 23
- Isotyping Of Monoclonal Antibodies 23
- Ouchterlony Assay 23
- ELISA 24

Results 27
- General Characterization Of The Monoclonal Antibodies 27
- Recognition Of Viral Antigens In Plant Extracts 29
- Sensitivity Of Virus Antigen Recognition 32
- Recognition Of Altered Conformations Of Viral Antigens 32
- Selectivity Of Monoclonal Antibodies For Denatured Virus 41
- Recognition Of Separated Viral Components 45
- Recognition Of Dissociated Viral Components By

Monoclonal Antibodies.. 52
Effects of Ribonuclease Treatment On The Antigenicity Of Virus.. 53

Discussion.. 56

Chapter 2: Characterization Of The Interference Between CPMV-SB and CPSMV-DG In Co-inoculated *Vigna unguiculata*.. 63

Introduction.. 63

Methods.. 82
Virus and plant stocks.. 82
ELISAs.. 82
Viral RNA Analysis From Plant Extracts.. 82
Extraction Of RNA From Virus.. 83
UV Irradiation Of Viral RNA.. 85
Labeling Of Virus RNA.. 85
Isolation of M13.. 86
Infection Of Bacteria With M13.. 87
Plasmid Isolation.. 87
Oligo-labeling Of DNA.. 89
Electrophoretic Transfer.. 89
UV Irradiation of Filters.. 90
cDNA Cloning of CPMV and CPSMV.. 93
Screening of CPMV and CPSMV Libraries.. 94
Subcloning Of Restriction Enzyme Fragments.. 95
DNA Sequencing.. 95
Synthesis Of SP6 and T7 Transcripts.. 96
Hybridization Of Nucleic Acids.. 95

Results.. 99
cDNA Synthesis.. 99
Viral RNA Cloning.. 99
Replication Of CPMV In Cowpeas Seedlings.. 102
Modulation Of Symptom Expression In Co-inoculated Plants.. 111
Optimal Conditions For Interference.. 114
Co-inoculation With Various Components Of CPMV and CPSMV.. 118
Accumulation Of Viral RNAs and Capsid Polypeptides Within Individual Co-inoculated Cowpeas.. 123
Requirement Of Infectious CPMV RNA For Interference. 129
Sequence Of The 3'-terminal Of CPSMV RNA.. 132
Interference As A Genetic Trait Of The Plant.. 135
Co-inoculation Of Plants With CPMV, CPSMV and SHMV.. 137
Acquired Systemic Resistance Induced By CPMV.. 139
Interference In Blackeye-5 Cowpeas.. 139

Discussion.. 143

List of References.. 152

Appendix A **175**

LIST OF TABLES

TABLE	CONTENT	PAGE
1	Characterization of monoclonal antibodies produced against CPMV	28
2	Characterization of monoclonal antibodies produced against CPSMV	30
3	Detection of viral antigens in plant extracts by antibody-trapped indirect ELISA assays	33
4	Monoclonal antibodies specific for CPMV binding to altered virus conformations in plate-trapped ELISAs	37
5	Monoclonal antibodies specific for CPSMV binding to altered virus conformations in plate-trapped ELISAs	38
6	Reactivity of monoclonal antibody SB2 in plate-trapped ELISAs with virus proteins disrupted with guanidine-LiCl	43
7	Effect of Ribonuclease A1 treatment on the antigenicity of comoviruses	54
8	Summary of antibody binding activity	59
9	Bio-assay of sap from Blackeye-5, Black and Arlington cowpeas inoculated with CPMV	112
10	Co-inoculation of Black and Arlington cowpeas with CPSMV and CPMV at various stages of plant development	115
11	Co-inoculation of Black cowpeas with varying ratios of CPSMV to CPMV	117
12	Delayed inoculation of CPSMV to Black and Arlington cowpeas previously infected with CPMV	119
13	Co-inoculation of Black and Arlington cowpeas with CPSMV and fractionated components of CPMV	121
14	Comparison between the co-inoculation of Black cowpeas with CPSMV and CPMV, and co-inoculation with ssRNA purified from the two viruses	122

15 Distribution of CPMV and CPSMV in primary infected leaves of Black and Arlington cowpeas co-inoculated with CPMV and CPSMV.............. 133

16 Effects of UV-irradiation on the ability of CPMV ssRNA to interfere with the replication of CPSMV ssRNA in Black cowpeas........................ 134

17 Co-inoculation of Black and Arlington cowpeas with SHMV, CPSMV and CPMV........................ 138

18 Challenge by CPSMV of secondary leaves of plants with primary leaves infected by CPMV............ 141

LIST OF FIGURES

FIGURE	CONTENT	PAGE
1	Liquid phase competition assays demonstrating the specificity of monoclonal antibodies for immunogen	31
2	Sensitivity of monoclonal antibodies in antibody-trapped ELISA	36
3	Gel filtration chromatography of virions disrupted by exposure to SDS plus urea	40
4	Separation of untreated and disrupted virions by equilibrium gradient centrifugation	42
5	Immunoblots of capsid proteins	44
6	Comparison of monoclonal antibody cross-reactivity with virus in altered conformations	46
7	Comparison of affinity ELISAs and liquid phase competition assays using monoclonal antibodies specific for CPMV	47
8	Comparison of affinity ELISAs and liquid phase competition assays using monoclonal antibodies specific for CPSMV	49
9	Comparison of affinity ELISAs and liquid phase competition assays using polyclonal antibodies specific for either CPMV or CPSMV	51
10	Specificity of viral cDNA clones	101
11	Partial sequence of CPMV and CPSMV clones	103
12	Replication of CPMV genome in inoculated and secondary leaves of BE-5 cowpeas	104
13	Replication of CPMV genome in primary inoculated leaves of Black and Arlington cowpeas	105
14	Analysis by denaturing agarose gel electrophoresis of CPMV replication fourteen days p.i. in different varieties of cowpeas	108

15 Accumulation of CPMV virions in BE-5, Black and Arlington cowpeas.............................. 109

16 Accumulation of CPMV virions in BE-5 and Black cowpeas inoculated with purified viral RNAs... 110

17 Co-inoculated Black cowpeas with infected and uninfected secondary leaves..................... 113

18 Comparison of the accumulation of CPMV and CPSMV plus sense RNA in primary co-inoculated leaves of Black cowpeas.............. 125

19 Accumulation of CPSMV plus sense RNA in primary inoculated leaves of Black and Arlington cowpeas.............................. 126

20 Comparison of the accumulation of CPSMV genomic RNAs in co-inoculated Black cowpeas which produced uninfected or infected secondary leaves.................................. 128

21 Comparison of the accumulation of plus-sense CPSMV RNA1 and RNA2 between, symptom-free and collapsed secondary leaves of Black cowpeas co-inoculated with CPMV and CPSMV.................. 130

22 Comparison of the accumulation of plus-sense CPSMV RNA1 and RNA2 in primary leaves of Black cowpeas co-inoculated with CPMV and CPSMV....... 131

23 Comparison of the 3'-terminal sequences of CPSMV genomic RNAs.............................. 136

24 Comparison of virus accumulation in BE-5 cowpeas singularly and co-inoculated with CPMV and CPSMV virus or RNA............................. 142

25 Accumulation of CPSMV and CPMV in secondary leaves of co-inoculated BE-5 cowpeas........... 143

Chapter 1: General Introduction

The Comoviruses

Fourteen viruses have been assigned to the comovirus group on the basis of similiar particle morphology, genomic organization and replication cycle, cytopathological effects and serological relatedness (Stace-Smith, 1981; Bruening, 1978; Francki et al., 1985). Comoviruses have been reviewed extensively (Van Kammen, 1972; Bruening, 1977; Stace-Smith, 1981; Francki et al., 1985; Goldbach & Van Kammen, 1985). Cowpea mosaic virus (CPMV) is the type member of the comovirus group.

Individual comoviruses have a narrow host range, the majority of them infecting Leguminaceae, the remainder infecting plants in the Solanaceace, Cucurbitaceae, Basellaceae and Cruciferae families. All comoviruses are transmissible mechanically and many are transmitted, in a non-specific manner, by leaf-feeding beetles (Fulton et al., 1987; Gergerich & Scott, 1988). Seed transmission has been recorded at a low (less than 10%), but significant level (Cockbain et al., 1976).

Electron micrographs indicate that the viral particles have a polyhedral structure, and a diameter of 24 to 30 nm (Francki et al., 1985). The capsids of all comoviruses examined are composed of two polypeptides present in equimolar amounts. The molecular weights of the capsid proteins of CPMV are 37 kDa and 23 kDa (Wu & Bruening, 1971; Geelen et al.,

1972). A three-dimensional model has been proposed for the structure of CPMV particle having 5:3:2 symmetry; consisting of 12 pentamers of the 37 kDa protein and 20 trimers of the 23 kDa protein at the fivefold and threefold axes of symmetry, respectively (Crowther et al., 1974; Bruening, 1977; Schmidt et al., 1983).

Two electrophoretic forms of the 23 kDa protein have been observed. Proteolytic cleavage in the host and after virus isolation resulted in the loss of a peptide of about 2.5 kDa from the 23 kDa polypeptide (Niblett & Semancik, 1969; Geelen et al., 1973). A relationship was inferred between proteolytic cleavage and increased infectivity based on the increased infectivity of a mutant which was more rapidly cleaved in vivo (Siler et al., 1976). However, the mutation was localized in the sequences encoding the capsid polypeptides, thus the decreased infectivity of the mutant could have been related to an altered three-dimensional structure of the mutant virion. Geelen et al. (1973) presented evidence indicating the infectivity of CPMV is not dependent on the electrophoretic forms of the virus.

Comoviruses have a bipartite genome of plus-sense RNA (ie. the genomic RNA functions directly as a template for translation). The primary sequence of both RNAs of CPMV-SB has been determined. RNA1 is 5889 nucleotides in length (Lomonossoff & Shanks, 1983) and RNA2 is 3481 nucleotides in length (Van Wezenbeek et al., 1983). In addition, both RNAs

have 3'-termini with 150 to 200 nucleotide polyadenylate tails, and a small viraly encoded polypeptide covalently linked to the 5'-termini (VPg) (Stanley et al., 1978). The primary structure of red clover mottle virus (RCMV) has also been determined (Shanks et al., 1986) and found to have a genomic organization similiar to RNA2 of CPMV. There is very little sequence homology between RNA1 and RNA2 of CPMV, except in the 5'- and 3'- non-coding regions.

Comoviruses can be separated into three components by equilibrium centrifugation: 58 S top component, devoid of nucleic acid; 98 S middle component and 118 S bottom components, each encapsidating one molecule of RNA2 and RNA1, respectively (Mazzone et al., 1962; Bruening, 1978). Both middle and bottom components are required for systemic infection of plants (Van Kammen, 1968). However, inoculation of protoplasts with bottom component alone results in the synthesis of some viral polypeptides, but capsid proteins are not synthesized. Infection of protoplasts with middle component alone does not result in detectable capsid protein being synthesized (Goldbach et al., 1980; Rezelman et al., 1982). Therefore, RNA1 exhibits limited independance, but RNA2 is dependent on RNA1 for replication, and both RNA1 and RNA2 are required for the production of virus particles. The independent replication of one RNA component of a bipartite virus has been observed in the tobravirus group also. RNA1 of the select tobacco rattle virus is able to replicate and

establish infections in plants and protoplasts (Harrison & Robinson, 1978). Virions are not formed as the coat protein is encoded by the RNA2.

Mixtures of RNA1 and RNA2 of strains of CPMV with different phenotypes (pseudorecombinants) have been used to assign specific functions to the individual viral RNAs. Bruening (1969) used two strains of CPMV differing in top component production, to demonstrate that the amount of top component produced was governed by RNA2. De Jager and van Kammen (1970) confirmed this observation and also associated the ability of the virus to spread systemically in plants with RNA2. Thongmeearkom and Goodman (1978) linked the genetic control of symptom expression and serotype to RNA1 and RNA2 of cowpea severe mosaic virus (CPSMV), respectively. Local lesion and systemic symptom expression were mapped to both RNA1 and RNA2 (Wood, 1972; De Jager, 1976). Evans (1985) identified a nitrous acid mutant of CPMV that infected _Phaseolus vulgaris_, but not _Vigna unguiculata_. This host range mutation was linked to RNA1.

The mode of polypeptide synthesis by both RNA1 and RNA2 of CPMV has been elucidated. Both viral RNAs direct the synthesis of large precursor polypeptides which are cleaved by a virally encoded protease to smaller, functional viral proteins (Vos et al., 1988: Figure 1). RNA1 is translated as a single 200 kDa polypeptide, from which intermediates of 170, 110, 84 and 60 kDa are produced. The five final cleavage

products are oriented in the initial translation product as: NH_2-32K-58K-4K(VPg)-24K-87K-COOH (Rezelman et al., 1980; Goldbach & Rezelman, 1983; Wellink et al., 1986). In vitro translation of RNA2 yielded two polypeptides (105 and 95 kDa) with overlapping carboxy-termini (Vos et al., 1984). These proteins were further processed to produce 48, 58 and 60 kDa intermediates (Franssen et al., 1982). The 60 kDa polypeptide was cleaved to form the viral capsid proteins (23 and 37 kDa) (Wellink et al., 1987). Viral proteolytic processing was done by the 24 kDa protein encoded for by RNA1 (Verver et al., 1987). This protease cleaved at glutamine-glycine and glutamine-serine dipeptides. The activity of the 24 kDa protease was modified by the 32 kDa polypeptide also encoded by RNA 1 to cleave at glutamine-methionine dipeptides (Vos et al., 1988). Proteolysis required adenosine triphosphate (ATP) and dithiothreitol (Pelham, 1979). Inhibition studies indicated that the 24 kDa protein was a thiol-type protease (Pelham, 1979).

The functions of other virally encoded polypeptides have been identified. Although RNA1 was replicated and expressed when singularly inoculated to cowpea protoplasts, there was no systemic spread in seedlings of the viral nucleic acid without RNA2 (Eggen & Van Kammen, 1988). This suggested that RNA2 encodes a polypeptide functional in facilitating transport of the virus. The RNA2 encoded 48 kDa and 58 kDa polypeptides may have been responsible for this function (Goldbach & Van

Kammen, 1985). It cannot be discounted that viral encapsidation alone induced systemic spread. However, a 30 kDa transport protein has been suggested for tobacco mosaic virus (TMV) (Zimmern & Hunter, 1983; Meshi et al., 1987), and the 48 kDa protein of CPMV has limited homology with the 30 kDa protein of TMV (Meyer et al., 1986).

An RNA-dependent RNA polymerase function has been associated with the 110 kDa precursor polypeptide encoded for by RNA1 of CPMV (Dorssers et al., 1984; Eggen et al., 1988b). The viral polymerase was found to be the core protein associated with two host proteins in a membrane bound polymerase complex. An endogenous RNA-dependent RNA polymerase has been found in uninfected cowpeas, the activity of which increases twenty-fold when plants were infected with CPMV (Dorssers et al., 1982). Biochemical and immunological evidence has shown that the host polymerase was not involved in virus replication. A membrane bound host polymerase was also identified, but it replicated only short, heterologous, minus sense strands of viral RNA. It exhibited no preference for viral RNA template and its template *in vivo* or function have not been identified (Dorssers et al., 1983; Van Der Meer et al., 1984). In a similar system, a host encoded RNA-dependent RNA polymerase has been purified from cucumbers infected with cucumber mosaic virus (CMV) (Kumarasamy & Symons, 1979). This polymerase also synthesized small heterologous RNA molecules.

The 60 kDa precursor polypeptide from which the VPg was cleaved was also membrane bound (Goldbach et al., 1982; Zabel et al., 1982). To date, VPg has only been identified covalently attached to viral RNA, and not as a free 4 kDa protein in the cytoplasm of infected cells. The VPg of poliovirus has been implicated in virus replication (Baron & Baltimore, 1982) where the protein may have been attached to the viral RNAs prior to cleavage from its precursor (reviewed in Semler et al., 1988). If CPMV VPg functions in a similar manner during replication, the 60 kDa polypeptide may be an attachment site in the host membrane for viral RNA replication. The majority of virus replication may be membrane associated, which is consistent with the large number of membranous vesicles found in plant tissues infected with comoviruses.

Cytopathological changes in all comovirus infections studied were very similiar (Francki et al., 1985). The most common, and visible changes were large vesiculate inclusions in the cytoplasm (Van der Scheer & Groenewegen, 1971). They were composed of interconnected membrane-bound vesicles, which occupied the majority of the cytoplasm and were surrounded by mitochondria and chloroplasts. Assembled virus particles have been observed in the vacuoles, and/in tube-like protrusions extending from the cell wall, which may pass through the plasmadesmata (Francki et al., 1985). Infection with fractionated RNA1 of CPMV resulted in the appearance of

cytoplasmic inclusions in inoculated tissue (Rezelman et al., 1982). Therefore RNA1 induced cytopathological changes in the absence of RNA2.

Double-stranded RNA (dsRNA), the replicative form of the viral genome, was found to co-sediment with virus inclusion bodies (Assink et al., 1973). Antibodies specific for non-structural proteins of CPMV have been used to locate proteins encoded by the viral genome in the cytoplasmic inclusions (Wellink et al., 1988). Chloramphenicol inhibition of the 70 S chloroplast ribosomes had no effect on viral protein synthesis, but cycloheximide inhibition of the 80 S cytoplasmic ribosomes inhibited viral protein synthesis (Owens & Bruening, 1975). Therefore, viral replication required the protein synthesis machinery of the cytoplasm, suggesting that viral replication occurred in the cytoplasm.

Animal picornaviruses and plant comoviruses have many similarities and sequence homologies, despite very different host ranges and biological properties. Analogous features have been recognized between the two virus families in genomic structure, and modes of replication and translation. In both virus groups viral replication is located in virally induced membranous vesicles. The genomes of comoviruses and picornaviruses are positive sense single-stranded RNA (ssRNA), with a 5'-linked VPg and a 3'-polyadenylate tail. The genomes of both viral groups are translated as large precursor polypeptides which are cleaved by a virally encoded protease

to form the functional viral proteins (Putnak & Phillips, 1981).

Comparison of the genomic organization and nucleotide sequences of the genomes of comoviruses and picornaviruses revealed striking similarities (Kiamura et al., 1981; Nomoto et al., 1981; Goldbach & Van Kammen, 1985). Picornaviruses do not have a split genome, however there is an analogous arrangement in the order of the functional domains of the non-structural viral proteins (Franssen et al., 1984). Considerable amino acid homology exists between the 110 kDa polypeptide of CPMV and the RNA-dependent RNA polymerases of poliovirus and foot-and-mouth disease virus (Franssen et al., 1984). The virally encoded proteases of poliovirus and CPMV both cleave glutamine-glycine dipeptides (Nicklin et al., 1986; Vos et al., 1988). These findings indicate there is an evolutionary relatedness between these two biologically distinct virus groups. It is not known which virus group preceded the other, but the two virus families appear to have evolved from common ancestry (reviewed by Zimmern, 1988).

A number of conserved amino acid motifs have been identified among the RNA-dependent RNA polymerases of both animal and plant RNA viruses, some of these amino acid motifs have been identified in the putative RNA-dependent RNA polymerase of CPMV (Zimmern, 1988). Recently, homologies to these amino acid motifs have been found in eukaryotic and prokaryotic helicases and nucleases (Gorbalenya et al., 1988),

giving rise to a new superfamily of replicative proteins. The conserved amino acid motifs within these replicases were postulated to form the functional sites for pyrophosphate, magnesium or nucleic acid binding (Argos, 1988; Hodgman, 1988).

Among the plant virus groups, comoviruses are related more closely to nepoviruses than any other. Both virus groups have a bipartite genome with a 5'-linked VPg and a 3'-linked polyadenylate tail. The RNAs of nepoviruses are also translated as one polypeptide and processed by cleavage with a virally encoded protease (Morris-Krsinich et al., 1983). However, apart from biological differences, the two virus groups have different capsid structures and genome sizes (Goldbach & Van Kammen, 1985). Presumably, the primary structures and nucleotide sequences of viral genomes from the two virus groups is different (Rott et al., 1988), although there may exist distinct regions of homology.

Tobacco etch virus (TEV), a member of the plant potyvirus family, has a single-stranded RNA (ssRNA) genome encapsidated helically in a long flexuous particle. The genome of TEV is translated as a single polypeptide (Allison et al., 1986), and post-translationally cleaved by a virus encoded protease at glutamine-serine or glutamine-glycine dipeptides (Carrington et al., 1988). Therefore, properties of the viral protease are conserved between unrelated plant viruses and between plant viruses and other eukaryotic viruses.

Bruening et al. (1973) observed that in mixed infections CPMV-SB interfered with the replication of CPSMV-DG when the two viruses were co-inoculated in cowpeas resistant to CPMV-SB infection. This work examined the extent of interference between the co-inoculated viruses. The results obtained provided the basis for postulating a theoretical mechanism to explain the interference phenomenon. To facilitate this work cDNA clones were prepared from the RNAs of both viral genomes. Hybridomas secreting monoclonal antibodies for either virus were isolated and used to study the accumulation of virus in infected plants. The monoclonal antibodies were also used to characterize the antigenic relatedness of CPMV and CPSMV.

CHAPTER 2: COMPARISON OF MONOCLONAL ANTIBODIES AGAINST TWO COMOVIRUSES

INTRODUCTION:

The members of the comovirus family have varying degrees of serological relatedness (Bruening, 1978; Stace-Smith, 1981). The serological relatedness of CPMV and CPSMV has been documented (Agrawal & Maat, 1964; Swaans & Van Kammen, 1973). These two comoviruses were classified originally as strains of the same virus (Agrawal, 1964); however, a re-evaluation of their relatedness based on thermal inactivation points, sedimentation patterns, host range and symptomology (Swaans & Van Kammen, 1973) led to their current classification as two distinct viruses.

Heterologous mixtures of the middle and bottom components of these two viruses were not successful in establishing infections on host plants (Van Kammen, 1968). Competition hybridization experiments showed little homology in nucleotide sequences between the RNAs of the Nigerian strain of CPMV and the Vs strain of CPSMV (Swaans & Van Kammen, 1973). Short segments of 60% homology, at the amino acid level, have been detected between the predicted translation products of RNA1 of CPMV and CPSMV (G. Bruening, personal communication). However, the degree of amino acid sequence homology shared by non-structural proteins was not necessarily indicative of serological relatedness. Overall, the nucleic acid sequence homology between RNA2 of the S strain of RCMV and RNA2 of

CPMV-SB was found to be 62% (Shanks et al., 1986). The regions coding for viral capsid proteins contained blocks of 80% and greater homology, yet the viruses shared only distant serological relatedness (Gibbs et al., 1968; Stace-Smith, 1981). The latter observation was attributed to the realization that the majority of epitopes of an antigen have secondary and tertiary structure, and represent a discontinuous amino acid sequence (Barlow, 1986).

Several isolates of CPMV have been identified, but few of these isolates have been compared serologically. However, isolates from Surinam (SB-isolate), Nigeria, Kenya and USA were found to have a close serological relatedness (Van Kammen & de Jager, 1978).

Isolates of CPSMV have been divided into four serotypes (Lin et al., 1984). The four sero-groups had one common antigenic determinant and each group was characterized by one unique epitope. Other antigenic determinants were shared to varying degrees among the sero-groups. CPSMV-DG was characterized by Beier et al. (1977), and found to possess physical and biochemical properties similar to other CPSMV isolates.

The separation of virus particles into three distinct bands by equilibrium-density gradient centrifugation was found to be characteristic of the comoviruses (Van Kammen, 1972). The bands corresponded to empty top component, and middle and bottom components encapsidating viral RNA2 and RNA1,

respectively. The particles appeared to be identical morphologically (Bruening, 1977; Schmidt et al., 1983). Both middle and bottom components were required for systemic infection of host plants (Bruening & Agrawal, 1967). CPMV-SB typically had an equal ratio of middle to bottom component (Bruening, 1969), while CPSMV-DG had a significantly greater amount of middle component than bottom component (de Jager, 1979). Top component of CPMV-SB was less than 5% of the total virus, while top component compromised an even lower percentage of CPSMV-DG preparations. Bruening (1969) suggested the formation of top component was determined by RNA2 based on pseudo-recombination experiments, using a wild type strain of CPMV and a mutant that was deficient for production of top component. De Jager and Van Kammen (1970) identified a nitrous acid mutant of CPMV-SB which produced more top than middle component. The mutation was localized to RNA2 by heterologous recombination with wild-type virus. Since RNA2 also coded for capsid polypeptides (Pelham, 1979), the formation of stable, empty virions could be determined by the primary amino acid sequence of the capsid polypeptides.

In order to examine the interaction between these two viruses in co-inoculated cowpea lines, it would be essential to identify either virus independent of the other. Monoclonal antibodies have been used to distinguish between serologically related viruses in plant sap (Massalski & Harrison, 1987; Dekker et al., 1988). Therefore, hybridoma cell lines were

generated that secrete antibodies against CPMV and CPSMV.

METHODS:

Virus and plant stocks. CPSMV-DG strain and CPMV-SB strain were obtained from the laboratory stocks of G. Bruening. Both viruses were maintained in the cowpea line *Vigna unguiculata* Walp. California Black Eye (BE-5; Vermont Bean Seed Co.). Viruses were isolated in our laboratory according to published procedures (Bruening, 1969). Primary and secondary leaves of BE-5 were harvested 7 and 14 days post inoculation, respectively. Infected tissue was frozen on dry ice and stored for up to 1 year at -20 C. All isolation procedures were performed at 4 C. Frozen tissue was ground in a Waring Blender with 3 ml per gram of leaves of ice cold grinding buffer (50 mM potassium phosphate, pH 7.0; 2 mM disodium ethylene diamine tetra-acetate (EDTA), 56 mM 2-mercaptoethanol, 79 mM sodium bicarbonate). The homogenate was filtered through Miracloth (CalBiochem). The solids were re-extracted with 0.5 ml per gram of leaves of grinding buffer and again filtered. The filtrates were combined and centrifuged at 15,000 g for 10 minutes. The supernate was collected, and stirred on ice for 20 minutes, with an equal volume of a 1:1 mixture of normal butanol and chloroform. The phases were resolved by centrifugation at 7000 g for 10 minutes. The upper aqueous phase was centrifuged again at 15,000 g for 10 minutes. The recovered supernatent layer was adjusted to 0.2 M sodium chloride and 7% (w/w) polyethylene glycol (PEG-8000, Sigma),

and stirred on ice until completely dissolved. The solution was stored for 5 hours to overnight.

The precipitate was collected by centrifugation at 8000 g for 10 minutes. The pellet was washed with 1 ml of 50 mM potassium phosphate, pH 7.0; 2 mM disodium EDTA per 20 grams of leaves. The extract was re-centrifuged, and the supernates were combined. The virus was recovered by high speed centrifugation in a 70Ti rotor (Beckman Instruments) for 2 hours at 45,000 rpm. The virus pellet was resuspended in 50 mM potassium phosphate, pH 7.0.

The concentration of virus was calculated using absorptivity values for 1 mg/ml of 8.1 at 260 nm (Geelen et al., 1972). Purity of virus stocks was confirmed by host range and RNA hybridization assays.

Separation of virus components. Approximately 5 mg of virus was diluted to 0.5 ml in gradient buffer (50 mM potassium phosphate; 2 mM disodium EDTA, pH 7.0) and layered onto 11 ml of 39% (w/w) caesium chloride in buffer (final concentration 37.3% w/w). The gradient was centrifuged for 36 hours at 4 C at 36,000 rpm in a SW 40Ti rotor (Beckman Instruments). Bands were visualized by diffraction of white light, and collected individually by piercing the side of the tube with a syringe needle. The gradient fractions containing virus components were diluted at least ten-fold with gradient buffer and pelleted by centrifugation at 45,000 rpm for 2 hours. The

pellets were resuspended in 50 mM potassium phosphate, pH 7.0. Concentration of the virus components was calculated using absorptivity values for 1 mg/ml of 6.2 and 10.0 at 260 nm for middle and bottom virus components, respectively, and 1.28 at 280 nm for top component (Geelen et al., 1972).

Plant extracts. Cowpeas were infected with virus and samples of primary infected leaves were collected 5 to 7 days after inoculation. A disc of tissue (approximately 100 mg) was taken with a #10 cork borer and stored at -20 C. Each disc was homogenized in 600 ul of phosphate-buffered saline (PBS: 140 mM NaCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 2.7 mM KCl, pH 7.4) plus 0.05% Tween-20, 1% ovalbumin (Sigma), and 0.1% polyvinyl-pyrrolidone. The suspension was centrifuged briefly. The supernate was diluted 1:100 in extraction buffer and 100 ul applied to a microtitre plate previously coated with the immunoglobulin fraction from rabbit serum (see below).

Virus particle disruption. Virus particles were subjected to one of two treatments. In the first procedure, virus was heated at 65 C for 1 hour in disruption buffer (8 M urea, 0.2% 2-mercaptoethanol, 1% sodium dodecyl sulfate (SDS)) (Wu & Bruening, 1971). The soluble product was dialyzed overnight against either PBS plus 0.1% 2-mercaptoethanol, or 8 M urea in PBS plus 0.1% 2-mercaptoethanol. Protein concentrations were determined by absorbance at 280 nm. Samples were further diluted in PBS as required, and used directly in plate-trapped

ELISAs. To assess the extent of virion dissociation induced by this procedure, a sample of treated virus was subjected to gel filtration chromatography. Approximately 5 mg of protein was applied to a column of Sephadex G-200 (45 X 2.5 cm) equilibrated with 5 M urea, 0.2% 2-mercaptoethanol in PBS. The protein peak was detected by absorbance at 280 nm and protein concentration in column fractions was determined by the method of Sedmak and Grossberg (1977). Samples from individual fractions within the protein peaks were subjected to denaturing gel electrophoresis (Laemmli, 1970).

In the alternate disruption method, virus was incubated overnight at 0 C in 2.65 M guanidine-HCl, 1.65 M LiCl, 5 mM 2-mercaptoethanol, 1 mM boric acid and 0.5 mM NaOH (Wu & Bruening, 1971). The precipitate was removed by centrifugation and the protein was recovered from the supernatent layer by exhaustive dialysis against distilled water at 4 C. The material that precipitated during dialysis was collected by centrifugation and redissolved in sample buffer (5 M urea, 0.2% 2-mercaptoethanol, in one-quarter strength PBS) by heating at 37 C. Samples of disrupted virus were applied to a Sephadex G-200 column (90 X 1.5 cm) equilibrated with sample buffer. The peaks were detected by absorbance at 280 nm and the two protein peaks collected, dialyzed against water, and concentrated by lyophilization. The identification of protein peaks as the large and small capsid protein subunits was confirmed (data not shown) by denaturing polyacrylamide gel

electrophoresis (Laemmli, 1970). The dried samples were redissolved in sample buffer and used for plate-trapped ELISAs.

Ribonuclease treatment of virus. Unfractionated virus and isolated, intact or dissociated virus components were dialyzed against TE buffer (10 mM Tris-HCl, pH 7.5; 1mM EDTA) and exposed to Ribonuclease A1 (1 mg/ml; Sigma) for 30 minutes. Samples were used for plate- or antibody-trapped ELISAs.

Immuno-blotting. Samples of virus were disrupted in urea, SDS and 2-mercaptoethanol as described above. Proteins were resolved by denaturing gel electrophoresis (Laemmli, 1970) and the protein transferred to nylon membranes (GeneScreen) by standard procedures (Burnette, 1981). Electroblotting was done at 4 ^{o}C at 250 mA overnight in transfer buffer (25 mM Tris-HCl, pH 8.3; 192 mM Glycine).

Non-specific binding sites were blocked with blocking buffer (PBS, 0.05% Tween-20, 2% fetal calf serum (FCS)) at room temperature for 2-4 hours with gentle agitation. Probing monoclonal antibodies were added in blocking buffer, and incubated as described above. The antibody dilution used was selected to give maximum signal relative to background. Bound monoclonal antibodies were detected with peroxidase-labelled goat anti-mouse-(IgG & IgM) antibodies (Kirkegard & Perry Lab Inc.) and substrate solution (0.5 mg 4-chloro-1-naphtol and 0.03% hydrogen peroxide in 1:5 methanol:PBS). Between

incubation with each antibody, the blots were washed for 10 minutes: once with PBS plus 0.05% Tween-20 (PBS-tween), thrice with blocking buffer, and once with PBS-tween.

Production of rabbit immunoglobulins. Antiserum was prepared from rabbits (New Zealand White) following one subcutaneous injection of 100 ug virus in Freund's complete adjuvant and three bi-weekly injections of virus in Freund's incomplete adjuvant. Sera R90 and R94 were prepared from rabbits immunized with CPSMV-DG and CPMV-SB, respectively. Serum was stored at -20 C as a 1:1 solution with glycerol. The serum was dialyzed against PBS and the immunoglobulin fraction was purified on a column of DEAE-Sephacel (Tremaine et al., 1985a) before being used for trapping.

Production of hybridoma cell lines. The production and culture of hybridomas was essentially as previously described (Goding, 1980; Oi & Herzenberg, 1980). Balb/c mice (Charles River) were immunized with four to six bi-weekly intraperitoneal injections of 50 ug virus in Freund's incomplete adjuvant. Three to 5 days before the fusion, the mice were primed with a final injection of antigen without adjuvant.

All further manipulations were done aseptically in a bio-hazard laminar-flow hood. Cells were cultured at 37 C in a 10% CO_2 environment. The media was supplemented Dulbecco's Modified Eagles Medium (DMEM, supplemented with 40 mM sodium

bicarbonate, 2 mM L-glutamate, 1 mM pyruvate, 35 mg/ml gentamycin sulfate) plus 10-20% heat-treated (60 C, 30 minutes) FCS. NS-1 myeloma cells were harvested at a log phase of growth. The spleens of immunized mice were macerated through cheesecloth in DMEM (no FCS) and the cell suspension collected with a pasteur pipet. The NS-1 and spleen cells were centrifuged at 800 g for 10 minutes; the pelleted cells were resuspended in DMEM, and the wash repeated twice. The cells were combined in a conical centrifuge tube and pelleted. The supernatant fluid was removed and the slurry of cells agitated in a tube mixer (Vortex). Cell fusion was done at 37 C by adding 1 ml of 50% PEG-8000 in DMEM slowly over 1 minute with a pasteur pipette, used also to stir the cell pellet. The cell pellet was stirred for an additional minute. An additional 1 ml of DMEM was added over 1 minute with stirring, then 7 ml of DMEM was stirred in over the next 4 minutes. The suspension was centrifuged as described above and the supernate discarded. The pellet was resuspended in 160 ml of HAT medium (80% DMEM, 20% FCS, 1.6 mM thymidine, 10 mM hypoxanthine, 0.04 mM aminopterin, 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.0), fortified with thymocytes (obtained from two 4 to 6-week old mice, and washed as described for the spleen cells above). Aliquots (200 ul) of the suspension were then dispersed into eight 96-well culture plates and incubated at 37 C. The approximate ratio of spleen cells to myeloma cells at fusion was 4:1.

Ten to 14 days post-fusion, the media was removed from the wells, and replaced with 200 ul of HT (DMEM, 20% FCS, 1.6 mM thymidine, 10 mM hypoxanthine). The plates were re-incubated for approximately 2 days, after which the hybridoma culture fluids were screened individually for secretion of desired antibodies by both an antibody-trapped and a plate-trapped indirect ELISA as described below. Hybridomas that tested positive for desired antibody secretion were subjected to a minimum of two cycles of limiting dilutions and rescreening to ensure monoclonality and stability of the cell lines. Clones which still tested positive were cryo-preserved in freezing buffer (DMEM, 20% FCS, 10% dimethylsulfoxide) at -70 C for 24 hours, then transferred to liquid nitrogen for long-term storage.

Ascites fluid collection. Approximately 10^6 cells were collected from culture media and injected intraperitoneally into 4 to 8 month old mice treated 1 to 2 weeks previous with Pristane (Sigma). The ascites fluid was collected after 7 to 12 days, depending on the rate of tumour development.

Isotyping of monoclonal antibodies. The sub-isotypes or sub-classes of antibodies were determined by ELISA. The reagents and test antibodies were in kit form (CalBiochem) and used according to the manufacturers recommendations.

Ouchterlony assay. The double-diffusion immunoassay was performed in 0.7% agar in PBS (Stollar & Levine, 1963).

bicarbonate, 2 mM L-glutamate, 1 mM pyruvate, 35 mg/ml gentamycin sulfate) plus 10-20% heat-treated (60 C, 30 minutes) FCS. NS-1 myeloma cells were harvested at a log phase of growth. The spleens of immunized mice were macerated through cheesecloth in DMEM (no FCS) and the cell suspension collected with a pasteur pipet. The NS-1 and spleen cells were centrifuged at 800 g for 10 minutes; the pelleted cells were resuspended in DMEM, and the wash repeated twice. The cells were combined in a conical centrifuge tube and pelleted. The supernatant fluid was removed and the slurry of cells agitated in a tube mixer (Vortex). Cell fusion was done at 37 C by adding 1 ml of 50% PEG-8000 in DMEM slowly over 1 minute with a pasteur pipette, used also to stir the cell pellet. The cell pellet was stirred for an additional minute. An additional 1 ml of DMEM was added over 1 minute with stirring, then 7 ml of DMEM was stirred in over the next 4 minutes. The suspension was centrifuged as described above and the supernate discarded. The pellet was resuspended in 160 ml of HAT medium (80% DMEM, 20% FCS, 1.6 mM thymidine, 10 mM hypoxanthine, 0.04 mM aminopterin, 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.0), fortified with thymocytes (obtained from two 4 to 6-week old mice, and washed as described for the spleen cells above). Aliquots (200 ul) of the suspension were then dispersed into eight 96-well culture plates and incubated at 37 C. The approximate ratio of spleen cells to myeloma cells at fusion was 4:1.

Ten to 14 days post-fusion, the media was removed from the wells, and replaced with 200 ul of HT (DMEM, 20% FCS, 1.6 mM thymidine, 10 mM hypoxanthine). The plates were re-incubated for approximately 2 days, after which the hybridoma culture fluids were screened individually for secretion of desired antibodies by both an antibody-trapped and a plate-trapped indirect ELISA as described below. Hybridomas that tested positive for desired antibody secretion were subjected to a minimum of two cycles of limiting dilutions and rescreening to ensure monoclonality and stability of the cell lines. Clones which still tested positive were cryo-preserved in freezing buffer (DMEM, 20% FCS, 10% dimethylsulfoxide) at -70 C for 24 hours, then transferred to liquid nitrogen for long-term storage.

Ascites fluid collection. Approximately 10^6 cells were collected from culture media and injected intraperitoneally into 4 to 8 month old mice treated 1 to 2 weeks previous with Pristane (Sigma). The ascites fluid was collected after 7 to 12 days, depending on the rate of tumour development.

Isotyping of monoclonal antibodies. The sub-isotypes or sub-classes of antibodies were determined by ELISA. The reagents and test antibodies were in kit form (CalBiochem) and used according to the manufacturers recommendations.

Ouchterlony assay. The double-diffusion immunoassay was performed in 0.7% agar in PBS (Stollar & Levine, 1963).

Antigen (40 ul of virus at 200 ug/ml) was pipetted into wells adjacent to wells containing 40 ul of serially diluted antibody solutions in PBS.

ELISA. Three forms of indirect ELISA were used, antibody-trapped, plate-trapped and liquid-phase competition assays (Tremaine et al., 1985b). For the antibody-trapped assay, rabbit immunoglobulins were diluted 1:100 in PBS, and 100 ul was incubated in each well of a Linbro microtitre plate (Flow Laboratories) overnight at 4 C. Plates were washed with tap water, between each successive binding step. The plates were blocked for 2 hours at 37 C with 200 ul of 1% ovalbumin in PBS-tween. If the sample was to contain plant extract, 0.1% soluble polyvinylpyrrolidone was included in the blocking buffer (Clark et al., 1986). The sample antigen was added in 100 ul of blocking buffer and incubated 2 hours at 37 C or overnight at 4 C. For plate-trapped assays, antigen in the desired buffer was added directly to microtitre plates and incubated overnight at 4 C. The remainder of the assays were identical for both antibody- and plate-trapped ELISAs. Antibody was added in 100 ul of blocking buffer and incubated 2 hours at 37 C. The antibody dilution used was selected to give maximum absorbance relative to background with 30 ug/ml antigen. Rabbit immunoglobulins were diluted 1:100. Monoclonal antibodies DG5, DG8, SB3, SB7, SB8, and SB9 were obtained in culture fluid and used diluted 1:1 with PBS. Ascites fluid

containing DG3 was diluted 1:2000; DG4 was diluted 1:4000 and all others were diluted 1:5000.

Liquid-phase competition assays were done as described by Tremaine et al. (1985a) and Muller et al. (1986). Antigen and murine monoclonal antibody were incubated together in PBS-tween overnight at 23 C. The concentration of monoclonal antibody was adjusted to give approximately 75% saturation by plate- or antibody-trapped ELISAs. The resulting mix was added (100 ul per well) to microtitre plates with saturating amounts of homologous antigen bound to rabbit immunoglobulins coated in the wells (antibody-trapped liquid-phase competition assay), or to microtitre plates with saturating amounts of antigen bound directly to the wells (plate-trapped liquid-phase competition assay). The plates were then incubated 2 hours at 37 C. In this way, a relative measure of the free murine antibody versus murine antibody bound to the competing antigen was obtained.

In all cases, the quantity of antibody bound was detected by incubating the plates for 2 hours with 100 ul per well of either goat antimouse-(IgG + IgM) or goat antirabbit-(IgG + IgM) conjugated to alkaline phosphatase (Kirkegard & Perry Lab Inc.). Colour was developed was based on the conversion of p-nitrophenol phosphate to p-nitophenol (Sigma) in 10% diethanolamine-HCl, pH 9.8. Absorbance values were determined with a microtitre plate reader (Bio-Tek) and the reported values are the difference between readings at 405 and 495 nm.

Each antigen was tested on one plate with all antibodies. Each experiment consisted of two to three duplicate plates and the experiments were repeated three times with the exception of tests with guanidine-HCl disrupted virus proteins, these were only performed twice.

RESULTS:

General characterization of the monoclonal antibodies. The fusion and screening processes yielded a number of hybridomas that were stable upon successive recloning and limiting dilutions. Antibodies in subclasses IgG, IgM and IgA were obtained (Tables 1 and 2). Each of the antibodies was tested for its ability to cause the formation of visible precipitin lines in the Ouchterlony double-diffusion assay. A total of four antibodies (SB5, DG7, DG8 and DG11) gave obvious precipitin lines.

Tables 1 and 2 summarize data from antibody-trapped and plate-trapped indirect ELISAs. Both homologous and heterologous assay results are presented to indicate the specificity of the antibodies in each form of assay. Monoclonal antibodies SB2, SB5, DG7, DG9 and DG11 exhibited the greatest selectivity as indicated by much higher assay yields in homologous ELISAs as compared to heterologous assays. The ability to discriminate between these comoviruses was particularly evident in antibody-trapped ELISAs. With the exception of antibodies SB2 and DG4, the recognition of antigen and the ability to discriminate between the viruses was diminished severely in plate-trapped ELISAs.

The selectivity of several monoclonal antibodies was examined, and confirmed by liquid-phase competition assays. The soluble phase most closely approximates the native virus

TABLE 1. Characterization of monoclonal antibodies produced against CPMV.

MONOCLONAL ANTIBODY	ISOTYPE	OUCHTERLONY	ELISA RESULTS[1]			
			ANTIBODY-TRAPPED[2]		PLATE-TRAPPED[3]	
			CPMV	CPSMV	CPMV	CPSMV
SB1	IgG_3	–	+	–	+	–
SB2	IgG_{2a}	–	++++++	+	+++++	+
SB3	IgG_{2b}	–	–	–	+	+
SB4	IgG_{2a}	–	++	+	+	+
SB5	IgG_{2b}	+	++++	–	+	–
SB7	IgM	–	+	+	+	+
SB8	IgM	–	+	+	+	+
SB9	IgM	–	–	–	+	+

1. The ELISA absorbance readings above background were grouped as follows: >2.53, ++++++; 2.04 - 2.53, +++++; 1.54 - 2.03, ++++; 1.04 - 1.53, +++; 0.54 - 1.03, ++; 0.04 - 0.53, + and < 0.04, -.

2. Plates were coated with homologous rabbit immunoglobulins; background = 0.080.

3. Background = 0.095.

condition (Muller et al., 1986), as binding to polystyrene plates or antibodies can induce distortion of the antigen (Tainer et al., 1984). Liquid-phase competition assays revealed that in this form of assay, antibodies SB2, SB5, DG4 and DG11 were very selective (Figure 1). Antibodies SB4, DG7 and DG9 demonstrated similiar selectivity for immunizing antigen in this assay (data not shown). These data are consistent with the plate- and antibody-trapped ELISAs. These results confirm the isolation of hybridomas secreting monoclonal antibodies highly selective for each of the immunizing comoviruses. Results obtained with rabbit immunoglobulins are presented for comparison.

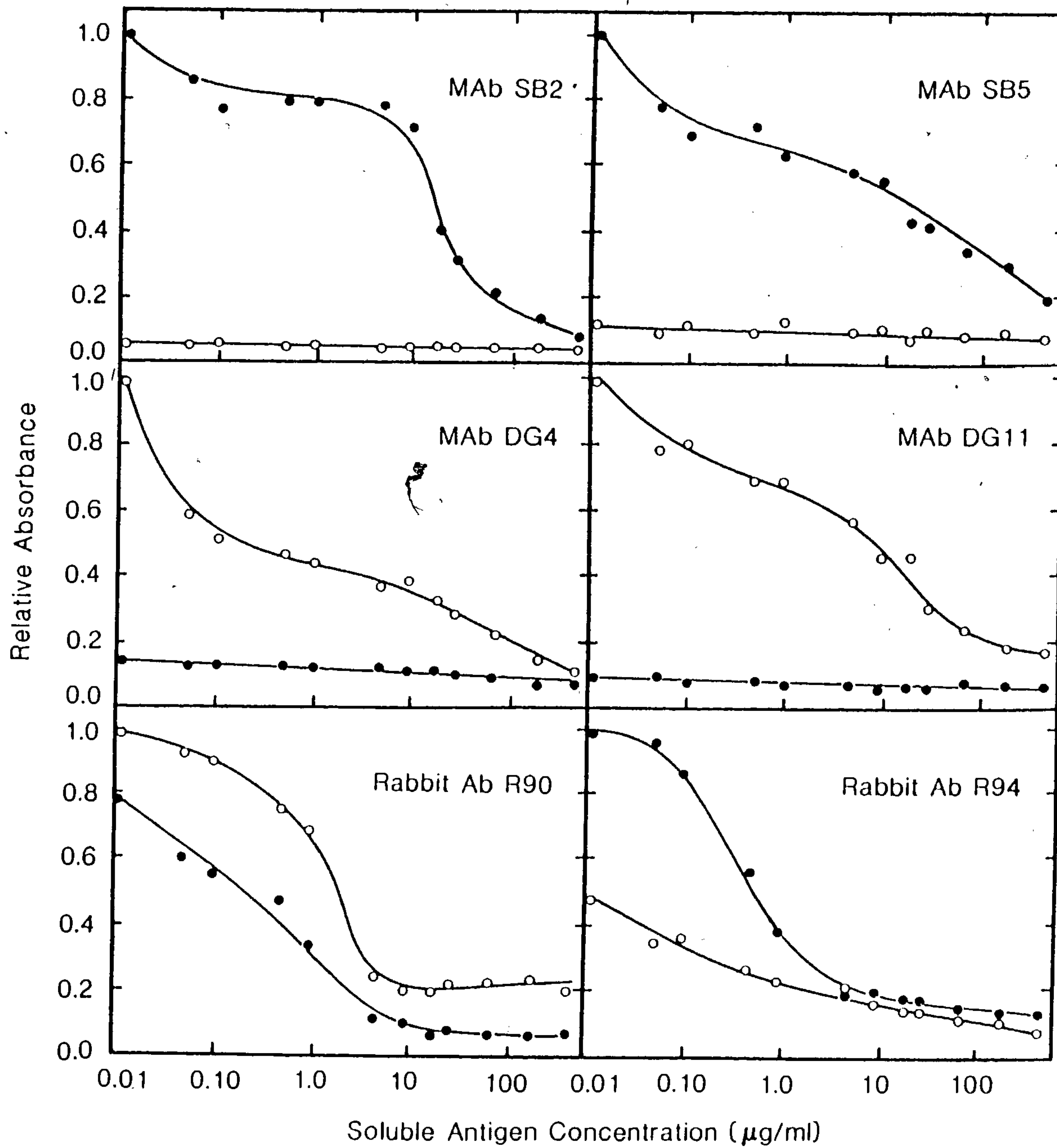
Recognition of viral antigens in plant extracts. One of the major incentives for the preparation of monoclonal antibodies was to develop an assay system that was capable of discriminating between serologically related viruses in extracts of infected plants. Table 3 demonstrates the ability of the panel of monoclonal antibodies to recognize the appropriate viral immunogen in diluted extracts (1:100) of virus-infected cowpeas. Undiluted extracts give a proportionally higher absorbance value, but for the purposes of comparison, the diluted values are presented. The non-specific retention of monoclonal antibodies in sample wells containing extracts from uninfected plants is very low, suggesting that the antigens recognized by the antibodies are not of host origin. Only antibody DG6 produces an

TABLE 2. Characterization of monoclonal antibodies produced against CPSMV.

MONOCLONAL ANTIBODY	ISOTYPE	OUCHTERLONY	ELISA RESULTS[1]			
			ANTIBODY-TRAPPED[2]		PLATE-TRAPPED[3]	
			CPMV	CPSMV	CPMV	CPSMV
DG3	IgM	-	+	+++	++	++
DG4	IgG_1	-	-	+	-	+
DG5	IgM	-	++	++++	+	+++
DG6	IgM	-	+	+	-	-
DG7	IgA	+	-	+++	+	+
DG8	IgG_{2a}	+	-	+	-	-
DG9	IgG_{2a}	-	-	++	-	+
DG11	IgG_{2a}	+	-	++++++	-	+

1. For the ranges of ELISA absorbances, refer to Table 1.
2. Antigen was trapped with homologous rabbit immunoglobulins; background = 0.050.
3. Background = 0.080.

Figure 1. Liquid-phase competition assays demonstrate the specificity of monoclonal antibodies for the indicated immunogen. Rabbit antibody data are presented for comparison. Antibody is incubated with soluble antigen; the antibody remaining unbound at this time is then allowed to react with antigen bound to the microtitre plate. The relative ELISA absorbance is therefore a direct reflection of the amount of unbound antibody after the initial incubation period. Soluble competing antigen: CPMV (●); CPSMV (o). All assays represented are antibody-trapped ELISAs.


MAb SB2
MAb SB5
MAb DG4
MAb DG11
Rabbit Ab R90
Rabbit Ab R94
Relative Absorbance
1.0
0.8
0.6
0.4
0.2
0.0
0.01
0.10
1.0
10
100
Soluble Antigen Concentration (μg/ml)

unacceptable, high background value in plant extracts, whereas reasonable relative signal and background values are found in assays of purified virus (Table 2). This high background in healthy plant extracts suggests that antibody DG6 may be against a plant protein contamination of isolated virus. No further characterization of this antibody was performed.

Sensitivity of virus antigen recognition. The presence of antigen in plant samples is routinely assayed via an antibody-trapped ELISA. To evaluate the utility of these monoclonal antibodies for such assays, the limits of detection for the antibody-trapped ELISA were determined. Virus was diluted in tissue extracts from healthy plants and the mixture assayed (Figure 2). As anticipated, the sensitivity of ELISA was highly dependent on the particular antibody employed. Under these assay conditions, monoclonal antibodies SB2, SB5 and DG11 enabled detection of 0.05 ug/ml or more of virus, but antibody DG4 did not detect virus at less than 5 ug/ml. (However, if DG4 was employed in a plate-trapped ELISA, the sensitivity was found to increase significantly to 0.05 ug/ml (data not shown).) SB4 and DG9 were of intermediate sensitivity with detection limits of approximately 0.5 ug/ml.

Recognition of altered conformations of viral antigens. During screening and recloning of hybridomas, the secreted antibodies were tested for their ability to recognize the antigen in either an antibody-trapped or a plate-trapped

TABLE 3. Detection of viral antigens in plant extracts by antibody-trapped indirect ELISA assays.

MONOCLONAL ANTIBODY	HEALTHY CONTROL	CPMV INFECTED	CPSMV INFECTED	MONOCLONAL ANTIBODY	HEALTHY CONTROL	CPMV INFECTED	CPSMV INFECTED
SB1	0.058	0.067	0.077	DG3	0.077	0.085	0.866
SB2	0.095	0.992	0.112	DG4	0.071	0.063	0.127
SB3	0.063	0.067	0.078	DG5	0.088	0.076	0.100
SB4	0.060	0.434	0.077	DG6	0.133	0.114	0.151
SB5	0.060	0.378	0.073	DG7	0.064	0.071	0.858
SB7	0.057	0.061	0.073	DG8	0.064	0.066	0.137
SB8	0.070	0.093	0.086	DG9	0.064	0.068	0.964
SB9	0.066	0.066	0.076	DG11	0.069	0.059	1.794

indirect ELISA (Tables 1 and 2). Significant differences between the recognition of antigen in the antibody-trapped versus plate-trapped ELISAs were evident. Binding of several monoclonal antibodies to antigen was reduced substantially in a plate-trapped ELISA relative to an antibody-trapped ELISA. Antibody from hybridomas SB4, SB5, DG3, DG7, DG9, and DG11 exhibited striking decreases in binding. Conversely, binding of SB1, SB3 and SB9 increased, although the differences were not as pronounced. Little or no change was observed with assays using other antibodies (SB2, SB7, SB8, DG4, DG5 and DG8).

The physical binding of antigen to the polystyrene plate in a plate-trapped type of immunoassay can cause pronounced alteration in particle structure and antigenic character (Friguet et al., 1984; Halk, 1986; Muller et al., 1986). Thus, antibodies SB4, SB5, DG3, DG7, DG9 and DG11 were specific for epitopes that were sensitive to the integrity of the virus particle; the distortion of virus structure that occurred upon binding to the plate modified or concealed the respective antigenic determinants and thereby reduced recognition of the epitope by antibody. It was also evident that antibodies SB1, SB3, and SB9 recognized epitopes that were normally shielded from antibody binding in the intact particle, but were more accessible as a result of particle deformation on binding to the microtitre plate.

Antibody DG5 bound to CPSMV with similar avidity in both

antibody- and plate-trapped ELISAs (Table 2). However, DG5 bound to CPMV only in an antibody-trapped ELISA (but with lesser affinity compared to CPSMV). In a plate-trapped ELISA, DG5 did not recognize CPMV. These observations suggested that DG5 recognized an external epitope of CPSMV. CPMV displayed an epitope of similiar configuration, but more labile. These results may have arisen from differences in the primary amino acid sequence that formed the epitope directly and/or determined the higher-order structure of the epitope (Al Moudallal et al., 1985; Motte et al., 1987).

Recognition of virus particles with altered conformation was examined further by increasing the severity of protein denaturation conditions. When virions were incubated with SDS plus urea, the affinity of the monoclonal antibodies decreased with some notable exceptions (Tables 4 and 5). SB2 continued to bind strongly to the treated virus particles. The binding of antibodies SB9 and DG4 increased significantly upon denaturation of CPMV-SB in SDS plus urea. These data suggest that the epitopes recognized by SB9 and DG4 were shielded by a relatively stable structure in the intact virion.

Gel filtration was used to assess the state of virion disruption induced by exposure to SDS plus urea. This treatment of either CPMV or CPSMV caused a dramatic change in the elution profile to components of lower molecular weight (Figure 3). Intact virions yielded a single peak (maximum absorbance in fraction 20), while virus treated with SDS plus

Figure 2. Sensitivity of monoclonal antibodies in antibody-trapped ELISA. A) CPMV was serially diluted in plant extract and assayed by ELISA. The antibodies tested were: SB2 (●); SB4 (■) and SB5 (o). B) Serial dilutions of CPSMV in plant extract were assayed by ELISA. The antibodies tested were: DG4 (●); DG9 (■) and DG11 (o). The inset axis refers to antibody DG4.

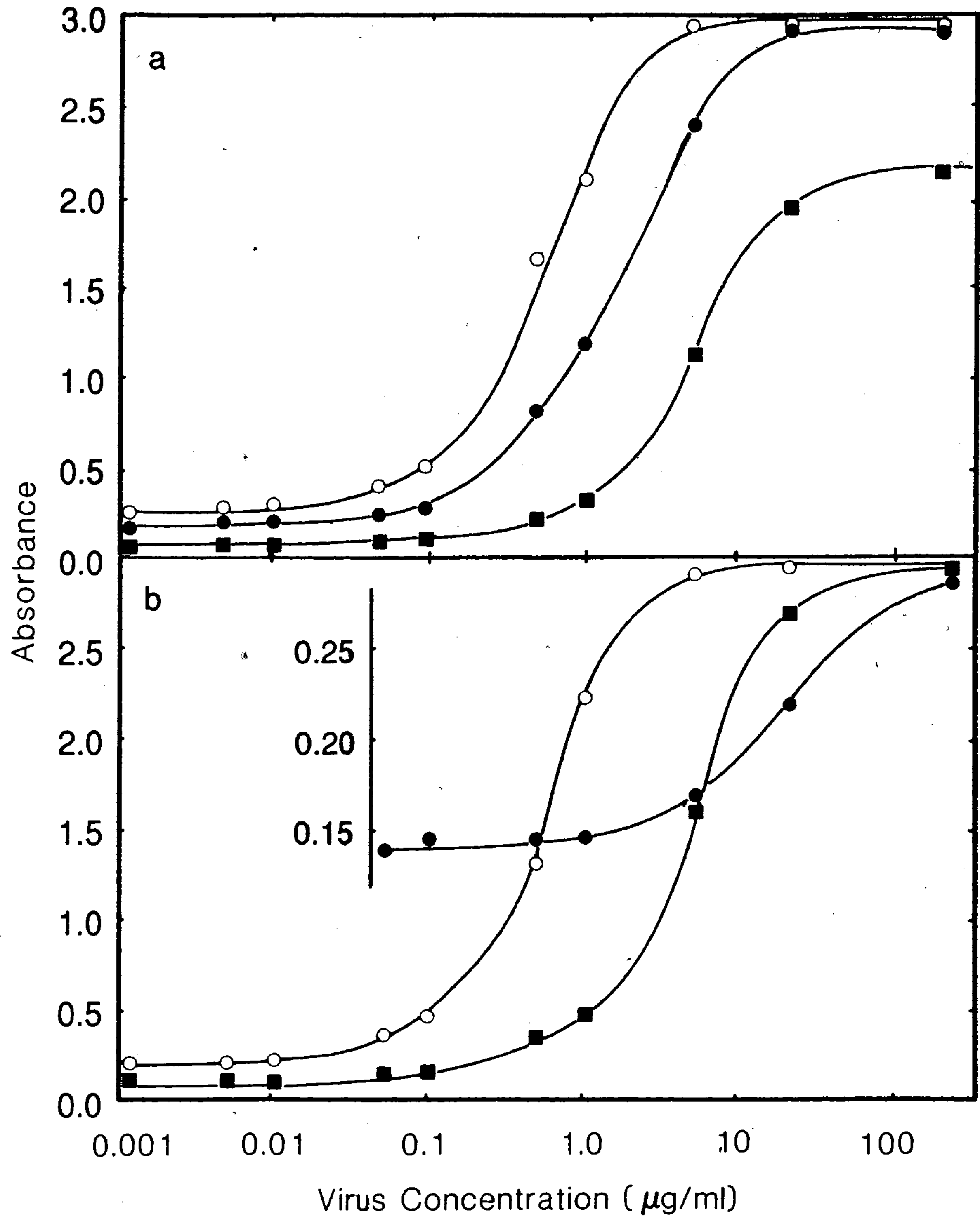

a
b
Absorbance
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.25
0.20
0.15
0.001
0.01
0.1
1.0
10
100
Virus Concentration (µg/ml)

TABLE 4. Monoclonal antibody binding to altered virus conformations in plate-trapped ELISAs.

MONOCLONAL ANTIBODY	ELISA RESULTS[1]			
	DENATURANT			
	SDS+UREA		GUANIDINE	
	CPMV	CPSMV	CPMV	CPSMV
SB1	+	-	+	+
SB2	++++	+	++++++	-
SB3	+	+	+	-
SB4	+	+	-	-
SB5	-	-	-	-
SB7	+	+	-	-
SB8	+	+	+	+
SB9	+	+	-	-

1. For the ranges of ELISA absorbances, refer to Table 1; background = 0.095.

TABLE 5. Monoclonal antibody binding to altered virus conformations in plate-trapped ELISAs.

MONOCLONAL ANTIBODY	ELISA RESULTS[1] DENATURANT			
	SDS+UREA		GUANIDINE	
	CPMV	CPSMV	CPMV	CPSMV
DG3	++	++	+	+
DG4	–	++	+	++
DG5	+	+++	+	–
DG7	+	+	+	–
DG8	–	–	+	–
DG9	–	–	–	–
DG11	–	–	–	–

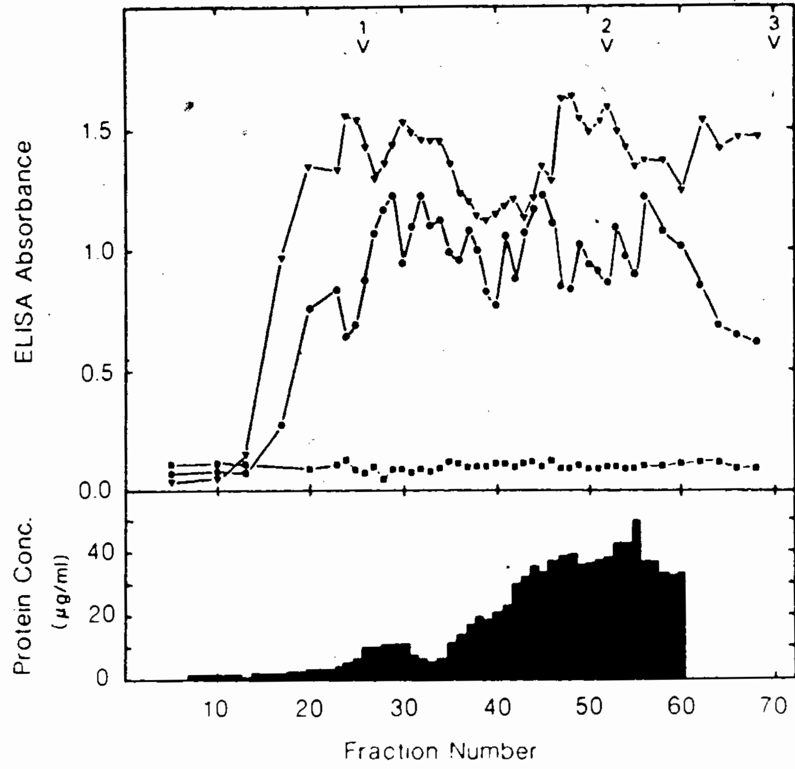
1. For the ranges of ELISA absorbances, refer to Table 1; background = 0.085.

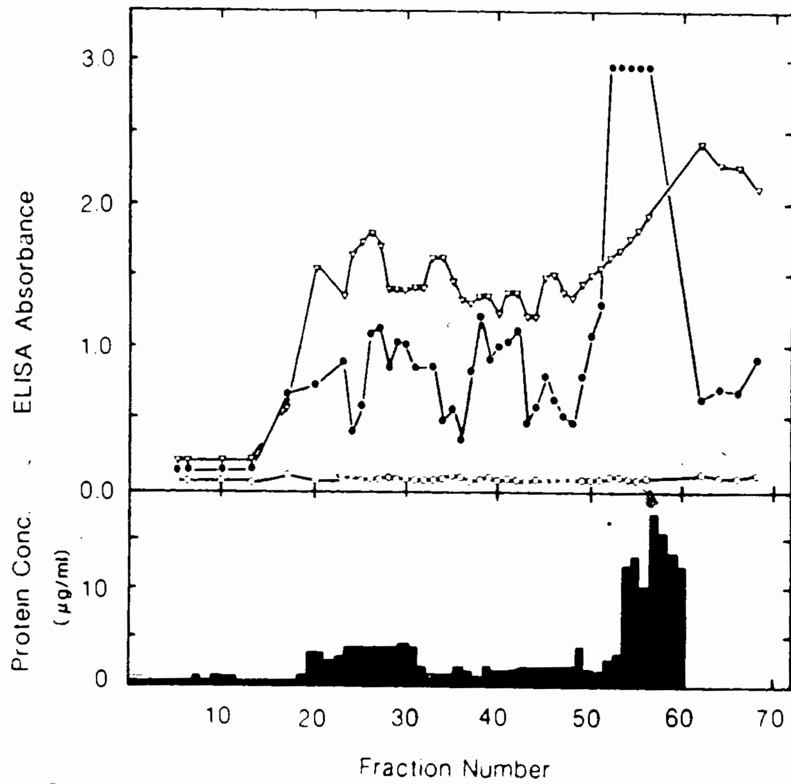
urea produced a range of molecular weights suggested by the broad distribution of protein containing-fractions, with the maximum occurring at fraction 55. This behavior implied extensive aggregation of the proteins; Wu & Bruening (1971) showed that the large capsid protein (VP37) was prone to aggregation, even in the presence of 8M urea. The integrity of virions disrupted by exposure to SDS plus urea was also analyzed by equilibrium-density gradient centrifugation (Figure 4). The treated virus particles yielded a single low density band suggesting that the disrupted virus particles were devoid of nucleic acid.

Individual column fractions were tested for the ability to bind antibodies. Antibody SB2 recognized protein fractions representing all molecular weight fractions of CPMV (Figure 3A). Rabbit antibodies R91 also recognized the entire spectrum of fractions, in keeping with the polyclonal nature of this preparation. Antibodies SB4 and SB5 were specific for intact virions, and failed to recognize any of the column fractions. An identical pattern emerged from analysis of CPSMV treated with SDS plus urea. Antibody DG4 and polyclonal rabbit serum bound to all column fractions while antibodies DG7, DG9 and DG11 did not recognize any of the column fractions (Figure 3B). Neither SB2 nor DG4 exhibited reactivity in heterologous assays of column fractions.

Guanidine is a more powerful protein denaturant than is urea-SDS, and is particularly effective in dissociating

Figure 3. CPMV (A) and CPSMV (B) were denatured with SDS plus urea and the products subjected to gel filtration through Sephadex G200 equilibrated with urea. Aliquots of the individual fractions were assayed by plate-trapped ELISA utilizing monoclonal antibodies. (A): SB2 (●), SB5 (■) and rabbit antibodies from pool R94 (▼). (B): DG4 (●), DG11 (o) and rabbit antibodies from pool R90 (▽). Background was $<$ 0.100. Protein was also determined in each fraction by calibration to a BSA standard. The indicated points correspond to the elution of molecular weight standards: 1) blue dextran, 2×10^6; 2) yellow dextran, 2×10^4; 3) vitamin B_{12}.


A
1
2
3
1.5
1.0
0.5
0.0
ELISA Absorbance
Protein Conc. (μg/ml)
40
20
0
10
20
30
40
50
60
70
Fraction Number


B
3.0
2.0
1.0
0.0
ELISA Absorbance
Protein Conc. (μg/ml)
10
0
10
20
30
40
50
60
70
Fraction Number

nucleoprotein complexes (Cox, 1968). Only monoclonal antibodies SB2 and DG4 recognized immunogen after guanidine treatment (Tables 4 and 5). The ability of these antigenic determinants to withstand extensive protein denaturation suggests that contiguous epitopes are involved (Sober et al., 1988). SB2 clearly reacted with VP37 subunit of CPMV with little or no recognition of denatured CPSMV proteins (Table 6).

Epitopes were further examined by immuno-blots of denaturing polyacrylamide gels (Figure 5). Antibodies SB2 and DG4 reacted in homologous assays with VP37. Antibodies SB4, SB5, DG7, DG9 and DG11 did not yield a detectable signal.

Selectivity of monoclonal antibodies for denatured virus. Many of the selected antibodies were able to distinguish between CPMV and CPSMV in plant sap (Table 3). The degree of virus disruption had a marked influence on the ability of some monoclonal antibodies to differentiate between viruses (Figure 6). Antibody SB1 discriminated between CPMV and CPSMV only if virus structure was modified. Structural perturbation increased the accessibility of a CPMV epitope, while binding to CPSMV remained unchanged. Antibodies SB4 and DG3 lost their power of differentiation upon denaturation of the virus. Antibody DG3 was of particular interest. With increasing degrees of perturbation, the ability to recognize CPSMV particles diminished, while binding to CPMV was enhanced. In SDS plus urea, both viruses bound this antibody equally. These

Figure 4. Separation of CPMV-SB viral components by equilibrium gradient centrifugation. A) Separation of native virus (T=top, M=middle, and B=bottom). B) Separation of virus heated in the presence of urea and SDS (D=dissociated virus).

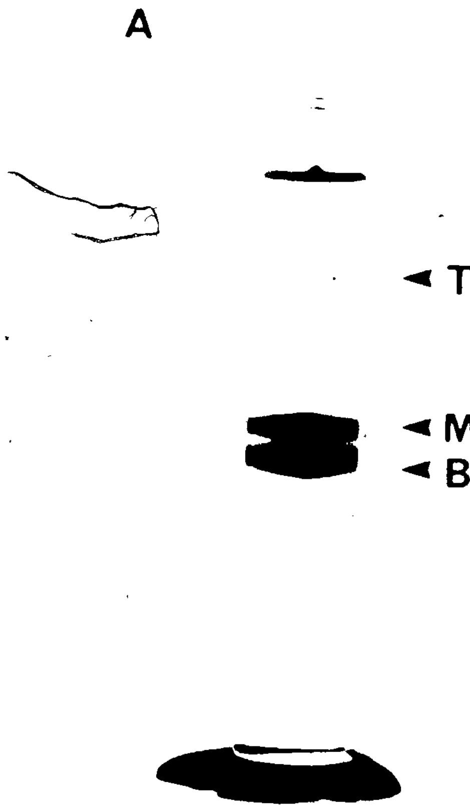
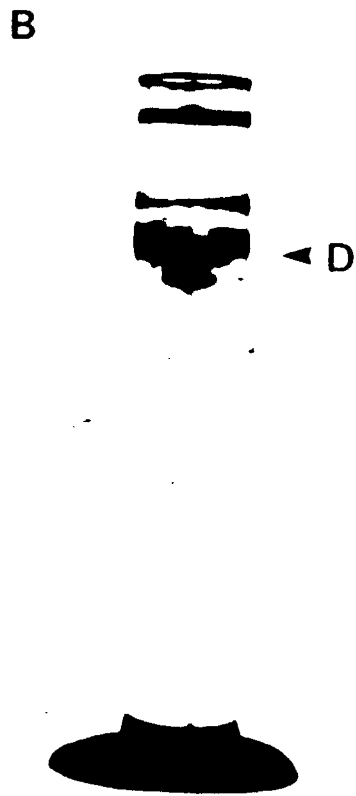
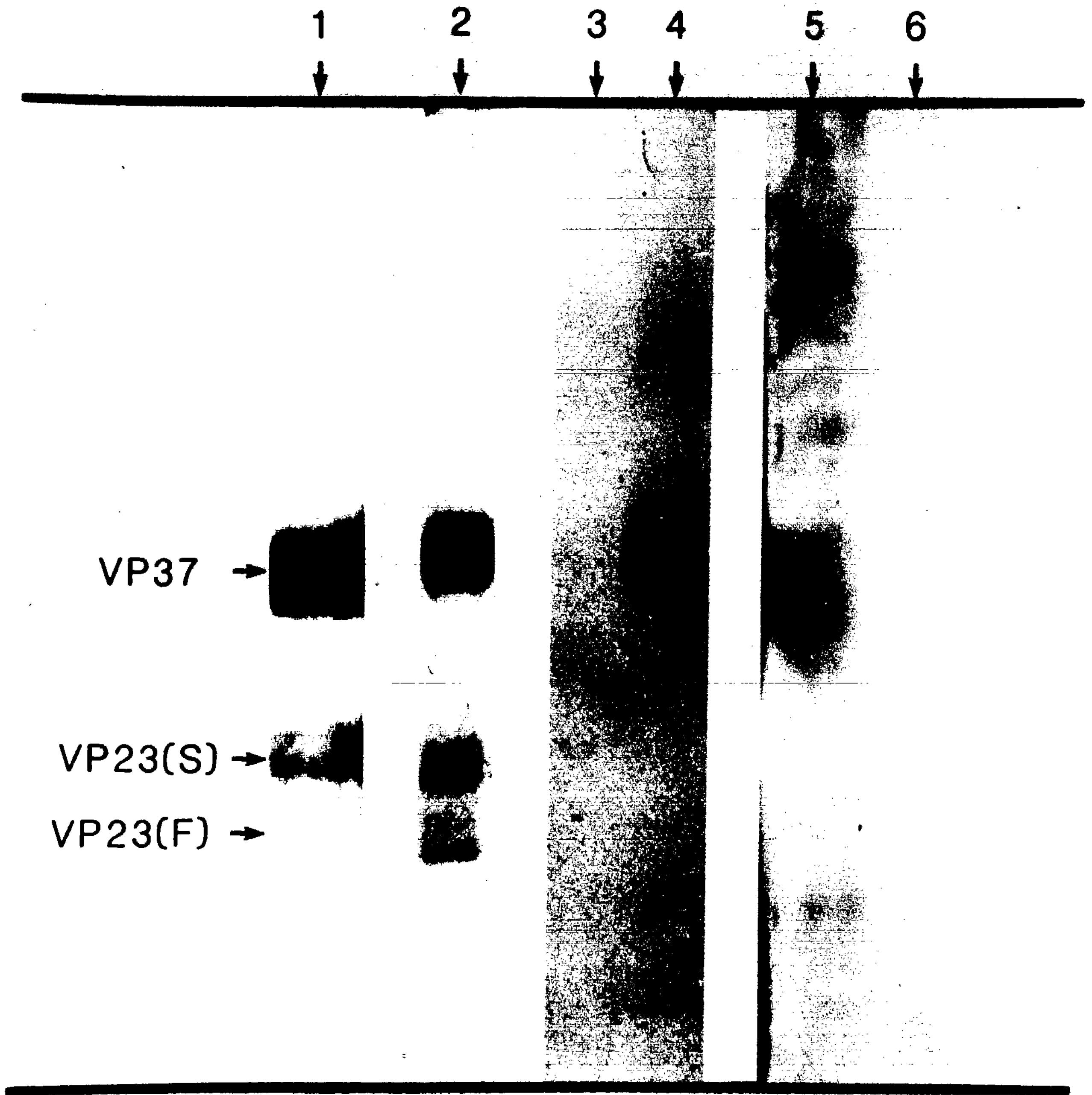

A
T
M
B

B
D

TABLE 6. Reactivity of monoclonal antibody SB2 in plate-trapped ELISAs with virus proteins disrupted with guanidine-LiCl.

PROTEIN SAMPLE (0.3 µg/ml)		ELISA RESULTS[1]
CPMV	- DISRUPTED VIRUS	++++++
	- LARGE SUBUNIT (VP37)	+++
	- SMALL SUBUNIT (VP23)	+
CPSMV	- DISRUPTED VIRUS	-
	- LARGE SUBUNIT	-
	- SMALL SUBUNIT	-

1. For the ranges of ELISA absorbances, refer to Table 1; background = 0.132.

Figure 5. Immunoblots of capsid proteins. Viral structural proteins from CPMV and CPSMV were denatured and resolved by discontinuous polyacrylamide gel electrophoresis. Representative lanes were stained for total protein while the remaining lanes were transferred to nylon membranes in a Western blot. Lanes 1 and 2 contain CPSMV and CPMV respectively, and were stained with Coomassie Brilliant Blue. The large (VP37) and small (VP23) subunits are visible. The two electrophoretic forms of VP23 are clearly evident. Lanes 3 and 4 contain CPSMV and CPMV respectively and were incubated with monoclonal antibody SB2. Lanes 5 and 6 were CPSMV and CPMV incubated with monoclonal antibody DG4.


1
2
3
4
5
6
VP37
VP23(S)
VP23(F)

data suggested that CPMV and CPSMV share a common epitope that is modified or shielded by neighboring groups in the intact virus particles of CPMV. However, as the degree of denaturation of the virus particle increased, the antibody exhibits preferential binding to CPSMV.

Recognition of separated viral components. The panel of fifteen monoclonal antibodies were tested for their ability to distinguish, in homologous binding assays, unfractionated virus and the individual density components. The vast majority of monoclonal antibodies bound to the unfractionated virus and the virus components with equal affinity (data not shown). However, a subgroup of monoclonal antibodies demonstrated distinct preferences in their binding patterns for individual virus components. In comparing binding affinities of antibody to top component versus middle or bottom components, the concentration at half maximal absorbance was shifted 1 to 2 log units (Figure 7: SB5; Figure 8: DG4, DG9 & DG11). Homologous plate-trapped ELISAs utilizing antibodies SB5, DG9 and DG11 yielded greater absorbance values for middle and bottom components than for top component. In contrast, plate-trapped ELISAs with antibody DG4 displayed enhanced signal strength for top component on a molar basis. No distinction in binding affinities were observed for any of the viral components in antibody-trapped ELISAs with antibody DG4. The data indicated that there was no significant difference in

Figure 6. Comparison of antibody cross-reactivity with virus of altered conformations. Shaded bars were ELISA results obtained with CPMV antigen, and the open bars were obtained with CPSMV antigen. These values were derived from Tables 1, 2, 4 and 5. The absorbance values indicated are net absorbances; the background absorbances was less than < 0.100.

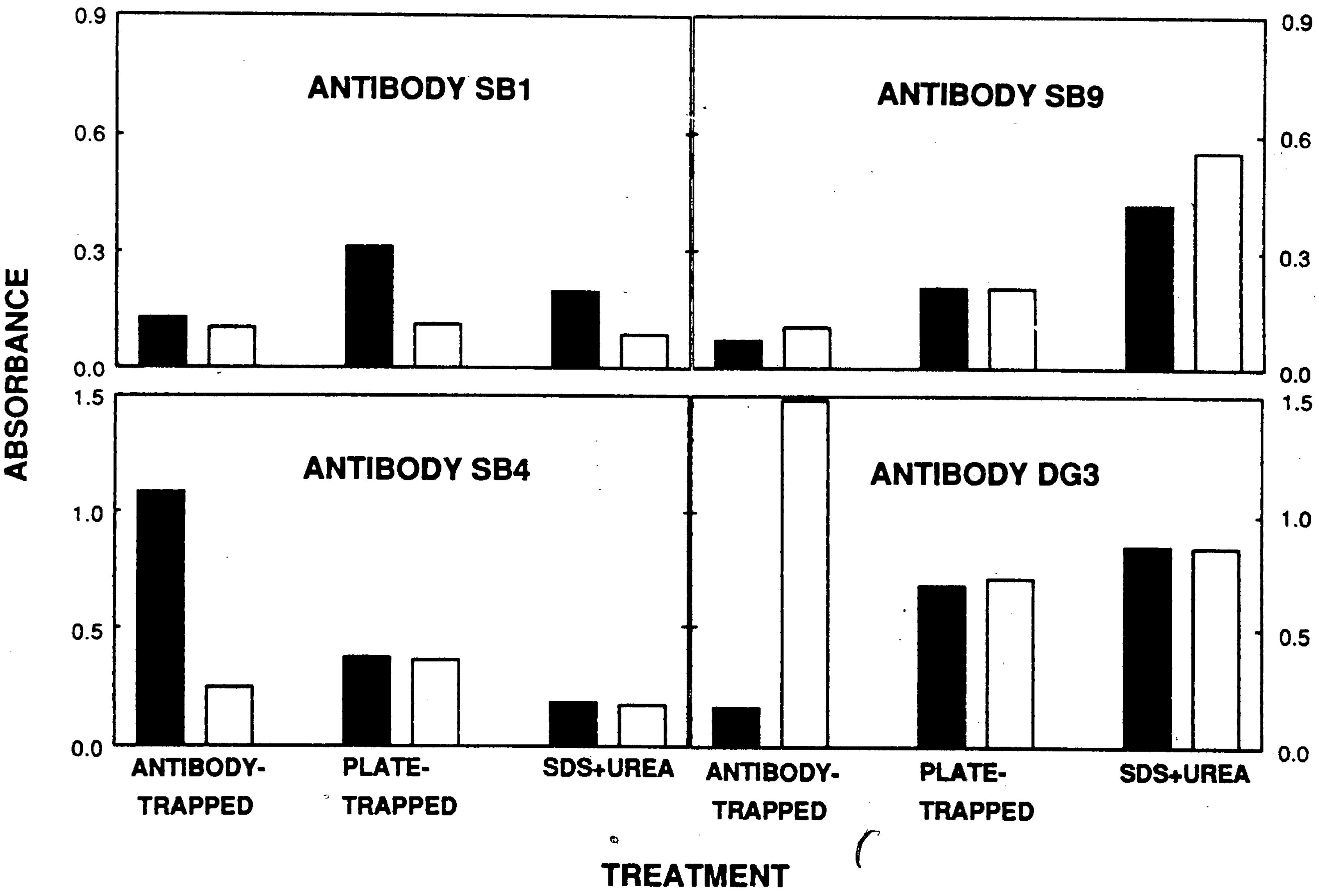
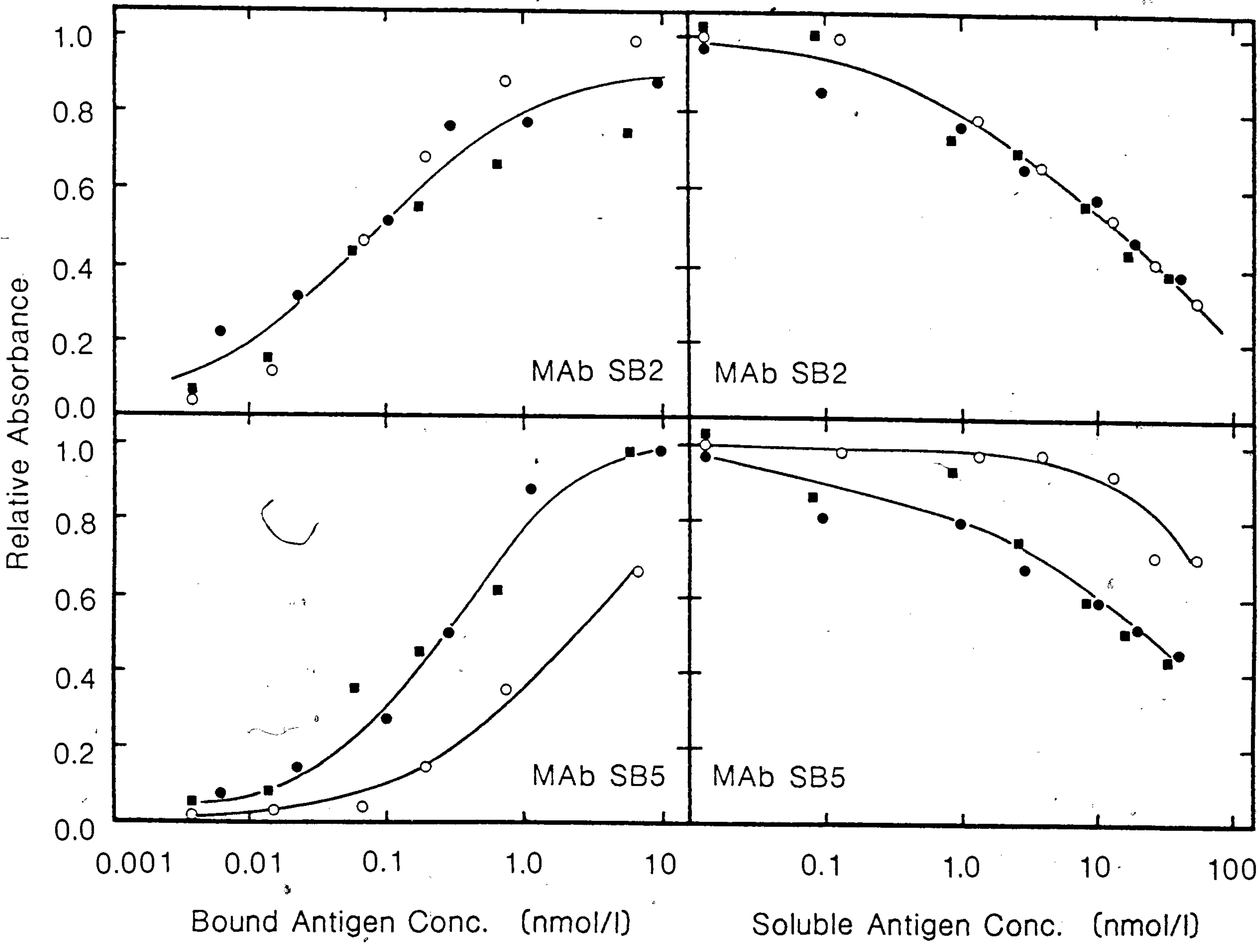

ANTIBODY SB1
ANTIBODY SB9
ANTIBODY SB4
ANTIBODY DG3
ABSORBANCE
0.9
0.6
0.3
0.0
1.5
1.0
0.5
0.0
ANTIBODY-TRAPPED
PLATE-TRAPPED
SDS+UREA
TREATMENT

Figure 7. A comparison of affinity ELISA assays and liquid phase competition assays using monoclonal antibodies against CPMV. Affinity assay (left panel): Each CPMV component is serially diluted and assayed by plate-trapped ELISA. Liquid-phase competition assay (right panel): The monoclonal antibody is incubated with the indicated antigen; the antibody remaining unreacted at the end of the reaction period is allowed to bind to whole virus preparation bound to microtitre plates pre-coated with homologous polyclonal antibodies. Antigen: top component (○); middle component (■) and bottom component (●). Standard deviation = =/- 10 %.



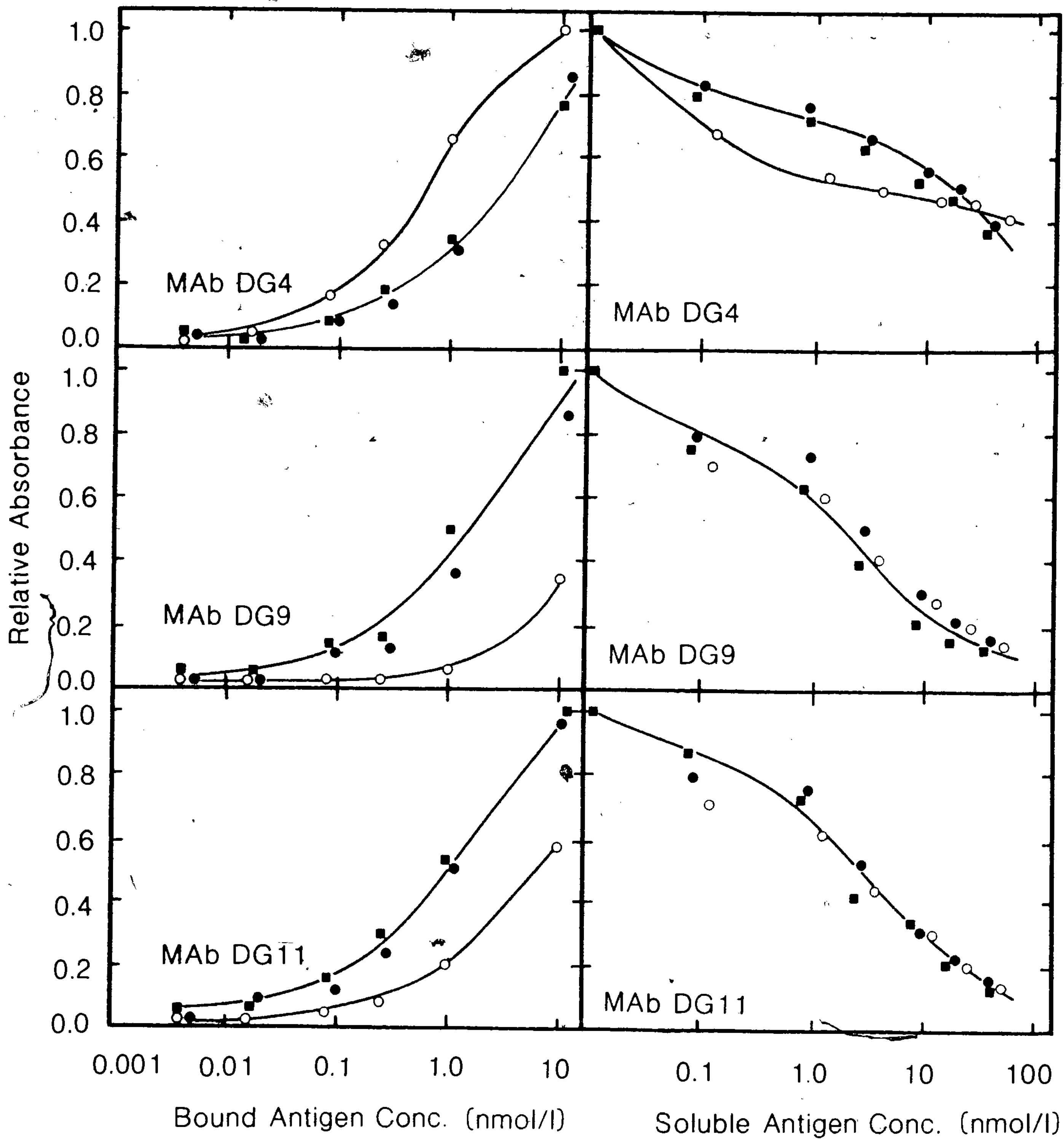
Relative Absorbance
1.0
0.8
0.6
0.4
0.2
0.0
MAb SB2
MAb SB2
MAb SB5
MAb SB5
0.001
0.01
0.1
1.0
10
100
Bound Antigen Conc. (nmol/l)
Soluble Antigen Conc. (nmol/l)

recognition of middle versus bottom components by this group of monoclonal antibodies. The concentration of virus component at one-half maximum absorbance was constant for top, middle and bottom components with antibody SB2 in plate- or antibody-trapped ELISAs. This was consistent with the previous conclusion that the epitope recognized by SB2 was displayed on the virus surface and was not appreciably altered by subtle virus conformational changes.

The possibilities that the rabbit immunoglobulins used for trapping preferentially bound one component, or that the virus components exhibited differential binding to the polystyrene microtitre plates were investigated. Individual virus components were allowed to bind directly to microtitre plates, and then challenged with the rabbit immunoglobulins; the bound rabbit antibody was then quantitated. The results for all three virus components were co-linear (Figure 9), suggesting that the virus components bound to the plates and to the rabbit immunoglobulins with equal affinities.

As previously noted, binding of antigen to polystyrene plates altered the conformation and subsequently, the antigenic properties of proteins. To minimize the influence of these factors on measurement of antibody specificity, liquid-phase competition assays were performed, utilizing the fractionated viral components as competing antigens. Recognition of unfractionated CPMV in an antibody-trapped ELISA by antibody SB5 was more sensitive to inhibition by

Figure 8. A comparison of affinity ELISA and liquid phase competion assays using monoclonal antibodies against CPSMV. Affinity assay (left panel): Each CPSMV component is serially diluted and assayed by plate-trapped ELISA. Liquid-phase competition assay (right panel): The monoclonal antibody is incubated with the indicated antigen; the antibody remaining unreacted at the end of the reaction period is allowed to bind to whole virus preparation bound to microtitre plates pre-coated with homologous polyclonal antibodies. Antigen: top component (○); middle component (■) and bottom component (●). Standard deviation = +/- 10 %.

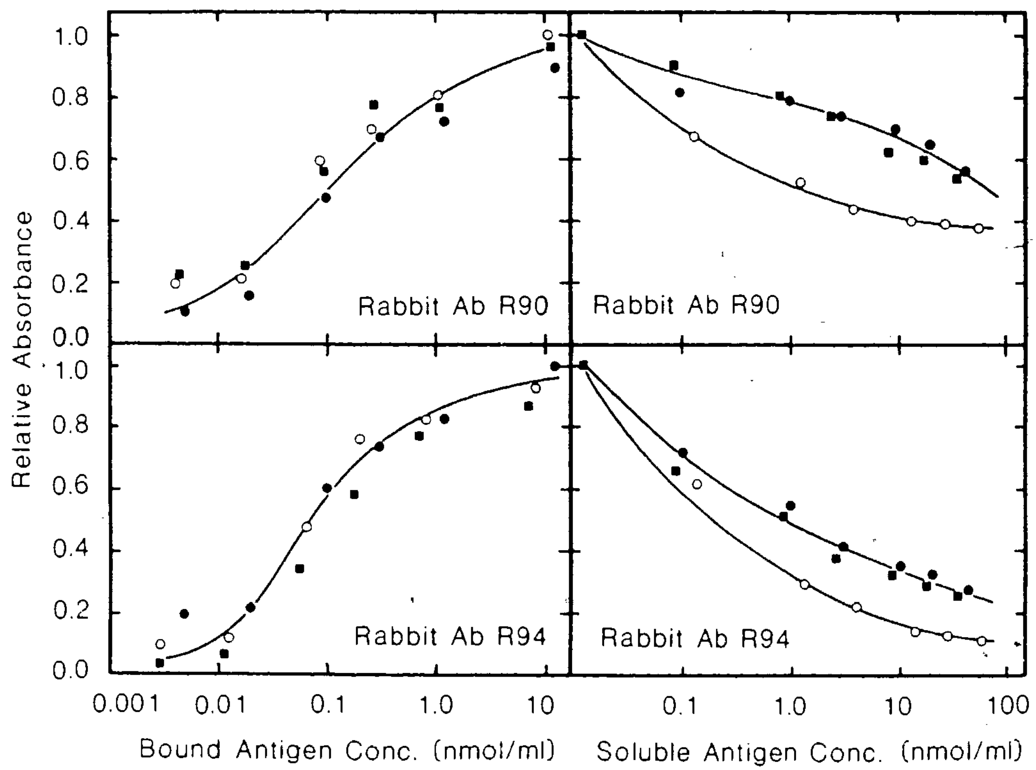

Relative Absorbance
1.0
0.8
0.6
0.4
0.2
0.0
MAb DG4
MAb DG4
MAb DG9
MAb DG9
MAb DG11
MAb DG11
0.001
0.01
0.1
1.0
10
100
Bound Antigen Conc. (nmol/l)
Soluble Antigen Conc. (nmol/l)

middle or bottom components than by top component (Figure 7). This was in accord with the diminished affinity for top component noted in simple affinity assays. Similarly, binding of DG4 to CPSMV-DG in a liquid-phase competition assay was inhibited at lower molar concentrations of top, than middle or bottom components (Figure 8), reflecting a greater affinity for empty capsids. These observations corroborate the results of the affinity assays. Liquid-phase competition assays with DG4 were analyzed by plate-trapped ELISA, because DG4 bound unfractionated CPSMV at 100-fold lower concentration in antibody-trapped compared to plate-trapped ELISAs (Figure 2B).

None of the virus components exhibited preferential inhibition of the binding of monoclonal antibodies DG9 and DG11 to CPSMV in antibody-trapped, liquid-phase competition ELISAs, although differences had been observed in the affinity assays (Figure 8). It has been demonstrated that DG9 and DG11 did not recognize the dissociated virus (Tables 2 & 5). These observations suggested that the binding of top component, but not middle and bottom components, to the ELISA plates caused deformation of the viral component. This, in turn, implied that encapsidation of RNA stabilized virion conformational integrity. Structural differences induced by antibody binding to a solid support have been demonstrated in other serological assay systems (Tainer et al., 1984; Koenig & Torrance, 1986).

Through the experiments designed to evaluate antibody recognition of altered virus conformation, SB2 provided a

Figure 9. A comparison of affinity ELISA and liquid phase competition assays using rabbit antibodies against either CPMV or CPSMV. Affinity assay (left panel): Each CPMV and CPSMV component is serially diluted and assayed by plate-trapped ELISA. Liquid-phase competition assay (right panel): The monoclonal antibody is incubated with the indicated antigen; the antibody remaining unreacted at the end of the reaction period is allowed to bind to whole virus preparation bound to microtitre plates pre-coated with homologous polyclonal antibodies. Antigen: top component (○); middle component (■) and bottom component (●). Standard deviation = +/- 10 %.


Relative Absorbance
1.0
0.8
0.6
0.4
0.2
0.0
Rabbit Ab R90
Rabbit Ab R90
Rabbit Ab R94
Rabbit Ab R94
0.001
0.01
0.1
1.0
10
100
Bound Antigen Conc. (nmol/ml)
Soluble Antigen Conc. (nmol/ml)

negative control with no differentiation between top, middle or bottom components in the affinity or the liquid-phase competition assay, using either plate- or antibody-trapped ELISAs.

Early reports of double-diffusion assays (Bruening & Agrawal, 1967) suggested that rabbit antisera did not distinguish between virus fractions. These observations were supported by simple dilution curves (Figure 9). However, liquid-phase competition assays revealed a difference in affinity: top > middle, bottom. Differences between dilution curves and competition assays have been observed in other protein systems (Muller et al., 1986) and have been attributed to extensive deformation of the antigen caused by antibody-antigen binding (Getzoff et al., 1987). Formation of the antigen-antibody complex results in conformational changes to the structure of the antigen, exposing new antigenic sites on the viral top component. It has been documented that immunizing antigens can dissociate when injected into the animal (Van Regenmortel, 1982), and antibodies are produced against epitopes not identified in the native structure of the antigen. These antibodies can result in multiple parallel lines in Ouchtelony assays (Van Regenmortel & Lelarge, 1973).

Recognition of dissociated viral components by monoclonal antibodies. Monoclonal antibodies SB2 and DG4 have been shown to recognize antigenic determinants after the unfractionated virus was dissociated by heating in the presence of SDS plus

urea (Figures 4 & 5, respectively). The individual top, middle and bottom components were dissociated by this procedure and evaluated for their ability to bind monoclonal antibodies. Neither antibodies SB2 nor DG4 demonstrated a differential affinity for any one of the three dissociated viral components. This supported the understanding that, upon the encapsidation of RNA, there was no alteration in the primary structure of the capsid proteins. Although these data did not preclude proteolytic cleavage remote from these epitopes, it was more probable that differences in secondary and tertiary structure occurred as a consequence of RNA encapsidation.

Effect of ribonuclease treatment on the antigenicity of virus. The direct participation of RNA in defining antigenic sites was evaluated. There was no alteration in the specificity of the monoclonal antibodies as a result of ribonuclease treatment. This was consistent with RNA being completely shielded within the protein shell. Dissociated virus particles were also subjected to ribonuclease treatment. Nuclease digestion did not alter the antigenicity of the dissociated virus (Table 7), suggesting the RNA did not participate directly in any of the antigenic determinants recognized by the panel of monoclonal antibodies. Alternatively, the protein subunits may have bound RNA very tightly and protected short segments of RNA from nuclease attack. The latter regions, if they occurred, could be part of the quaternary structure of an

antigenic determinant. The present data did not distinguish between these possibilities.

Table 7. Effect of Ribonuclease A1 treatment[1] on the antigenicity of comoviruses.

TREATMENT[1]	ELISA RESULTS[2]			
Antibody:	SB2	R91	DG4	R90
i) undissociated virus:				
virus + BSA	++++++	+++++	++	++++++
virus + RNase	++++++	++++	++	+++++
ii) dissociated virus:				
» TE + RNase; » PBS	+	+	-	++
» TE; » PBS	-	-	-	++
» TE + RNase; » PBS/urea	+++	++	++	+++
» TE; » PBS/urea	+++	++	++	+++
» TE	+++++	++	++++	++++
» PBS	++++	++	+++	+++
» PBS/urea	+++	++	+	+++

1. Unfractionated intact CPMV at 30 µg/ml was treated with either BSA or Ribonuclease A1 at 1 mg/ml. Virus was dissociated with SDS plus urea and then dialysed against the indicated buffer. See text for buffer composition and assay conditions. » indicates a dialysis step.

2. The ELISA absorbance readings above background were grouped as follows: >2.53, ++++++; 2.04 - 2.53, +++++; 1.54 - 2.03, ++++; 1.04 - 1.53, +++; 0.54 - 1.03, ++; 0.04 - 0.53, + and <0.04, -. Background = 0.090.

DISCUSSION:

Monoclonal antibodies frequently form the cornerstone of ELISAs. As for many other virus groups, monoclonal antibodies against two comoviruses provide a means of distinguishing between two serologically related viruses, and offer increased sensitivity in evaluation of crude plant extracts (Dekker et al., 1988; Massalski & Harrison, 1987; Torrance et al., 1986).

Monoclonal antibodies have been used successfully for the antigenic analysis of many viruses (Icenogle et al., 1981; Koenig & Torrance, 1986; Tremaine et al., 1985a). While virtually the entire surface of an antigen is available as a potential epitope (Benjamin et al., 1984; Muller et al., 1986), monoclonal antibodies permit unique topographic features to be examined and compared individually.

Treatment of comoviruses with SDS plus urea or guanidine-lithium chloride compromised the structural integrity of the particle. However, the ability of the subunit proteins to reassociate differs dramatically, depending on the method of virus dissociation. Denaturation with SDS plus urea yields protein aggregates intermediate between individual protein subunits and intact virions. The aggregates eluted as a single broad peak from gel filtration, and consisted of both VP23 and VP37, as indicated by gel electrophoresis (data not shown). In contrast, guanidine-lithium chloride allowed individual protein subunits VP23 and VP37 to be resolved by gel filtration.

Several monoclonal antibodies recognized virions denatured with SDS plus urea, but failed to bind to particles treated with guanidine-lithium chloride. Antibodies SB2 and DG4 recognized epitopes not directly involved in the interaction between subunits. Moreover, the data indicated that the sites of SB2 and DG4 binding are defined by linear amino acid sequences.

The ability of monoclonal antibodies to exhibit differential binding in antibody- and plate-trapped ELISAs is a scenario seen with many virus groups (Dekker et al., 1987; McCullough et al., 1985; Tremaine et al., 1985b). It has been postulated that the native conformation of the antigen is altered upon binding to the polystyrene plate in a plate-trapped ELISA. Antibody-trapped ELISA procedures, where the antigen is bound to a primary, homologous antibody coat, are preferable for studying the antigen in a conformation more closely related to its native structure (Altschuh et al., 1985). The preferred reactivities of antibodies SB4, SB5, DG6, DG7, DG8, DG9, and DG11 in antibody-trapped compared to plate-trapped ELISAs is demonstrative of this observation. The influence that antigen conformation exerts on cross-reactivity must be recognized in designing experimental protocols where serologically related viruses may be encountered. Present data and data from other plant virus studies demonstrated that the assay system can influence the ability of monoclonal antibodies to distinguish between viruses (Altschuh et al.,

1985; Dore et al., 1987).

Using the sum of the information presented above, antibodies were classified in accordance with characteristics of their antigen binding properties. Based on observations of whether the antibody reacted preferentially with native or disrupted virions, the antibody recognition sites were classified as either external or internal sites (i.e. sites not exposed in the intact, native conformation of the virion) (Table 8). Within the group of external antigenic determinants, some were stable and unaffected by conditions of protein denaturation. Others were labile; recognition of these sites was lost by modification of virus structure. These epitopes were likely defined by discontinuous amino acid sequences. It is expected that within any panel of monoclonal antibodies, many will be specific for discontinuous sites (Barlow, 1986). This was clearly demonstrated by monoclonal antibodies elicited against tobamoviruses (Altschuh et al., 1985), potexviruses (Koenig & Torrance, 1986) and now for these representative comoviruses.

Antibody DG5 has unique characteristics. DG5 bound to a stable, external epitope on CPSMV, but recognized a labile, external epitope on CPMV, indicating the two viruses share an external epitope with partial structural homology.

The cross-reactivity of a number of monoclonal antibodies suggested many antigenic sites are conserved. All antibodies that recognized internal epitopes were cross-reactive, while

TABLE 8. **Summary of antibody binding activity.**

EPITOPE	MONOCLONAL ANTIBODY	
	ANTI-CPMV	ANTI-CPSMV
EXTERNAL, STABLE:	SB2, SB7, SB8	DG3, DG4, DG5
LABILE:	SB4, SB5	DG7, DG8, DG9, DG11
INTERNAL:	SB1, SB3, SB9	

antibodies against exposed sites represented both cross-reactive and non-cross-reactive antibodies. This pattern is similar to many animal virus systems where the surface antigens tend to be variable in an attempt to overcome the host's immune system, while the internal or structural components are more highly conserved (Sheshberadaran et al., 1983; Van Wyke et al., 1984). The conservation of these regions within plant viruses may reflect important functional roles in virus survival.

Characteristic of many comoviruses, empty virion shells of CPMV can be assembled devoid of viral RNA. Morphologically, empty virions cannot be differentiated from middle and bottom components. The affinity of this panel of monoclonal antibodies for the separated viral components was assessed to determine if RNA encapsidation altered the three-dimensional profile of the virus particle and consequently, the antigenic structure of the viral capsid. Antibodies SB5 and DG4 had a greater affinity for RNA-containing virions and empty viral shells, respectively. Incorporation of RNA into the capsid modified the surface antigenicity of the virion. Ribonuclease digestion had no effect on these observations. As expected, the RNA does not directly define the antigenic sites.

One possibility to account for differences in the antigenic behavior of different viral centrifugal components would be the association of viral capsid protein processing with the encapsidation of RNA. Further modification of intact

capsids has been observed, and was the result of proteolytic cleavage of a fragment from the C-terminal of the small capsid protein (VP23) (Geelen et al., 1972; Kridl & Bruening, 1983; Niblett & Semancik, 1969). However, all three virus components were equally susceptible to this form of processing (Siler et al., 1976), and the protein patterns obtained through polyacrylamide gel electrophoresis were identical for top, middle and bottom components (Geelen et al., 1972). Similiar proteolytic modification of the capsid proteins of CPSMV has been reported (Thongmeearkom & Goodman, 1978). Moreover, low level neutron scattering suggested that there was no major modification of electron density profiles of the protein shells of top, middle and bottom components (Schmidt et al., 1983). None of the antibodies generated for the present study recognized VP23 in immunoblot analysis. Therefore, it was not feasible to distinguish between antigenicity of VP23(F) and VP23(S).

It has been observed, however, that through neutral pH discontinuous polyacrylamide gel electrophoresis, top component could be resolved from ribonucleoproteins (Siler et al., 1976). This suggested an altered surface charge, and was the first indication that the capsid structure of top component may be altered with respect to that of middle or bottom components.

Based on immunological evidence, the present work suggests there is modification of the capsid surface in

response to RNA encapsidation. Although these are novel observations for the comoviruses, similiar traits have been demonstrated in related virus systems. Artificially induced empty capsids of picornaviruses (Frassen et al., 1984) showed definite modification of their antigenic determinants (Icenogle et al., 1981; Meloen et al., 1979; Rombaut et al., 1982). Circular dichroism analysis of the isometric plant virus turnip yellow mosaic virus provided clear evidence that encapsidation of RNA was associated with conformational changes within the protein shell (Tamburro et al., 1978).

The monoclonal antibodies produced against CPMV and CPSMV recognized external epitopes specific for the immunizing virus, and internal epitopes, some of which were present on both of the viruses. The majority of the monoclonal antibodies recognized antigenic determinants formed by discontinuous amino acid sequence. The specificity of these monoclonal antibodies was utilized to distinguish between these related viruses without interference or background signal from the other. Two monoclonal antibodies, one each specific for CPMV and CPSMV, exhibited differential binding between fractionated top, and middle and bottom viral components. Therefore, encapsidation of viral RNA by the capsid polypeptides of both CPMV and CPSMV apparently alters the three-dimensional conformation of the viral particles.

CHAPTER 3: CHARACTERIZATION OF THE INTERFERENCE BETWEEN CPMV-SB AND CPSMV-DG IN CO-INOCULATED *Vigna unguiculata*

INTRODUCTION:

McKinney (1929) demonstrated that infection of a plant with one strain of a virus can induce resistance in the plant against infection by another strain of the same virus. This observation has lead to many studies and numerous theories to explain the phenomena of acquired resistance, cross-protection and interference (reviews by: Dodds & Hamilton, 1976; Fraser, 1987; Hamilton, 1980; Zaitlin, 1976).

The terminology pertaining to these biological phenomena has been inconsistent. Therefore, the terms are defined as they apply in this work. In many examples infection of a plant with one strain of a virus (inducer virus) protects the same plant against infection by another strain of the same virus, or against infection by a different virus (challenger virus) (Hamilton, 1980). This is known as acquired resistance; the plant has gained resistance against the challenger virus as a result of prior infection by the inducer virus. If the inducer and challenger virus can be used reciprocally, the term cross-protection best describes the phenomenon (Hamilton, 1980).

Protection is a measure of the delay of symptoms elicited by the challenger virus. The extent of protection can be expressed as: 1) a decrease in the number of lesions (Thomson, 1959); 2) a decrease in the mean size of the

lesions (Davis and Ross, 1968); 3) a decrease in the concentration of challenger virus in the infected plant (Zaitlin, 1976); or 4) a delay or total prevention in the expression of symptoms by the challenger virus (Fulton, 1951).

The response initiated by an inducer virus can lead to either local or systemic acquired resistance. Local acquired resistance refers to tissue primarily inoculated with the inducer virus showing varying degrees of resistance to subsequent inoculation by the same or a different virus (Ross, 1961a). Systemic acquired resistance is the same phenomenon as the former, only manifested systemically (Ross, 1961b).

In systems exhibiting either acquired resistance or cross-protection the challenger virus is inoculated after the inducer virus has initiated replication in the primary inoculated leaves. If the symptoms caused by the challenger virus are suppressed when it is co-inoculated with the inducer virus, the phenomenon is described as interference. The biological system studied herein is interference. The majority of systems studied are representative of either acquired resistance or cross-protection; however, there is no reason to assume some of the molecular mechanisms which characterize the former cannot apply also to the phenomenon of interference.

Initial studies of acquired resistance were conducted with different strains of the same virus (Fulton, 1951; Thung, 1932), however acquired resistance has since been demonstrated

using two different viruses (Fulton, 1975; Kassanis et al., 1974). In general, the extent of protection against the challenger strain was accepted as a reflection of the relatedness of the two virus strains (Hamilton, 1980). Initial studies examined the biological properties of these mixed infection experiments. The optimal conditions for many virus duets exibiting acquired immunity were determined based on symptom expression (Fulton, 1951), and from these studies theories of cross-protection were formulated to explain the biological observations. It was not until recently that some of the underlying molecular mechanisms responsible for these observations have become understood (Hemenway et al., 1988; Loesch-Fries et al., 1987).

Metabolic stress in the plant as a result of inducer virus replication has been suggested as the cause for the decreased infectivity of the challenge virus (Hamilton, 1980). Although stress has been accepted as an underlying condition in inoculated plants, little or no direct evidence has been presented to indicate stress was specifically responsible for acquired resistance against the challenging virus. In some cases of mixed infections a synergistic increase in the accumulation of either or both viruses has been reported (Goodman & Ross, 1974). Furthermore, metabolic stress has not been coordinated with the observed specificity of the inducer/challenge virus combination.

Inhibitors induced by virus infection have been implicated in protecting the infected plant from further infection (Hamilton, 1980). Acquired resistance or systemic resistance was induced in tobacco plants containing the N gene. Infection of these plants with necrosis-inducing viruses resulted in the synthesis of pathogenesis-related (PR) or "b" proteins (Van Loon & Van Kammen, 1970). PR-proteins have since been found to accumulate in response to a variety of stimuli (Gianinazzi & Kassanis, 1974; Van Loon, 1977), in many different plants (Van Loon, 1985). Some PR-proteins have been localized to the intercellular spaces surrounding virus induced local lesions (Antoniw & White, 1986; Carr et al., 1987). Some of the genes coding for PR-proteins have been identified and characterized (Cornelissen et al., 1987; Pfitzner & Goodman, 1987), and at least one of these genes contained the consensus sequence of a heat shock protein regulatory element (Pfitzner et al., 1988). The biochemical activity of some PR-proteins has been identified as acidic and basic 1,3-B-glucanases (Kauffmann et al., 1987) and chitinases (Hooft van Huijsduijnen et al., 1987). These enzymes have been identified as components of defence reactions in plants infected with fungi (Darvill & Albersheim, 1984).

Another inhibitor, unrelated to PR-proteins, has been identified in hypersensitive and systemic tissues of tobacco mosaic virus (TMV) infected tobacco containing the N gene (Sela et al., 1966). The antiviral factor (AVF) is a 22 kDa

phosphoglycoprotein activated, in plants containing the *N* gene, from a precursor. All *Nicotiana* species synthesized the precursor irrespective of whether they contain the *N* gene (Mozes et al., 1978; Sela et al., 1978). Mozes et al. (1978) suggested AVF interferes with the replication of TMV.

Although PR-proteins and AVF are associated with the acquired resistance induced by necrosis inducing viruses, there is no evidence to indicate they are the primary cause of resistance. The expression of PR-proteins and AVF may be part of the larger plant response to pathogen invasion, and not a specific response to an inducer virus.

Different isolates of tobacco streak virus (TSV), an ilarvirus, differ in their ability to cross-protect in *Nicotiana clevelandii* (Fulton, 1978). TSV has a ssRNA tripartite genome and sediments into three components during equilibrium density gradient centrifugation. After examining all possible combinations of middle and bottom components of different strains, the extent of symptom expression elicited by the challenger virus was found to be determined by the bottom RNA component of the inducer virus. This indicated a viral function was directly involved in overcoming the acquired resistance established by the challenge virus.

It was postulated that the RNA-dependent RNA polymerase of the inducer virus may bind the RNA of the challenger virus and either not be able to replicate it, or not replicate it

faithfully (Gibbs, 1969). In either cases the genome of the challenger virus would be tightly bound to a non-functional RNA-protein complex and be unavailable for replication. There is no evidence presently to support or reject the involvement of this mechanism in acquired immunity. Recently a core amino acid sequence was found in the RNA-dependent RNA polymerases of many unrelated RNA viruses (Zimmern, 1988). Pseudorecombinants of RNAs of two strains of tobacco rattle virus have demonstrated that heterologous RNA can be replicated (Robinson, 1977).

Palukaitis and Zaitlin (1984) proposed a mechanism to explain cross-protection between two closely related viruses. They suggested plus-sense RNA of the inducer virus, produced during virus replication, may hybridize to minus-sense RNA of the challenger virus. Formation of a stable RNA duplex would prevent replication of the challenger virus. The existence of such hybrid structures is not questioned, but demonstration of the mechanism *in vivo* was marginally successful (Hemenway et al., 1988). The anti-sense RNA of potato virus X (PVX) coat protein gene was expressed in transgenic tobacco plants. The transformed plants were resistant to inoculation by PVX at low virus concentrations. Hemenway et al. (1988) postulated that higher levels of expression of endogenous antisense transcripts were required. Ali Rezaian et al. (1988) transformed tobacco plants with regions of the cucumber mosaic virus (CMV) genome such that anti-sense transcripts were

produced. Excess levels of endogenous anti-sense transcripts were present in the leaves, yet the transgenic plants were susceptible to infection by CMV. Two possible explanations could have accounted for the negative results: 1) viral RNA bound translational factors during uncoating making it unavailable for RNA duplex formation with anti-sense transcripts; 2) anti-sense transcripts were expressed in the nucleus but virus replication occurred in the cytoplasm. However, two other recent discoveries indicated that the replication of some plant viruses can be regulated by the formation of anti-sense RNA hybrids (Ali Rezaian & Symons, 1986; Hillman et al., 1987).

CMV is a tripartite ssRNA virus which also encapsidates a fourth subgenomic RNA coding for the viral coat protein, derived from the 3'-end of RNA3. CMV is also associated with a satellite RNA (sat-RNA) which requires CMV-encoded polypeptides for its replication. Sat-RNAs associated with plant viruses are characterized by their lack of extensive nucleotide homology with the genome of the parent virus (Francki, 1985). However, Ali Rezaian and Symons (1986) found that short complementary sequences between the sat-RNA and the coat protein gene on RNA3 and 4 formed stable anti-sense hybrids <u>in vitro</u>. This may be the mechanism by which viral coat protein synthesis is regulated. There are many examples to illustrate the regulation of protein synthesis by the

hybridization of anti-sense RNAs with cellular mRNAs (Pestka et al., 1984; Melton, 1985).

Viral sat-RNAs are often responsible for moderating the symptoms induced by the associated virus (Francki, 1985). Animal viruses also have symptom-modulating RNAs associated with infection, known as defective interfering RNA (DI RNA) (Lazzarini et al., 1981). Hillman et al. (1987) have identified DI RNAs associated with tomato bushy stunt virus (TBSV) which modulates the symptoms induced by the virus. The DI RNAs associated with TBSV are approximately 400 bases in length and have slightly different nucleotide sequences. These sat-RNAs are composed of blocks of sequence homologous to the parent virus genome. The DI particles appear to be generated from the genome of TBSV, utilizing a linear copy choice pattern of replication by the viral RNA polymerase utilized in the synthesis of other DI RNAs (Kirkgaard & Baltimore, 1986). The DI RNAs may interfere with viral replication by competition for the components of the viral replication machinery (Barrett & Dimmock, 1986), or by forming a stable anti-sense RNA hybrid with the viral genome.

De Zoeten and Fulton (1976) postulated a different mechanism for cross-protection with two strains of TMV. They theorized that the RNA of the challenger virus was encapsidated, before it was replicated, by the coat protein of the inducer virus. Heterologous encapsidation has been demonstrated in mixed virus infections (Dodds & Hamilton,

1974). However, when cross-protection experiments were repeated using a mutant of the inducing strain of TMV defective in capsid protein production, cross-protection was still observed (Sarkar & Smitamana, 1981; Zaitlin, 1976). This suggested that genomic masking was not the principle mechanism of cross-protection.

However, there is considerable evidence from other systems with two closely related viruses that the coat protein of the inducer virus binds to the RNA of the challenger virus, thereby preventing its replication (Zinnen & Fulton, 1986). Tobacco plants initially inoculated with sunn-hemp mosaic virus (SHMV) were resistant to subsequent systemic inoculation by a necrotizing strain of SHMV (SHMV-n), or RNA isolated from SHMV-n. These plants were susceptible to infection by the common strain of TMV (TMV-C), but the severity of infection was decreased if TMV-C RNA was encapsidated in SHMV coat protein. Sherwood and Fulton (1982) made similar observations using two strains of TMV. Dodds et al. (1985) used the mild strain of CMV (CMV-S) to protect against secondary infection by the severe strain of CMV (CMV-P). Acquired resistance was induced in leaves inoculated with CMV-S and systemically infected leaves; neither the accumulation of CMV-P virions nor the dsRNA replicative form of CMV-P could be detected. The acquired resistance was overcome when protected plants were challenged with RNA of CMV-P. Both dsRNA and virions of CMV-P

were detected, but only in leaves inoculated with the challenge virus.

Recently, the gene encoding TMV coat protein has been expressed in transgenic tobacco plants (Abel et al., 1986; Nelson et al., 1987a). Approximately 50% of the transformed plants expressing the coat protein gene were resistant to infection by TMV, and the remainder exhibited a delay in developing systemic symptoms compared to plants not expressing the TMV coat protein gene. Register and Beachy (1988) transferred the gene coding for TMV coat protein to tobacco plants, and examined the early stages of TMV infection of protoplasts isolated from transformed plants. Their observations suggested that the expression of the TMV coat protein gene in the transformed plants prevented uncoating of the infecting virus. The protection could be overcome with infection by isolated TMV RNA.

Alfalfa mosaic virus (AMV) is a tripartite ssRNA virus with a replicative strategy similar to viruses in the ilarvirus group, the biology of which is distinct from TMV. In the absence of RNA4, the coat protein of AMV is required to initiate infection by the viral RNA, and to control viral transcription (Houwing & Jaspars, 1986). Transgenic plants expressing AMV coat protein gene were resistant to infection by AMV, and developed systemic symptoms slower than non-transformed plants (Loesch-Fries et al., 1987; Tumer et al., 1987). The protection could be overcome with infection either

by isolated AMV RNA, or by TMV. Transgenic plants expressing the coat protein gene of TSV (an ilarvirus) were resistant to infection by TSV but not AMV (Van Dun et al., 1988). However, AMV RNA infected the transformed plants but not the nontransformed plants, demonstrating that heterologous coat protein can maintain virus replication but cannot induce acquired resistance, even between two similar viruses. Plants transformed with a mutant AMV coat protein gene accumulated coat protein RNA transcripts at a level similar to plants transformed with wild-type coat protein gene; however, coat protein was not expressed. These plants were susceptible to infection by AMV, indicating that viral coat protein and not the transcript of the gene is responsible for inducing acquired immunity (Van Dun et al., 1988).

Potato virus X (PVX) is the type member of the potex virus family. The PVX ssRNA genome is encapsidated in a long, flexuous, rod-shaped virion assembled from 5' to 3' and disassembled from 3' to 5' (Lok & Abou Haidar, 1981); both assembly and disassembly being opposite to that of TMV particles (Shaw et al., 1986). Transgenic tobacco plants expressing either the coat protein gene of PVX or an antisense transcript of the coat protein gene were constructed. Both types of plants exhibited a delay and reduction in systemic symptom development after inoculation with PVX (Hemenway et al., 1988). Unlike previous examples, inoculation with PVX RNA

did not break resistance. The data from the above three examples suggests that the mechanism by which coat protein induces resistance is likely to differ in each case. A single hypothesis cannot reconcile the variation observed in each system. However, coat protein played an essential role in establishing acquired resistance against these three viruses.

Viroids are small (250-400 bases), un-encapsidated, highly infectious ssRNA molecules, which can induce acquired immunity against other viroids (Fernow, 1967). A mild strain of potato spindle tuber viroid (PSTV) prevented the development of severe symptoms in plants inoculated two weeks later with a severe strain of PSTV. Niblett et al. (1978), demonstrated that a viroid could induce acquired resistance against subsequent infection by an unrelated viroid. Recently, interference was demonstrated between two strains of PSTV, and between PSTV and hop stunt viroid co-inoculated to plants as a cDNA dimer (Branch et al., 1988).

In plants, induction of acquired resistance against further infection was not restricted to viruses alone (Kuc, 1983). Inoculating the roots or infesting the soil of cotton plants with a mild strain of *Verticillium albo-atrum* protected against subsequent infection by a severe strain of the fungus (Schnathorst & Mathre, 1966). Cotton seedlings previously exposed to spider mites exhibited resistance to infection by *Verticillium dahliae* (Karban et al., 1987). A cultivar of tobacco resistant to TMV infection was inoculated with TMV on

the three to four lower leaves, and subsequently the upper leaves became protected against blue mold caused by Peronospora tabacina (Ye & Kuc, 1988).

Little research has been done to investigate the induction of acquired resistance by comoviruses. Two related strains of squash mosaic virus (SMV) were able to induce reciprocal cross-protection in cantaloupe, but only unidirectional acquired resistance was observed in pumpkin (Albersio et al., 1975). The difference was attributed to a higher multiplication rate of the mild inducer strain in pumpkin than in cantaloupe.

As previously noted, one virus could interfere with the replication of another when the two viruses were co-inoculated on a host plant (Ponz & Bruening, 1986). There are considerably fewer examples of this phenomenon than of induced acquired resistance. In all reported examples of this phenomenon, the mild protecting or inducing virus did not induce detectable symptoms on the test plant. The U2 strain of TMV did not induce symptoms on pinto bean; however, U2 co-inoculated with the TMV strain U1 interfered with the induction of U1 induced local lesions (Wu & Rappaport, 1961). The interference between related comoviruses, CPMV and CPSMV, co-inoculated on hosts resistant to CPMV has been examined here.

The SB strain of CPMV was infectious when inoculated on

Vigna unguiculata cv. BE-5, but was not infectious on cultivars Black and Arlington (Beier et al., 1977). However, Eastwell et al. (1983) confirmed very low levels of CPMV RNA replication in the latter two cultivars. CPSMV replicated in all three cultivars with severity of symptom induction being: Arlington > Black > BE-5 (Beier et al., 1977). Only two cultivars of cowpeas have exhibited resistance to the exceptionally virulent CPSMV-DG (Rios & Das Neves, 1982)

The resistance to virus infection demonstrated by some plants has been studied. Cucumber cv. Elem accumulated one-tenth the level of CMV than the nearly isogenic cultivar Bet Alpha (Nachman et al., 1971). Resistance to bean common mosaic virus in two cultivars of beans has been examined (Zaumeyer & Meiners, 1975). A gene (*Tm-1*) has been identified in tomatoes which regulates resistance to some strains of TMV (Fraser & Loughlin, 1980). Recently, two point mutations were identified in the genomes of resistance breaking TMV strains (Meshi et al., 1988). The mutations resulted in amino acid changes in the 130 and 180 kDa viral proteins. These amino acid changes altered the net charge of the viral proteins, suggesting an electrostatic interaction between the viral proteins and a host resistance factor.

The most visible and most studied response of plants to virus infection is the hypersensitive response (HSR) (Loebenstein & Stein, 1985; Ponz & Bruening, 1986). The HSR is characterized by the appearance of necrotic local lesions at

the site of infection, and often localization of the virus at the infection site. However, the area in which the virus is localized and the necrotic region do not necessarily coincide. Plant breeders have often used the HSR as a phenotypic genetic marker in resistance breeding programs (Patel, 1982). The inheritance of the trait often follows simple Mendelian genetics. However, resistance based on the HSR is often overcome by spontaneous mutations in the virus (de Jager & Wesseling, 1981) or by environmental stress (Weststeijn, 1984).

The HSR could be induced by bacteria and fungi (Ponz & Bruening, 1986) as well as by non-pathogenic chemical agents and stress (Loebenstein & Stein, 1986). A number of the physiological and biochemical changes in plant tissues associated with the HSR were elicited by ethephon treatment of the tissues (Van Loon, 1977). Ethephon is converted into the growth regulator ethylene in plants. Large amounts of ethylene were detected at a time coinciding with the appearance of necrosis in virus infected hypersensitive plants (Van Loon, 1983). Ecker and Davis (1987) recently demonstrated that ethylene regulates plant defense genes. Therefore, the HSR is probably a general defense response of plants against pathogenic or environmental stress.

The HSR was associated with numerous physiological and biochemical alterations in the plant tissue. Several

structural changes were seen in mesophyll cells surrounding the necrotic area; these cells had smaller vacuoles, and more cytoplasm and ribosomes (Loebenstein & Stein, 1986). Lignification, and the deposition of suberin (Faulkner & Kimmins, 1975) and callose (Wu & Dimitman, 1970) were among the barrier substances found in the necrotic region resulting in a general thickening of the cell wall and blocking of the plasmodesmata. These mechanical barriers were once thought to localize virus infection (Esau, 1967), but were later considered to be the borders of the necrotic lesion (Wu, 1973). Increased membrane permeability was observed early in the HSR (Ruzicska et al., 1983). An increase in oxidative enzyme activities (Wagih & Coutts, 1982) and the appearance of the PR-proteins (Van Loon, 1983) coincided with the formation of necrotic local lesions.

The resistance of cowpeas to infection by CPMV was shown to correspond to the appearance of the HSR on some resistant varieties (Robertson, 1965). Beier et al. (1977) assayed over 1000 lines of cowpeas for susceptibility to infection by CPMV-SB. Sixty-five cultivars, including Black and Arlington, were found to be immune to infection by CPMV-SB at 100 times the concentration of virus required to uniformly infect susceptible varieties and the virus could not be transferred to these cultivars by graft inoculation. The immunity associated with Arlington was unique. Protoplasts were made from 54 of the immune cowpea lines and only Arlington

protoplasts retained significant resistance to virus infection (Beier et al., 1979; Kiefer et al., 1984). The immune factor in Arlington was inherited as a single dominant trait (Kiefer et al., 1984). Sanderson et al. (1985) demonstrated that a constituent of Arlington extracts inhibited the cleavage of CPMV-SB RNA2 translation products. Further characterization of this factor indicated that it was proteinaceous and was highly specific for the 24 kDa protease encoded by CPMV-SB (cleavage of the translation products of CPSMV-DG were not inhibited) (Ponz et al., 1988a; Sanderson et al., 1985).

Bruening et al. (1979) observed that in mixed infections, CPMV-SB interfered with the replication of CPSMV-DG. The extent of interference, assayed by the number of local lesions formed on inoculated leaves, was dependent on the concentration of CPMV-SB applied. The two viruses had to be co-inoculated and mixed inoculation of CPMV-SB capsid protein with CPSMV did not cause interference. However, co-inoculation with CPMV-SB RNA and CPSMV-DG resulted in interference, unless the CPMV-SB RNA had been previously irradiated with ultra-violet light. Therefore, biologically active CPMV-SB RNA was required for the interference response. Infectious CPMV-SB particles could not be recovered from the mixed inoculated plants, consequently CPSMV-DG was not facilitating the replication of CPMV-SB in the immune plants.

Ponz et al. (1988b) reported that Arlington, but not BE-

5, was immune to tobacco ringspot virus (TobRV), a nepovirus. However, the inhibitor of the 24 kDa protease of CPMV found in Arlington (Sanderson et al., 1985) did not inhibit the protease of TobRV. Furthermore co-inoculation of Arlington, but not BE-5, with TobRV and CPSMV-DG interfered with the replication of CPSMV-DG. The most effective protection against CPSMV-DG replication was not by co-inoculation, but with a 15 minute delay of CPSMV-DG infection following inoculation of TobRV. Thus, the conditions for interference between TobRV and CPSMV-DG were similar in several respects compared to the interference between CPMV-SB and CPSMV-DG, but differed in the timing of the inoculations (Ponz et al., 1988b).

Vigna unguiculata cv. TVu 470 was is also immune to CPMV-SB, with immunity being determined by a single dominant gene (Sterk & de Jager, 1987). However, protoplasts from TVu 470, like Black, were susceptible to infection by CPMV-SB. Because of the difference in susceptibility of protoplasts to CPMV-SB from Arlington compared to Black and TVu 470, the mechanism of resistance to the virus in seedlings may also differ. Sterk and de Jager (1987) observed interference between CPMV-SB and CPSMV-Vs co-inoculated on TVu 470. CPMV-SB also interfered with the replication of SHMV and CMV when co-inoculated on TVu 470, and CPMV-SB interfered with SHMV when co-inoculated on Arlington (Saaijer-Riep & de Jager, 1988). The replication of neither SHMV nor CMV were impeded when co-inoculated with CPMV-SB on TVu 1948, a line of cowpea also immune to CPMV-SB,

but immunity was found to be a single gene recessive trait (Patel, 1982). Therefore, interference by CPMV-SB may be dependent on the mechanism of immunity the plant has against CPMV-SB infection.

The spread of comoviruses from primary inoculated cells to neighboring cells is postulated to be dependent on the 58 KDa polypeptide encoded by RNA2. When RCMV RNA1 is inoculated independently of RNA2 viral RNA replication is restricted to the primary inoculated cells and does not spread throughout the primary inoculated leaves. However, co-inoculation of RCMV RNA1 and TMV facilitates the spread of RCMV RNA1 throughout the primary inoculated leaves (Malyshenko et al., 1988); suggesting that the putative 30 KDa transport protein of TMV initiated the spread of RCMV RNA1.

Interference between CPMV-SB and CPSMV-DG is examined in three cultivars of _Vigna unguiculata_: 1) BE-5, susceptible to CPMV-SB in both seedlings and protoplast; 2) Black, immune to CPMV-SB in seedlings and susceptible to CPMV-SB in protoplasts; 3) Arlington, immune to CPMV-SB in both seedlings and protoplasts. Replication of both viruses is examined independently by serological and nucleic acid hybridization assays.

METHODS:

Virus and plant stocks. The same strains of CPMV and CPSMV, and the same cultivars of *Vigna unguiculata* were used as described above. SHMV was isolated from a natural infection (provided by G. Bruening). Seeds of Chinese Red X Iron (CRXI), an indicator plant for CPMV and CPSMV (Bruening et al., 1979), were obtained from G. Bruening.

Cowpeas were grown at 23 C with fluorescent lighting during a 16 hour daily light period. Prior to inoculation the plants were placed in the dark for approximately 1 hour. Leaves dusted with carborundum were inoculated with virus or viral RNA diluted in inoculation buffer (0.05 M potassium phosphate, pH 7.0). The inoculated leaves were rinsed with a spray of distilled water, and returned to the dark for thirty minutes prior to resumption of normal light cycle.

ELISAs. The preparation of monoclonal antibodies against CPMV and CPSMV has been detailed in Chapter 1. Monoclonal antibodies against SHMV were prepared and screened in a similar manner. Infected plant tissue was assayed for virus capsid polypeptides. Preparation and analysis of plant extracts for determination of virus capsid polypeptides has been described in Chapter 1. Monoclonal antibodies SB2, SB5 and DG11 were used in ELISA analysis.

Viral RNA analysis from plant extracts. Plant extracts were prepared to examine the replication of viral RNA. A disk of

tissue (approximately 100 mg) was taken with a #10 cork borer and stored at -20 C or used fresh. Each disc was homogenized in 425 ul of grinding buffer (0.2 M glycine; 0.1 M sodium phosphate; 0.6 M sodium chloride; 1% SDS; 1% 2-mercaptoethanol). The solution was extracted with an equal volume of phenol and briefly centrifuged to separate the phases. The aqueous phase was recovered and extracted with 200 ul each of phenol and chloroform; and then twice with 400 ul of chloroform.

The extracted aqueous phase was diluted with 1.5 volumes of 10X SSC (0.15 M trisodium citrate; 1.5 M sodium chloride, pH 7.2) and bound to Gene-Screen (GS) hybridization membrane (New England Nuclear) according to the manufacturer's instructions, using a slot-blotter (Schliecher & Schuell). Briefly, GS was soaked for 10 minutes each in distilled water and 10X SSC. One-fifth of the plant extract was applied to the membrane and diluted 1:10 and 1:100 with 10XSSC in the slot-blotter. The sample was drawn through the GS under vacuum provided by a water-tap aspirator. Each well was washed with 200 ul of 10X SSC under vacuum. Nucleic acid was covalently bound to GS by cross-linking with UV irradiation as described below.

Extraction of RNA from virus. RNA was extracted from virus by modification of the procedure described by Daubert et al. (1978). All solutions and glassware used in this procedure

were autoclaved. Virus was diluted with distilled water to 1 mg/ml in a final volume of 1.9 ml and 0.1 ml of 20% SDS (BDH Chemicals) was added. The solution was heated at 80 C for 90 seconds, then cooled in an ice-water bath to lower the temperature of the dissociated virus solution below 20 C. The solution was adjusted to pH 8, by adding 0.4 ml of buffer (1 M Tris-HCl; 40 mM EDTA, pH 8.0). All subsequent manipulations were done at room temperature unless otherwise indicated.

The solution containing the viral RNA was extracted with two volumes of water saturated phenol, containing 0.1% 8-hydroxyquinoline (phenol). Following agitation for 5 minutes, 1 ml of a solution of chloroform and isoamyl alcohol (24:1) was added and the mixture was centrifuged at 10,000 g for 15 minutes at 4 C. The aqueous phase was removed and extracted three more times with: 1) 2 ml of phenol and 2 ml of chloroform and isoamyl alcohol; 2) 1 ml of phenol and 3 ml of chloroform and isoamyl alcohol; and 3) 4 ml of chloroform and isoamyl alcohol. One-tenth volume 3 M sodium acetate (pH 5.0) and 2.5 to 3 volumes of 95% ethanol were added. The RNA was allowed to precipitate overnight at -20 C.

The precipitate was collected by centrifugation at 10,000 g for 15 minutes at -10 C, and dried _in vacuo_. The RNA was dissolved at 4 C in 0.4 ml of TEN (10 mM Tris-HCl; 1 mM EDTA; 10 mM sodium chloride, pH 7.6) per 1 mg of original virus concentration. The RNA was further purified on a 1 ml spin-column of Sepharose CL-2B-300 (Pharmacia). The matrix was

packed in a 1 ml disposable syringe and equilibrated with at least 4 applications of 0.2 ml TEN. The column was centrifuged at 2,000 g for 2 minutes between additions of buffer, and sample (Maniatis et al., 1982). The purified RNA was precipitated as above.

The integrity and purity of the RNA was determined by electrophoresis in a 1% agarose gel containing electrophoresis buffer (50 mM boric acid; 5 mM sodium borate; 10 mM sodium sulfate) plus 1 mM of methylmercuric hydroxide (Alfa Chemicals) (Bailey & Davidson, 1976). Electrophoresis was performed in a ventilated fume hood. The gels were stained for 15 minutes in 14 mM 2-mercaptoethanol plus 0.3 ug/ml of ethidium bromide and visualized by UV light.

The concentration of RNA was calculated using absorptivity values for 1 mg/ml of 25 at 260 nm (Davis et al., 1986).

UV irradiation of viral RNA. Up to 125 ug of purified viral RNA was dissolved in 200 ul of sterile distilled water and placed on a wax membrane (Parafilm) over a plate of glass. The RNA was irradiated at a distance of 10 cm with 1200 uW/cm^2 of UV light source (254 nm) for 5 minutes. Degraded RNA fragments with a poly(A) tail were removed from the total RNA population by purification on an oligo-dT cellulose (Type 7; Pharmacia) column (Maniatis et al., 1982).

Labeling of virus RNA. Viral RNA was 5'-terminal labeled with

polynucleotide kinase (PNKase) (Negruk et al., 1980). Up to 2 ug of purified viral RNA was partially degraded in 10 ul of hydrolysis buffer (5 mM sodium carbonate; 45 mM sodium bicarbonate; 2 mM EDTA) for 1.5 minutes at 90 C. The solution was cooled in ice and the reaction mixture was assembled in the stated order: 8 ul of distilled water; 5 ul of PNKase buffer (100 mM Tris-HCl, pH 7.6; 100 mM dithiothreitol; 100 mM magnesium chloride; 40% glycerol); 12 U of PNKase; 50 uCi gamma-^{32}P-ATP. The mixture was incubated for 45 minutes at 37 C, and stopped with the addition of 1 ul of 20% SDS and heating for 5 minutes at 65 C. The labeled RNA was recovered from the reaction mixture by purification on a spin-column of Sephadex G-50-coarse (Pharmacia).

Isolation of M13. Bacteria containing recombinant bacteriophage M13mp11 (Messing, 1983) were grown in 0.5 ml YT medium (5 g/l yeast extract; 5 g/l tryptone; 5 g/l sodium chloride) at 37 C overnight, with aeration. The bacterial culture was diluted with 10 ml of YT and grown for 3 to 5 hours. Growth was stopped by rapid chilling in an ice-water bath for 30 seconds. The bacteria were removed from the solution by centrifugation at 7,000 g for 10 minutes at 4 C. Phage was precipitated from 8 ml of the supernate with the addition of 2 ml each of 2.5 M sodium chloride and 40% polyethylene glycol-6000 (PEG). The precipitating solution was mixed by inversion and left to stand for 1 hour at 4 C. The precipitated phage were pelleted at 10,000 g for 15 minutes at

4 C. The pellet was solubilized in 2 mM Tris-HCl (pH 7.5) at 4 C, and re-precipitated with PEG/sodium chloride. The phage were stored at 4 C as a PEG precipitate.

Infection of bacteria with M13. Bacteria (JM105 or JM109; Yanisch-Perron et al., 1985) were grown in 0.5 ml YT overnight. From the overnight culture 50 ul and 250 ul were transferred to 0.5 ml and 25 ml of YT, respectively. The 25 ml culture was grown for 6-8 hours, and the 0.5 ml culture was grown to turbidity. From the 0.5 ml culture 50 ul were again diluted with 0.5 ml of YT the bacteria incubated for 1.5 hours. The bacterial culture was diluted with four volumes of YT and split into four equal parts. Each fraction was inoculated with phage from either: 1) a stab from an M13 plaque; or 2) a PEG precipitate. The bacteria and phage were incubated for 3 hours. From the latter culture and the previously inoculated 25 ml culture 0.2 ml and 2 ml, respectively, were transferred to 100 ml of YT and incubated overnight.

Plasmid isolation. Plasmids and the replicative form (RF) of bacteriophage M13 were purified from bacterial hosts by an alkali-lysis procedure (Birnboim & Doiley, 1979), modified by D. Schaeffer and K.C. Eastwell (personal communication). Single bacterial colonies containing recombinant DNA were grown in 100 ml of YT media (plus 35 ug/ml ampicillin for plasmid DNA). The cultures were placed on ice for 1 hour or

overnight. The cells were collected by centrifugation at 7000 g for 10 minutes at 4 C, and resuspended in 25 ml of wash buffer (10 mM Tris-HCl; 20 mM sodium chloride, pH 7.5). The cells were again collected by centrifugation, the pellet resuspended in 15 ml TES (10 mM Tris-HCl, pH 7.5; 50 mM EDTA; 45 mM sodium hydroxide; 20% sucrose) and diluted with 35 ml of lysis buffer (0.18 M sodium hydroxide and 1% SDS) prior to being incubated for 40 minutes at 50 C, with agitation. The cells were then placed in an ice-water bath for 40 minutes, with agitation. The cell debris was precipitated from the solution by the rapid addition of 25 ml ice-cold 3M sodium acetate; 0.3 M acetic acid and incubated in ice-water for a further 20 minutes. The precipitated cell debris was removed by centrifugation at 7,000 g for 30 minutes at 4 C.

The supernate containing the plasmid was recovered and extracted with one-half volume each of phenol and chloroform. The phases were separated by centrifugation at 7,000 g for 10 minutes at 4 C. The aqueous layer was removed and the DNA precipitated with an equal volume of isopropanol. The precipitated DNA was pelleted at 7,000 g for 30 minutes at room temperature, re-dissolved with a minimal volume of TEN, and the isopropanol precipitation repeated. The plasmid was then further purified on a Sepharose CL-2B-300 spin-column and stored as an ethanol precipitate.

The integrity and purity of the plasmid was determined by electrophoresis in agarose gels (Maniatis et al., 1982).

Oligo-labeling of DNA. Plasmid and restriction enzyme fragments were labeled to high specific activity according to the procedure described by Feinberg and Vogelstein (1983). Plasmids were denatured by boiling in distilled water for 3 minutes and then kept at 37 C. Restriction enzyme fragments were resolved in low gelling temperature agarose by electrophoresis, stained with ethidium bromide and visualized by UV light. The desired bands were excised. The DNA in the gel matrix was dissolved and the DNA denatured by boiling in water for 7 minutes and diluted 1:3 with distilled water prior to labeling. The labeled DNA was removed from the reaction mixture by purification on a Sepharose G-50-coarse spin-column.

Electrophoretic transfer. Nucleic acids in agarose and denaturing agarose-methylmercury hydroxide gels were electroblotted to GS (Bittner et al., 1980). Non-denaturing agarose gels and the transfer membrane were soaked in electroblotting buffer (EBB: 0.025 M sodium phosphate, pH 6.5) for 15 minutes, while unstained agarose-methylmercury hydroxide gels were soaked for 15 minutes each in: 1) EBB plus 14 mM 2-mercaptoethanol; and 2) EBB. Electroblotting was done for 6 to 16 hours with 250 mA of direct current at 4 C. The GS membrane with bound nucleic acid was washed in EBB for 5 minutes.

UV irradiation of filters. Nucleic acid bound to the GS

membrane was cross-linked to the membrane by UV irradiation (Church & Gilbert, 1984). The membrane was placed on a plate of glass with the nucleic acid-coated side facing up. The membrane was covered with Saran Wrap and irradiated for 10 minutes at a distance of 15 cm with 1200 uW/cm^2 of UV light (254 nm) (Church & Gilbert, 1984). The membrane was air dried and baked for 1 to 3 hours at 90 C.

cDNA cloning of CPMV and CPSMV. Complementary DNA (cDNA) synthesis was based on the procedure described by Grunstein and Hogness (1975), and modified by D'Alessio et al. (1987). Up to 2 ug of purified CPMV and CPSMV RNA in 7.5 ul of distilled water was diluted with an equal volume of 40 mM methylmercury hydroxide. The solution was kept at room temperature for 10 minutes, then immersed in liquid nitrogen until frozen. The reaction mixture for first strand cDNA synthesis (50 mM Tris-HCl, pH 8.0; 75 mM potassium chloride; 3 mM magnesium chloride; 10 mM dithiothreitol; 5 uM each dATP, dTTP, dCTP, and dGTP; 70 ug/ml oligo $(dT)_{12-18}$; 10,000 U/ml M-MLV reverse transcriptase (Bethesda Research Laboratories); 1 uCi alpha-[^{32}P]-dCTP) was added in a total volume of 50 ul to the solution before it thawed. The mixture was incubated at 37 C for 1 hour and the reaction stopped by immersion in an ice-water bath.

For cDNA clones to be generated by cloning restriction enzyme fragments, the second strand cDNA synthesis was carried

out in the presence of RNase H (Gubler & Hoffman, 1983). To the first strand cDNA mixture were added the components of the second strand synthesis solution at 4 C in order, for a final composition of: 25 mM Tris-HCl, pH8.3; 100 mM potasium chloride; 5 mM magnessium chloride; 5 mM dithiothreitol; 250 uM each dATP, dTTP, dCTP and dGTP; 250 U/ml DNA polymerase 1 (Promega); 8.5 U/ml RNase H (Bethesda Research Laboratories) in a total volume of 350 ul. The mixture was incubated for 2 hours at 16 C. The reaction was stopped by placing on ice and adding 12 ul of 0.5 M EDTA (pH 8.0). The cDNA was purified from the other components of the reaction by passing the reaction mixture through a spin-column of Sepharose CL-2B-300 and stored overnight as an ethanol precipitate at -20 C.

The precipitated cDNA was collected by centrifugation and digested with restriction enzymes according to the manufacturers specifications (Bethesda Research Laboratories; Promega). Restriction enzyme fragments were ligated between promoters for the DNA-dependent RNA polymerases SP6 and T7, in the plasmid vectors pGEM2 and pGEM4Z (Promega) (Melton et al., 1984). The vector had been linearized with the appropriate restriction enzyme and dephosphorylated with calf intestinal phosphatase (Boerhinger-Mannheim) (Maxum & Gilbert, 1980). The linearized dephosphorylated vector was purified from low gel temperature agarose (Burns & Beacham, 1983). Restriction fragments of cDNA and the linearized and dephosphorylated vector were mixed at a 2 to 1 molar ratio and ligated (Crouce

et al., 1983). *E. coli* strains JM105 or JM109 were transformed with the ligation product (Peacock et al., 1981).

For cDNA clones to be generated by tailing with terminal transferase; the first strand cDNA solution was treated as described above, but RNase H was omitted from the second strand cDNA synthesis buffer. The precipitated double-stranded DNA was collected by centrifugation, dried, and dissolved in 20 ul of TE (10 mM Tris-HCl; 0.5 mM EDTA, pH 7.5). To this solution were added the following components of the RNase H reaction in order, for a final composition of: 20 mM Tris-HCl, pH 7.5; 10 mM magnesium chloride; 20 mM potassium chloride; 0.1 mM EDTA; 0.1 mM dithiothreitol; 20 U/ml RNase H, in a total volume of 100 ul. The mixture was incubated for 20 minutes at 37 C, then place on ice and the reaction stopped with 1 ul of 0.5 M EDTA (pH 8.0). The cDNA solution was extracted with phenol and chloroform, and ethanol precipitated. The cDNA was tailed with terminal deoxynucleotidyl transferase (Land et al., 1981) in a reaction that contained 100 mM potassium cacodylate (pH 7.2), 2 mM cobalt chloride, 1 mM dithiothreitol and 0.1 mM dCTP, in a final volume of 20 ul. After pre-incubation of the mixture for 5 minutes at 37 C, 30 U of terminal deoxynucleotidyl transferase (Bethesda Research Laboratories) were added and incubation continued for another 5 minutes at 37 C. The reaction was stopped with the addition of 1 ul of 0.5 M disodium EDTA (pH 8.0) and 6 ul of 5 M sodium chloride,

followed by heating at 65 C for 5 minutes. The tailed cDNA was phenol and chloroform extracted, and separated from the reaction mixture by passage through a spin-column of Sepharose CL-2B-300. The purified DNA was concentrated by ethanol precipitation.

Tailed cDNA and poly(G)-tailed pUC9 vector (Pharmacia) were mixed in a molar ratio of 1:1 in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA (pH 8.0), 10 mM ammonium sulfate and 80 mM potassium chloride, in a total volume of 20 ul. The DNA fragments were heated in a water bath for 5 minutes at 65 C. The heat source was then removed, and the sample cooled in the water bath for 3 hours allowing the DNA fragments to anneal. Competent bacteria were transformed with the recombinant plasmids.

Screening of CPMV and CPSMV libraries. The cDNA libraries of CPMV and CPSMV described above and those generated from restriction enzyme fragments of CPMV and CPSMV cDNAs previously cloned into the bacteriophage vector M13mp11 were screened for clones hybridizing specifically to a single RNA of the bipartite viral genome of either CPMV or CPSMV. (The latter M13 libraries were provided by K.C. Eastwell.) Colonies and plaques containing sequences homologous to CPMV or CPSMV were identified by hybridization with 5'-terminal labeled viral RNA.

Bacterial cells containing cDNA libraries in plasmids were grown overnight at 37 C on YT media plus 35 ug/ml ampicillin. The colonies were replica plated onto 0.45 um

nitrocellulose (NC: Schleicher & Schuell), and the NC with bound bacterial cells was layered over a plate of selection media and grown for 6 to 8 hours at 37 C. The cDNA libraries in M13mp11 were amplified in JM101 or JM105, and plaques were lifted onto NC. Bacteria in the colony and plaque lifts were lysed and their DNA bound to the NC (Grunstein & Hogness, 1975). Filter papers (Whatman 3MM) were soaked with each of the four disruption buffers: 1) 10% SDS; 2) 0.5 M sodium hydroxide, 1.5 M sodium chloride; 3) 0.5 M Tris-HCl, 1.5 M sodium chloride, pH 8.0; and 4) 25 mM sodium phosphate, 2 mM EDTA, pH 7.4. The NC disks were placed sequentially on each of the four buffer soaked filter papers for 10 minutes, then air dried and baked in a vacuum oven for 2 hours at 95 C. Prior to pre-hybridization, bacterial and phage debris was removed by washing the filters in wash buffer (50 mM Tris-HCl: 1 M sodium chloride; 1 mM EDTA, pH 8.0) for 1 hour at room temperature, with moderate agitation.

Subcloning of restriction enzyme fragments. Recombinant M13mp11 RF and pUC9 plasmids containing sequences homologous to the genomes of CPMV and CPSMV were digested with suitable restriction enzymes. Restriction enzyme fragments were resolved by electrophoresis in low gel temperature agarose and the desired bands recovered (Burns & Beacham, 1983). The plasmid vectors pGEM2 and pGEM4Z were linearized with the appropriate restriction enzyme and dephosphorylated. Recovered

restriction enzyme fragments and linearized, dephosphorylated vector were mixed at a ratio of 1:2, and ligated. Competent bacteria were transformed with the ligation product. Bacteria containing clones complementary to CPMV and CPSMV were identified by allowing replicated NC to hybridize with 5'-terminally labeled viral RNA as described above.

The orientation of the clones in the transcription vectors was determined by hybridizing transcripts generated by SP6 and T7 DNA-dependent RNA polymerase reaction to electroblots of gels containing positive sense viral RNA.

A recombinant plasmid containing a sequence homologous to 18S RNA cloned from *Costaria costata* (kindly provided by Dr. Debashish Bhattacharya) was digested with restriction enzyme (Eco R1). The desired 1.91 kilobase fragment was subcloned to pGEM4Z (as described above).

DNA sequencing. DNA inserts in pUC9, pGEM2 and pGEM4Z were sequenced by the dideoxyribonucleotide method (Sanger et al., 1977). Components of the sequencing reaction were obtained as a package and conditions for the sequencing reactions were according to the manufacturer's instructions (Promega); except, reactions with the large fragment of DNA polymerase were incubated at 42 C. DNA sequence was analyzed with the Pustell sequence analysis program (IBI).

Synthesis of SP6 and T7 transcripts. SP6 and T7 transcripts of cloned fragments were synthesized using a protocol modified

from Melton et al. (1984). All solutions and plasticware used in the reaction were sterilized. Recombinant plasmids were linearized with a suitable restriction enzyme to permit the synthesis of either plus or minus sense RNA transcripts.

Up to 1 ug of linearized DNA was dissolved in 3 ul of distilled water and denatured by heating at 65 C for 10 minutes. The reaction components were then added in order: 2.0 ul of 100 mM dithiothreitol; 4 ul of ribonucleotide triphosphates (2.5 uM each of ATP, GTP and UTP; 20 uM CTP, pH 7.0); 4 ul of 5X transcription buffer (200 mM Tris-HCl, pH 7.5; 30 mM magnesium chloride; 10 mM spermidine; 50 mM sodium chloride); 10-50 uCi alpha-^{32}P-CTP; 10 U of SP6 (Promega) or 5 U T7 DNA-dependent RNA polymerase (Promega or New England Biolabs). The reaction mixture was incubated for 1 hour at 37 C. The DNA template was degraded by the addition of RNase-free DNase 1 (Promega) at a concentration of 1 U/ug DNA, and the reaction incubated for 15 minutes at 37 C. The reaction was stopped by heating for 5 minutes at 65 C. The labeled transcripts were recovered from the reaction mixture by purification on a Sephadex-G50-coarse spin-column.

Hybridization of nucleic acids. All hybridization procedures were modifications of the protocol outlined by the manufacturer of GS. Hybridization membranes with bound nucleic acids were pre-hybridized and hybridized in 1 ml hybridization buffer/3 cm^2 GS (50 mM Tris-HCl, pH 7.5; 1 M sodium chloride; serum albumin; 0.2% polyvinyl-pyrrolidone (molecular weight

40,000); 0.2% ficoll (molecular weight 400,000); 0.1% sodium pyrophosphate; 1% SDS; 50% deionized formamide; 10% (w/v) dextran sulfate; 0.1 mg/ml of sonicated salmon sperm DNA). Hybridizations were incubated for 12 to 18 hours in heating baths with agitation. For hybridization the labeled probe was added to the pre-hybridization solution at 500,000 cpm/ml. The specific conditions for hybridization and washing varied depending on the hybrids being formed:

A) DNA:DNA hybridizations. Pre-hybridization and hybridization were performed at 50 C. The filters were washed with: two washes in 2X SSC, 5 minutes each at room temperature; three washes in 2X SSC and 1% SDS, 20 minutes each at 65 C; and three washes in 0.1X SSC and 0.1% SDS, 20 minutes each at room temperature.

B) DNA:RNA hybridizations. Pre-hybridization and hybridization were done at 55 C. The filters were washed with: two washes in 2X SSC, 5 minutes each at room temperature; three washes in 2X SSC and 1% SDS, 20 minutes each at 65 C; three washes in 0.1X SSC and 0.1% SDS, 20 minutes each at 50 C; three washes in 0.1X SSC and 0.1% SDS, 20 minutes each at room temperature.

C) RNA:RNA hybridizations. Pre-hybridization and hybridization were incubated at 60 C. The filters were washed with: two washes in 2X SSC, 5 minutes each at room temperature; three washes in 0.1X SSC and 0.1% SDS, 20 minutes each at 65 C; three washes in 0.1X SSC and 0.1% SDS, 20

minutes each at room temperature; 2X SSC and 1 ug/ml RNase A (Sigma) for 10 minutes at room temperature; and 0.1X SSC and 0.1% SDS for 40 minutes at 50 C.

The dried membranes were autoradiographed on XAR-5 or XK film (Kodak).

RESULTS:

cDNA synthesis. RNAs purified from CPMV and CPSMV were denatured with methylmercury hydroxide and used as templates for the synthesis of cDNA. Methylmercury hydroxide reacts reversibly with the N-H bonds of uridine and guanidine involved in Watson-Crick base pairing, thereby effectively denaturing the RNA molecule (Bailey & Davidson, 1976). Compounds with reactive sulfhydryl groups (eg. 2-mercaptoethanol and dithiothreitol) completely reverse the denaturation process (Simpson, 1964). Methylmercury hydroxide has been used as a denaturant of RNA for agarose gel electrophoresis (Bailey & Davidson, 1976; Lehrach et al., 1977), for *in vitro* translation of mRNA (Moore & Sharp, 1984) and for cloning of yeast dsRNA (Skipper, 1983).

Approximately 20% of the cDNA synthesized per reaction co-electrophoresed with viral RNA in an agarose-methylmercury hydroxide gel, indicating that near full-length cDNAs were being synthesized. Electrophoresis of cDNA digested with various restriction enzymes produced multiple, distinct bands in agarose gels (data not shown).

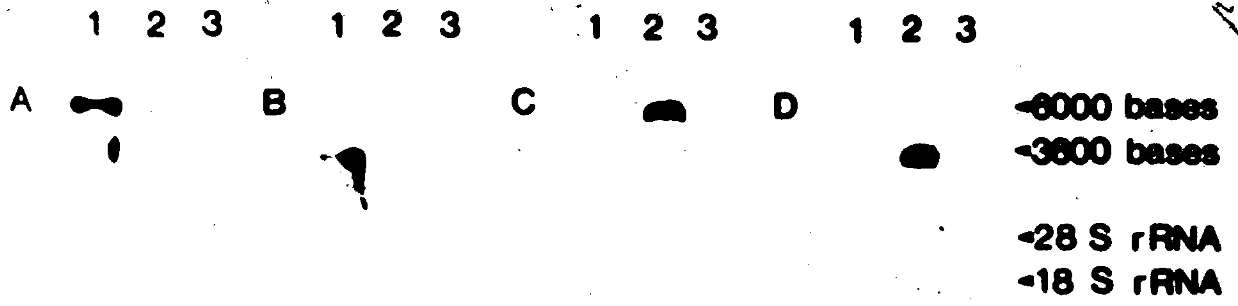
Viral RNA cloning. Clones with sequence complementary to RNA1 and RNA2 of CPMV, and RNA1 of CPSMV were identified in the cDNA libraries maintained in M13mp11. The libraries were screened by hybridization of 5'-terminal labeled viral RNAs to

plaque lifts. Clones were selected which hybridized to only one of the viral RNAs of either CPMV or CPSMV, but not both. These sequences were subcloned into the multiple cloning region of the transcription vector pGEM2 (Melton et al., 1984). Three distinct clones were identified: 1) pG2SB1, a 310 base Sau 3AI fragment of CPMV RNA1 cloned into the Bam HI site of pGEM2; 2) pG2SB2, a 342 base Taq I fragment of CPMV RNA2 cloned into the Acc I site of the vector, and 3) pG2DG1, a 740 base Taq I fragment of CPSMV RNA1 cloned into the Acc I site of the vector.

A clone homologous to CPSMV RNA2 was selected from the plasmid cDNA library, constructed by cloning restriction fragments generated from the cDNA of the viral RNA into pGEM4Z. The clone of CPSMV RNA2, pG4ZDG2, was an 845 base Bgl II restriction enzyme fragment cloned into a homologous restriction enzyme site in the multiple cloning region of the vector.

The SP6 and T7 DNA-dependent RNA polymerases were used to generate RNA transcripts complementary to the clones. These transcripts were hybridized to electroblots of the genomes of CPMV and CPSMV to determine the orientation of the clone and to confirm that each clone hybridized to a single RNA of either CPMV or CPSMV (Figure 10). Clones pG2SB1, pG2SB2, pG2DG1 and pG4ZDG2 were transcribed with RNA polymerase T7, SP6, T7 and T7, respectively, to generate RNA complementary to positive-sense viral RNA.

Figure 10. Minus sense transcripts of viral clones (A), pG2SB1; (B), pG2SB2; (C), pG2DG1; (D), pG4ZDG2) were hybridized to the genomes of CPMV (lane 1) and CPSMV (lane 2). Lane 3: RNA extracted from uninfected BE-5 cowpeas.


1 2 3
1 2 3
1 2 3
1 2 3
A
B
C
D
<6000 bases
<3600 bases
<28 S rRNA
<18 S rRNA

The sequence of each clone was determined by the chain termination method (Sanger et al., 1977) using primers complementary to the SP6 and T7 promoters in pGEM2 and pGEM4Z (Figure 11). Clones of CPMV RNA1 and RNA2 were aligned with the published sequences of the viral genome (Lomonossoff & Shanks, 1983; Van Wezenbeek et al., 1983). Clones pG2SB1 and pG2SB2 corresponded to bases 3547 to 3857 of CPMV RNA1 and 1622 to 1964 of CPMV RNA2, respectively. The nucleotide sequences at the 5'-end and 3'-end of the CPSMV clones were determined. However, the latter clones cannot be localized on the CPSMV genome as the complete sequence of the CPSMV genome is unknown. The sequences of the CPSMV clones were compared in blocks of 7 nucleotides to the sequence of the genome of CPMV, and analyzed for regions of 50% or greater homology. Regions of homology were not found. The latter was not unexpected because CPSMV clones were purified on the basis that they did not hybridize to CPMV.

Replication of CPMV in cowpea seedlings. As a foundation for further studies, the accumulation of CPMV RNA was determined in the susceptible cowpea variety BE-5 inoculated with 250 ug/ml intact CPMV (Figure 12). The accumulation of positive-sense viral RNA was determined in plant extracts at various time intervals post infection (p.i.). In BE-5, the quantity of viral RNA recovered from inoculated plants increased dramatically between 2 days and 5 days p.i. The viral RNA content of the inoculated leaves continued to increase up to

Figure 11. Partial sequences of CPMV and CPSMV clones.

```
            ---> Sp6 promoter
CPMV RNA1[1]  5'-GATCTCTTGTGATAGCACACATTGGTGGGAAGCACAAGATTGTGGGTGTT  3596
(pG2SB1)         CATGTTGCTGGTATTCAAGGTAAGATAGGATGTGCTTCCTTATTGCCACC  3646
                 ATTGGAGCCAATAGCACAAGCGCAAGGTGCTGAGGAATACTTT-...     3689

              ...-ATCTTCTGGAGTGGCTATGGTAGCAGGACTCAAACAAGGAGTTTACATCC  3765
                 ATTACCCACAAAAACAGCGCTAGTGGAGACCCCCTCCGAGTGGCATTTGG  3815
                 ACACACCATGTGACAAAGTTCCTAGCATTTTAGTTCCCACG-3'        3856
                                              T7 promoter <---
            ---> T7 promoter
CPMV RNA2[1]  5'-CGAGTTTTGTTGTCCAAGGCTATGGCTGGTGGTGATGTGTTATTGGATGA  1671
(pG2SB2)         GTATCTCTATGATGTGGTCAATGGACAAGATTTTAGAGCTACTGTCGCTT  1721
                 TTTTGCGCACCCATGTTATAACAGGCAAAATAAAGGTGACAGCTACCACC  1771
                 AACATTTCTGACAACTCGGGTTGTTGTTTGATGTTGGCCATAAATAGTGG  1821
                 TGTGAGGGGTAAGTATAGTACTGATGTTTATACTATCTGCTCTCAAGACT  1871
                 CCATGACGTGGAACCCAGGGTGCAAAAAGAACTTCTCGTTCACATTTAAT  1921
                 CCAAACCCTTGTGGGGATTCTTGGTCTGCTGAGATGATAAGT-3'       1963
                                              Sp6 promoter <---

            ---> Sp6 promoter
CPSMV RNA1    5'-CGACCCCAAAAAATTTGATTTGGGTGATGGTTCACGTAGTGGCCATCGCC    50
(pG2DG1)         TGnATAGACGGTTTTTCGCCCTTTGACGTTGGAGTCCACGTTTCTTTAAT   100
                 AGTGGACTCTTGTTCCAAACTGGAACAACACTCAACCCTATCTCGGGCTA   150
                 ATTCTTTTGATTTATAAGGGATTTTGCCGATTTCGGAACCACCATCAAAC   200
                 AGGATTTTCGCCTGCTGGGCAAATCC-...                       226

              ...-CGGATAACAATTTCACACAGAAACAGCTATGACCATAATTACGCCAAGCT   643
                 TGGCTACnGGTCGTACACCGTCTTAATACCAGCTGTATAGnTCACGCAAA   693
                 TCGTTTTCCGGTGACCGTTTTCCCACACCTTTTGTATATATACTTGT-3'   740
                                                   T7 promoter <---
            ---> Sp6 promoter
CPSMV RNA2    5'-TTGTTGGAGTTGAACTGGTTACTGATCCGGACCAGGGGGCAGTTTCTGTA    50
(pG4ZDG2)        TTGAGCAGCAGTCCAGTAGCAAATTTGCTACGCACAGCGGCTTGGAAGTG   100
                 TGGAAACCTGCATGTTAAAGTTGTTATGACTGGAGAGTTACTA-...      143

              ...-AAAGTCGTAGAAAACATAGAGTGAATCCAGAATACTTGGATCCCTGTTAG   800
                 GCAGTTGCCCAATCTGGACAGACACAGCAGGTTTGGAATAAGATC-3'     845
                                                 T7 promoter <---
```

1. Clones of CPMV RNA1 and RNA2 correspond to bases 3547 to 3856 and 1622 to 1963, of the respective viral RNAs.

Figure 12. Replication of CPMV RNA1 (A) and RNA2 (B) in inoculated and secondary leaves of Blackeye-5 cowpeas. CPMV RNA1 and RNA2 were assayed for with minus sense ssRNA transcribed from pG2SB1 and pG2SB2, respectively. The second column represented a 1/10 dilution of the first column.

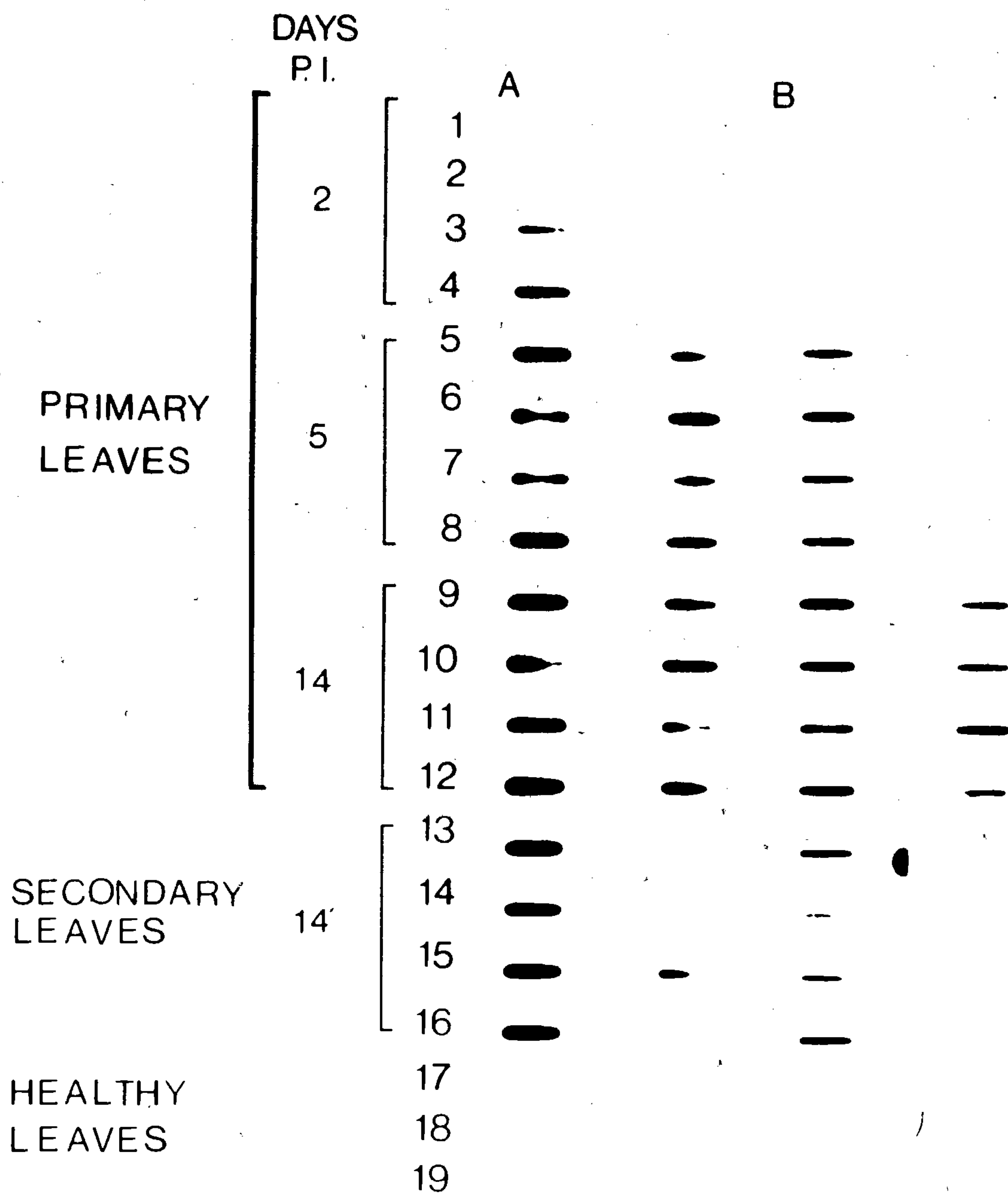
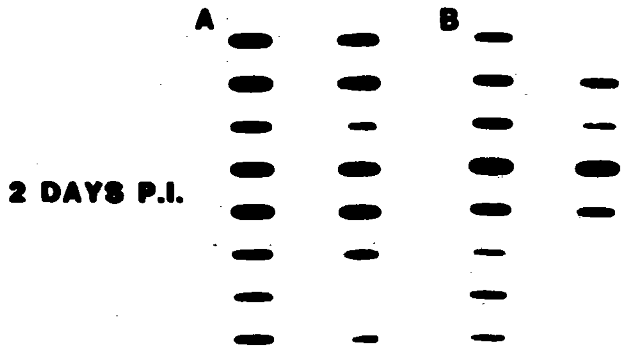
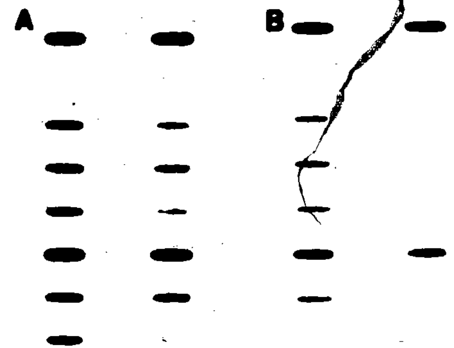

DAYS
P.I.
A
B
PRIMARY
LEAVES
2
5
14
SECONDARY
LEAVES
14'
HEALTHY
LEAVES
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19

Figure 13. Replication of CPMV RNA1 (A) and RNA2 (B) in inoculated leaves of Black and Arlington cowpeas. CPMV RNA1 and RNA2 were probed with minus sense ssRNA transcribed from pG2SB1 and pG2SB2, respectively. The second column represented a 1/10 dilution of the first column. Extracts from uninfected plants did not hybridize to the probes.

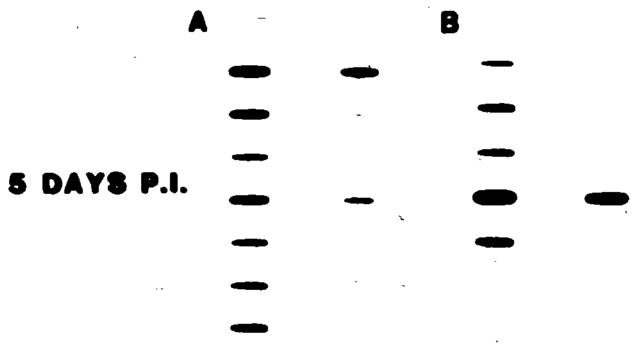
BLACK ARLINGTON

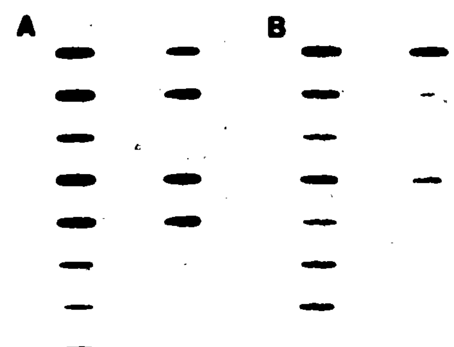
2 DAYS P.I.



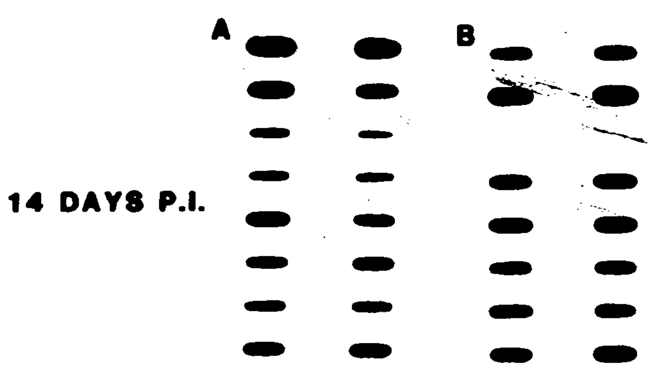


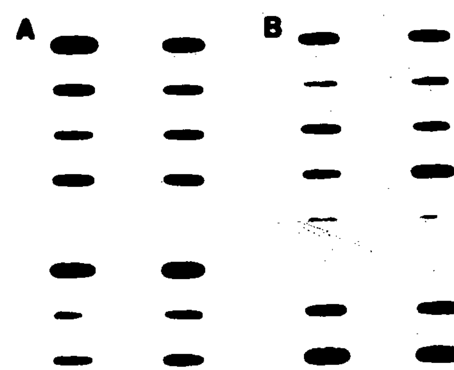
5 DAYS P.I.





14 DAYS P.I.





14 days p.i. No estimations were performed after 14 days p.i. because of the rapid onset of senescence. Viral RNA was found to accumulate in secondary leaves.

The accumulation of CPMV RNAs were also assayed in the immune cowpea cultivars Black and Arlington (Figure 13). Viral RNA replication was delayed considerably relative to the replication in BE-5. A net accumulation of viral RNA was not detected until after 5 days p.i. (Figure 13). Viral RNA was not detected in secondary leaves (data not shown). The viral RNA present at 2 days p.i. was apparently residual inoculum. The amount of RNA recovered continued to decline to that illustrated for 5 days p.i. Trials where all three cowpea cultivars were inoculated with 75 ug/ml CPMV gave parallel results (data not shown). Viral RNA was visualized in RNA preparations extracted 14 days p.i. from BE-5, Black and Arlington cowpeas infected with CPMV (Figure 14). Eastwell et al. (1983) detected the double-stranded RNA replicative form of CPMV RNAs in Black and Arlington cowpeas.

The accumulation of minus sense viral RNAs, the replicative forms of the viral genome, was not examined due to the sensitivity of the assay employed. Conditions for the analysis of virus replication were optimized to examine the replication of the viral genome in single plants at various times p.i., whereas previous experiments examining the accumulation of minus sense viral RNAs required 50 g of tissue per sample (Eastwell et al., 1983).

The accumulation of CPMV capsid polypeptides was examined at various time intervals p.i. in BE-5, Black and Arlington seedlings inoculated with 75 ug/ml and 250 ug/ml of intact CPMV (Figure 15), or inoculated with 50 ug/ml and 250 ug/ml of isolated CPMV RNA (Figure 16). Plant extracts were assayed by an antibody-trapped ELISA using monoclonal antibodies SB2 and SB5. Monoclonal antibody SB5 bound only intact virus capsid, while monoclonal antibody SB2 bound intact and dissociated capsid polypeptides (Chapter 2). Therefore, capsid polypeptides would have been detected by monoclonal antibody SB2 if they were translated but not assembled to form an infectious virus particle. CPMV capsid polypeptides did not accumulate in either Black or Arlington cowpeas. The absorbances recorded using monoclonal antibody SB5 were greater compared to values recorded using monoclonal antibody SB2 because SB5 had a greater affinity for antigen than SB2 (Chapter 2). In some of the CPMV infected plants, capsid polypeptides were detected 2 days or less p.i., and were presumed to be the inoculating virus. Residual inoculum was also observed in nucleic acid hybridization experiments (Figure 13). Viral capsid proteins were not detected in crude-sap extracts of Black and Arlington examined by polyacrylamide gel electrophoresis (as described by Evans, 1985).

Symptoms were not observed on Black and Arlington cowpeas inoculated with CPMV. Sap expressed from infected Black and Arlington seedlings was inoculated to BE-5 and the indicator

Figure 14. Analysis by denaturing agarose gel electrophoresis of CPMV replication 14 days p.i. in different varieties of cowpeas. Lanes 1, 2 and 3: 5.0 ug RNA extracted from uninfected Blackeye-5, Black and Arlington cowpeas, respectively. Lane 4: 0.5 ug of RNA extracted from BE-5 cowpeas infected with CPMV. Lanes 5 and 6: 1.0 ug of RNA extracted from Black and Arlington cowpeas infected with CPMV. Lane 7: 0.2 ug of RNA extracted from CPMV.

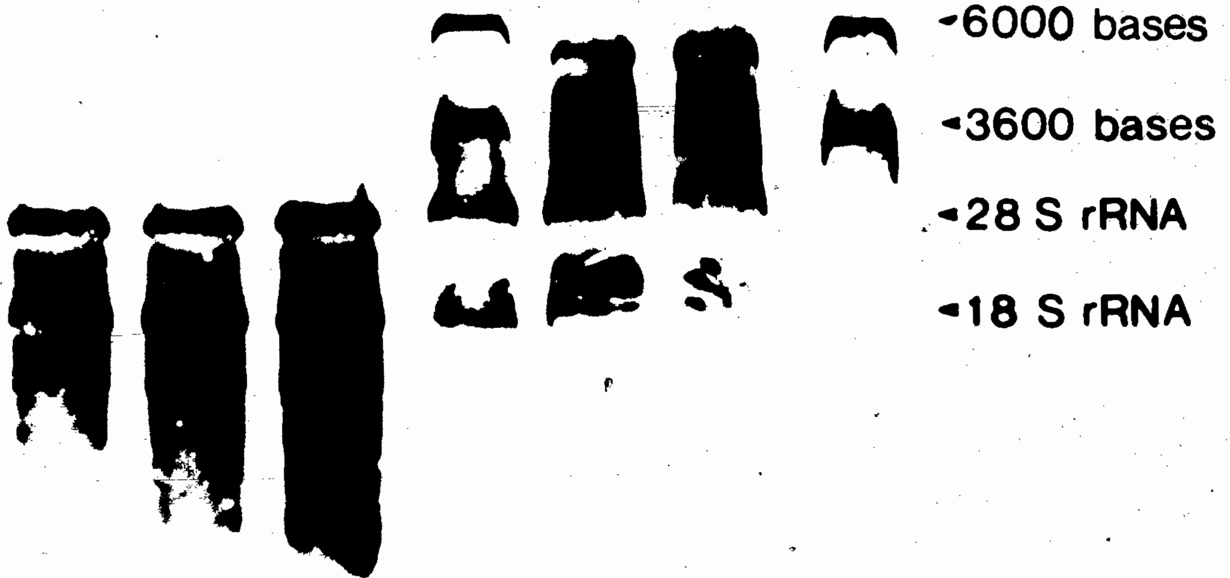
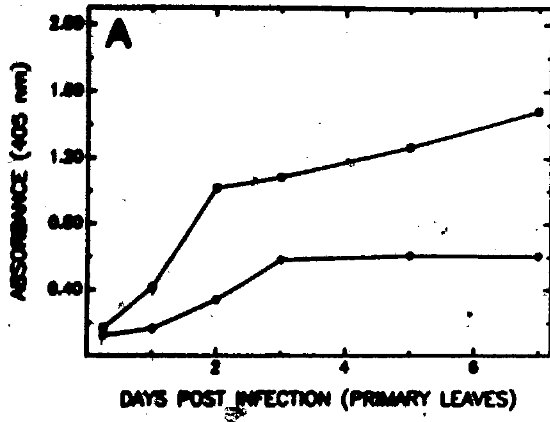
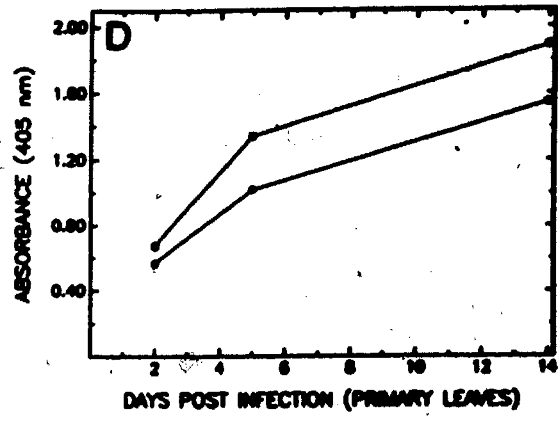
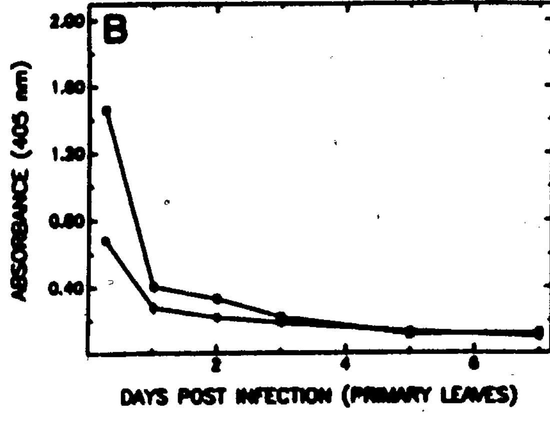
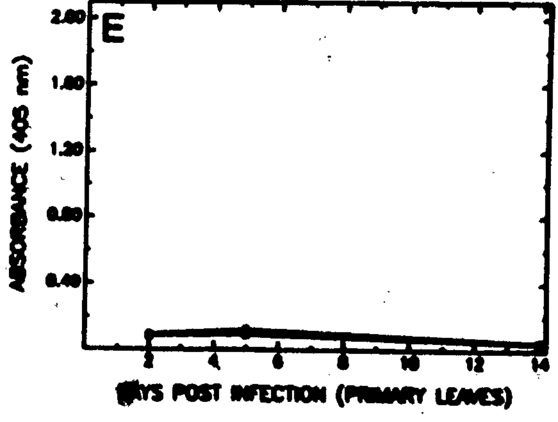

1
2
3
4
5
6
7
6000 bases
3600 bases
28 S rRNA
18 S rRNA

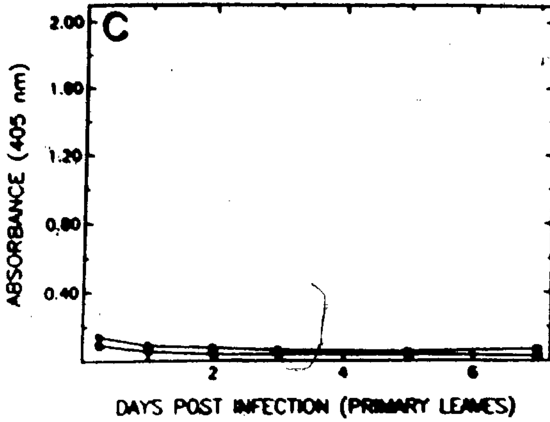
Figure 15. Accumulation of CPMV in Blackeye-5 (A & D), Black (B & E), and Arlington (C & F) cowpeas determined by antibody-trapped ELISA, using monoclonal antibodies SB2 (●) and SB5 (■). Plants were inoculated with 75 ug/ml (A-C) or 250 ug/ml (D-F) of CPMV. Each point represents an average of six test plants. Background < 0.100.


A
2.00
1.60
1.20
0.80
0.40
ABSORBANCE (405 nm)
2
4
6
DAYS POST INFECTION (PRIMARY LEAVES)


D
2.00
1.60
1.20
0.80
0.40
ABSORBANCE (405 nm)
2
4
6
8
10
12
14
DAYS POST INFECTION (PRIMARY LEAVES)


B
2.00
1.60
1.20
0.80
0.40
ABSORBANCE (405 nm)
2
4
6
DAYS POST INFECTION (PRIMARY LEAVES)


E
2.00
1.60
1.20
0.80
0.40
ABSORBANCE (405 nm)
2
4
6
8
10
12
14
DAYS POST INFECTION (PRIMARY LEAVES)


C
2.00
1.60
1.20
0.80
0.40
ABSORBANCE (405 nm)
2
4
6
DAYS POST INFECTION (PRIMARY LEAVES)

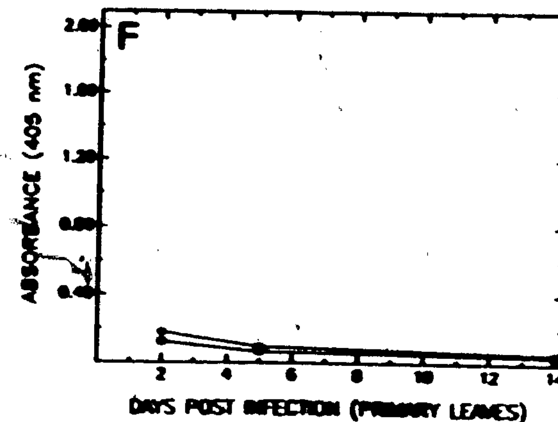
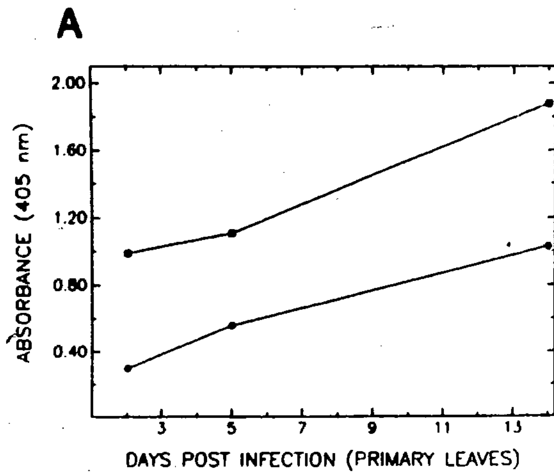
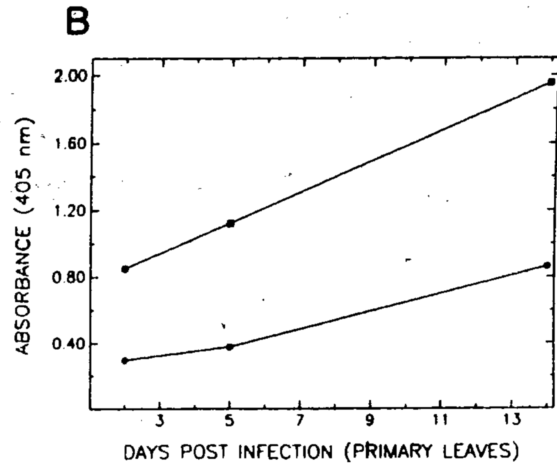
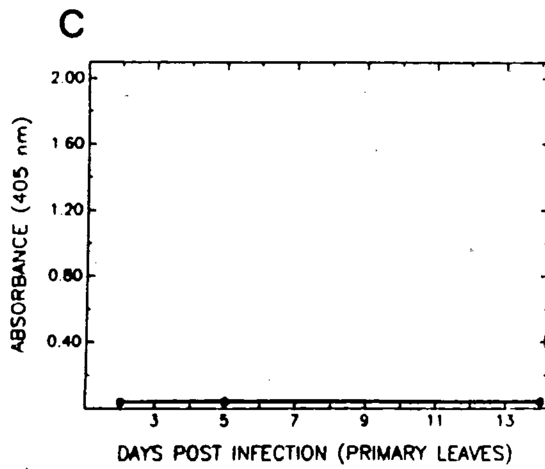
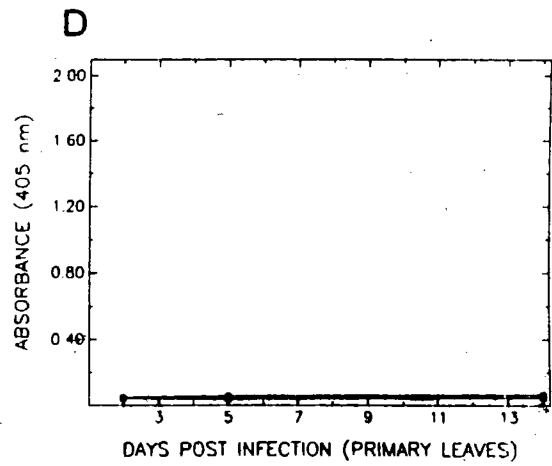

F
2.00
1.60
1.20
0.80
0.40
ABSORBANCE (405 nm)
2
4
6
8
10
12
14
DAYS POST INFECTION (PRIMARY LEAVES)

Figure 16. Accumulation of CPMV in Blackeye-5 (A-B) and Black (C-D) cowpeas determined by antibody-trapped ELISA, using monoclonal antibodies SB2 (●) and SB5 (■). Plants were inoculated with 50 ug/ml (A & C) and 250 ug/ml (B & D) of CPMV ssRNA. Each point represents an average of six test plants. Background < 0.100.


A
2.00
1.60
1.20
0.80
0.40
ABSORBANCE (405 nm)
3
5
7
9
11
13
DAYS POST INFECTION (PRIMARY LEAVES)


B
2.00
1.60
1.20
0.80
0.40
ABSORBANCE (405 nm)
3
5
7
9
11
13
DAYS POST INFECTION (PRIMARY LEAVES)


C
2.00
1.60
1.20
0.80
0.40
ABSORBANCE (405 nm)
3
5
7
9
11
13
DAYS POST INFECTION (PRIMARY LEAVES)


D
2.00
1.60
1.20
0.80
0.40
ABSORBANCE (405 nm)
3
5
7
9
11
13
DAYS POST INFECTION (PRIMARY LEAVES)

cowpea CRXI. The indicator plants were assayed for virus infection on the basis of an antibody-trapped ELISA (Table 9). Infectious CPMV was not transmitted from either Black or Arlington cowpeas. Furthermore, CPMV could not be purified from either Black or Arlington by the procedure used to recover CPMV from BE-5.

Modulation of symptom expression in co-inoculated plants. The symptoms induced in Black and Arlington cowpeas co-inoculated with CPMV and CPSMV were different from the symptoms induced in these cowpeas singularly inoculated with CPSMV. Initially, variations in symptoms were used to assess the extent of interference in the replication of CPSMV when co-inoculated with CPMV. Inoculation of Black or Arlington seedlings with a minimum of 1.0 ug/ml of CPSMV resulted in 100% of the inoculated plants developing symptoms. Large areas of necrosis were visible 3 to 4 days p.i., followed 6 to 8 days p.i. by vascular necrosis and collapse. Symptom expression was accelerated by increasing the temperature in the growth chamber above 25 C. Vascular collapse in Arlington seedlings was universal and irrevocable; however, approximately 5% of the collapsed Black cowpeas developed secondary leaves with chlorotic lesions after 21 days p.i.. Inoculation of Black and Arlington cowpeas with CPMV did not result in visible symptom expression.

Co-inoculation of Black and Arlington cowpeas with CPMV and CPSMV resulted in a 2 to 3 day delay in the appearance of

Table 9. Bio-assay of sap from Blackeye-5, Black and Arlington cowpeas inoculated with CPMV.

INOCULUM[1]	ASSAY PLANT	ANTIBODY-TRAPPED ELISA[2]	
		SB2	SB5
Healthy BE-5	BE-5	-	-
Healthy Blk	BE-5	-	-
Healthy Arl	BE-5	-	-
CPMV infected BE-5	BE-5	+++	++++
CPMV infected Blk	BE-5	-	-
CPMV infected Arl	BE-5	-	-
CPMV purified virus	BE-5	+++	+++
Healthy BE-5	CRXI	-	-
Healthy Blk	CRXI	-	-
Healthy Arl	CRXI	-	-
CPMV infected BE-5	CRXI	+++	+++
CPMV infected Blk	CRXI	-	-
CPMV infected Arl	CRXI	-	-
CPMV purified virus	CRXI	+++	++++

1. Blackeye-5 (BE-5), Black (BLK) and Arlington (Arl) cowpeas were initially inoculated with 75 ug/ml of CPMV-SB. Fourteen days post inoculation, 0.2 mg of primary leaves were pulverized in an equal volume (w/v) of inoculation buffer (50 mm potassium phosphate, pH 7.0), and bioassayed for CPMV infectivity on BE-5 and an indicator plant, Chinese Red X Iron (CRXI).

2. Infection of BE-5 and CRXI was assayed by antibody trapped ELISA using monoclonal antibodies SB2 and SB5. Absorbance at 405 nm was determined: 0.000-0.100 = -; 0.101-0.250 = +; 0.251-0.500 = ++; 0.501-1.000 = +++; >1.001 = ++++. Background < 0.100.

Figure 17. Co-inoculated Black cowpeas with infected (a) and uninfected (b) secondary leaves, twelve days p.i. Primary inoculated leaves of plants with uninfected secondary leaves show distinct necrotic lesions. Seedlings were co-inoculated with 75 ug/ml CPMV and 7.5 ug/ml CPSMV.

CPSMV symptoms on all the inoculated plants. An average of 25% of the co-inoculated plants developed distinct necrotic lesions and grew symptomless secondary leaves. The remainder of the plants developed large areas of necrosis and suffered vascular collapse by 9 to 10 days p.i. (Figure 17). Interference was considered to be complete in plants that developed symptomless secondary leaves. The distinct necrotic lesions which formed on the latter plants appeared to restrict further migration of CPSMV in the plant.

Optimal conditions for interference. Experiments were conducted to determine the optimal conditions for interference of CPSMV replication by CPMV. Plants were visually assayed for symptom development and/or by ELISA for virus replication 14 days p.i. unless otherwise stated. (Not all of the following experiments were conducted in both Black and Arlington cowpeas due to a shortage of Arlington cowpea seeds.)

Black and Arlington cowpeas were co-inoculated with 75 ug/ml of CPMV and 7.5 ug/ml of CPSMV at various stages of plant development. Initial observations indicated optimal interference would be observed if plants were inoculated when the secondary leaves had grown to a length greater than 2 cm (Table 10). However, 47% of those plants with uninfected secondary leaves at 14 days p.i. succumbed to vascular collapse by 21 days p.i. When plants were inoculated before the secondary leaves had formed or before the secondary leaves

Table 10. Co-inoculation of Black and Arlington cowpeas with CPSMV and CPMV at various stages of plant development.

STAGES OF PLANT DEVELOPMENT[1]	BLACK			ARLINGTON		
	SURVIVING PLANTS[2]	TOTAL NUMBER OF PLANTS INOCULATED	PERCENT SURVIVING	SURVIVING PLANTS[2]	TOTAL NUMBER OF PLANTS INOCULATED	PERCENT SURVIVING
no trifoliates	6	24	25	0	12	0
trifoliates < 1 cm	6	29	21	4	21	19
trifoliates > 2 cm	9	29	31	9	29	31

1. CPSMV and CPMV were co-inoculated at 7.5 ug/ml and 75 ug/ml, respectively.

2. Plants were assayed at fourteen days post infection

were greater than 1 cm in length, all the plants with uninfected secondary leaves at 14 days p.i. survived to 21 days p.i.. Therefore, Black and Arlington cowpeas were inoculated after the secondary leaves had formed, but before they were longer than 1 cm.

Black cowpeas were inoculated with 75 ug/ml of CPMV and various concentrations of CPSMV to determine the ratio of CPMV:CPSMV most efficient for interference. A concentration of CPSMV was chosen that resulted in vascular collapse of 100% of the seedlings singularly inoculated with CPSMV because the distinction between successful and unsuccessful interference was defined as the proportion of co-inoculated plants which developed uninfected secondary leaves compared to plants which suffered vascular collapse. Concentrations of less than 7.5 ug/ml of CPSMV did not result in universal vascular collapse of inoculated Black seedlings (Table 11). Similarly, inoculation with 7.5 ug/ml of CPSMV was required to induce vascular collapse in 100% inoculated Arlington seedlings (data not shown). Previously published experiments with Arlington seedlings had indicated a ratio of 75 ug/ml of CPMV to 7.5 ug/ml of CPSMV was the optimal ratio for interference (Bruening et al., 1979). Consequently, a ratio of 75 ug/ml CPMV to 7.5 ug/ml of CPSMV was determined to be the most suitable for interference for further experiments (Table 11).

The effect of the time interval between inoculation of Black and Arlington cowpeas with CPMV and CPSMV was evaluated

Table 11. Co-inoculation of Black cowpeas with varying ratios of CPSMV to CPMV.

INOCULUM [CPSMV] (ug/ml)	INOCULUM [CPMV] (ug/ml)	RATIO	SURVIVING PLANTS[1]	TOTAL NUMBER OF PLANTS INOCULATED	PERCENT SURVIVING
15	0	----	0	12	0
7.5	0	----	0	12	0
0.75	0	----	6[2]	12	50
0.18	0	----	9[2]	11	82
15	75	1:5	9	39	23
7.5	75	1:10	13	48	27
0.75	75	1:20	40	44	91
0.18	75	1:40	45	45	100

1. Plants were assayed 14 days post infection.
2. Every inoculated plant exhibited necrotic local lesions.

(Table 12). Plants were initially inoculated with 75 ug/ml of CPMV followed by inoculation with 7.5 ug/ml of CPSMV at various times. The results indicated that plants have to be co-inoculated for optimal interference. Bruening et al. (1979) conducted similar experiments with Arlington seedlings, but had observed a more gradual decrease in the efficiency of interference with respect to an increasing intervals between CPMV and CPSMV inoculation. In the present experiment with Black seedlings, the leaves initially inoculated with CPMV were thoroughly rinsed with distilled water and blotted dry with paper towel prior to inoculation with CPSMV, ensuring that residual CPMV was not co-inoculated with CPSMV. This combined with differences in the response of Black and Arlington cowpeas to viral inoculation, may account for the dramatic decrease in interference when CPMV and CPSMV are sequentially inoculated in Black seedlings, even with a brief time delay.

Co-inoculation with various components of CPMV and CPSMV. Top, middle and bottom components of CPMV were fractionated by equilibrium density gradient centrifugation, and co-inoculated individually with CPSMV to Black and Arlington cowpeas. Only the bottom component (containing RNA1) of CPMV interferred with the replication of CPSMV. Neither middle (containing RNA2) nor top (empty capsid) components of CPMV were able to attenuate the replication of CPSMV in Black or Arlington cowpeas (Table 13).

Table 12. Delayed inoculation of Black and Arlington with CPMV cowpeas following inoculation with CPSMV.

	BLACK			ARLINGTON		
TIME PRIOR TO CPSMV INOCULATION[1]	SURVIVING PLANTS[2]	TOTAL NUMBER OF PLANTS INOCULATED	PERCENT SURVIVING	SURVIVING PLANTS[2]	TOTAL NUMBER OF PLANTS INOCULATED	PERCENT SURVIVING
0	14	45	31	17	17	30
5 min.	2	20	10	2	20	10
4 hrs	1	22	5	3	25	12
1 day	1	38	3	1	21	5
3 days	1	22	5	2	32	6
6 days	1	25	4	3	42	7

1. CPSMV and CPMV were co-inoculated at 7.5 ug/ml and 75 ug/ml, respectively. All plants, except co-inoculated plants, were initially inoculated with CPMV, the leaves were rinsed with distilled-water and blotted dry.

2. Plants were assayed 14 days post infection.

The bottom component of CPMV interferred more efficiently with the replication of CPSMV than unfractionated virus. The average number of necrotic local lesions formed on the primary leaves of Arlington seedlings co-inoculated with CPMV-bottom component and CPSMV was one-tenth the number of lesions when plants were co-inoculation with unfractionated CPMV and CPSMV (Table 13). Therefore, RNA1 of the bottom component of CPMV determined the extent of interference with CPSMV replication in co-inoculated plants.

The efficiency of purified CPMV RNA relative to CPMV virions in interfering with the replication of CPSMV in Black seedlings was determined (Table 14). Plants were inoculated with: 1) 75 ug/ml of CPMV and either 7.5 ug/ml or 1.0 ug/ml of CPSMV; or 2) 15 ug/ml of CPMV RNA and either 1.5 ug/ml or 0.2 ug/ml of CPSMV RNA. In a parallel experiment, Black cowpeas were inoculated with the same molar concentrations of CPSMV and CPSMV RNA; 100% of the inoculated plants exhibited vascular collapse (Table 14, experiments 1 & 2). In co-inoculation experiments, CPMV RNA interferred more efficiently with CPSMV RNA than CPMV interferred with CPSMV.

An experiment was conducted to determine whether isolated CPSMV and CPSMV RNA were equally susceptible to interference by CPMV RNA. Purified CPMV RNA (15 ug/ml) and 1.0 ug/ml of CPSMV were co-inoculated on Black cowpeas. This ratio represents a 25:1 molar ratio of CPMV:CPSMV. Interference was observed in 25% of the inoculated plants (data not shown)

Table 13 . Co-inoculation of Black and Arlington cowpeas with CPSMV and fractionated components of CPMV.

INOCULUM[1]	LOCAL LESION NUMBER	SURVIVING PLANTS	TOTAL NUMBER OF PLANTS INOCULATED	PERCENT SURVIVING
Black[2]				
CPMV/CPSMV	ND[2]	16	64	25
CPMV-top comp./ CPSMV	ND	0	55	0
CPMV-middle comp./ CPSMV	ND	0	68	0
CPMV-bottom comp./ CPSMV	ND	24	67	36
Arlington				
CPMV/CPSMV	4	9	42	21
CPMV-top comp./ CPSMV	18	0	42	0
CPMV-middle comp./ CPSMV	18	0	36	0
CPMV-bottom comp./ CPSMV	0.4	24	67	36

1. Unfractionated CPMV and CPSMV were inoculated at 75 ug/ml and 7.5 ug/ml, respectively. Fractionated viral components were inoculated at 37.5 ug/ml. Plants were assayed 14 days post infection.

2. ND= not determined.

Table 9. Bio-assay of sap from Blackeye-5, Black and Arlington cowpeas inoculated with CPMV.

INOCULUM[1]	ASSAY PLANT	ANTIBODY-TRAPPED ELISA[2]	
		SB2	SB5
Healthy BE-5	BE-5	-	-
Healthy Blk	BE-5	-	-
Healthy Arl	BE-5	-	-
CPMV infected BE-5	BE-5	+++	++++
CPMV infected Blk	BE-5	-	-
CPMV infected Arl	BE-5	-	-
CPMV purified virus	BE-5	+++	+++
Healthy BE-5	CRXI	-	-
Healthy Blk	CRXI	-	-
Healthy Arl	CRXI	-	-
CPMV infected BE-5	CRXI	+++	+++
CPMV infected Blk	CRXI	-	-
CPMV infected Arl	CRXI	-	-
CPMV purified virus	CRXI	+++	++++

1. Blackeye-5 (BE-5), Black (BLK) and Arlington (Arl) cowpeas were initially inoculated with 75 ug/ml of CPMV-SB. Fourteen days post inoculation, 0.2 mg of primary leaves were pulverized in an equal volume (w/v) of inoculation buffer (50 mm potassium phosphate, pH 7.0), and bioassayed for CPMV infectivity on BE-5 and an indicator plant, Chinese Red X Iron (CRXI).

2. Infection of BE-5 and CRXI was assayed by antibody trapped ELISA using monoclonal antibodies SB2 and SB5. Absorbance at 405 nm was determined: 0.000-0.100 = -; 0.101-0.250 = +; 0.251-0.500 = ++; 0.501-1.000 = +++; >1.001 = ++++. Background < 0.100.

compared to 90% of the plants co-inoculated with 15 ug/ml of CPMV RNA and 0.2 ug/ml of CPSMV RNA. Therefore, co-inoculation of purified CPMV RNA and CPSMV RNA yielded more effective interference than co-inoculation of CPMV RNA and intact CPSMV. The purified CPSMV RNA may have been less infectious than encapsidated CPSMV RNA on a molar basis (Beier & Bruening, 1976) and consequently less effective. However, in the present study, a significant difference in infectivity between purified viral RNA and virus was not observed (data not shown).

Accumulation of viral RNAs and capsid polypeptides within individual co-inoculated cowpeas. Black seedlings were inoculated with 75 ug/ml CPMV and 7.5 ug/ml CPSMV. Plants were individually tagged at the time of inoculation, and the same plants were sampled at various times p.i.. At 14 days p.i. the plants were classified as either having uninfected secondary leaves (ie. interference) or infected secondary leaves (ie. vascular collapse). Replication of viral RNA in both groups of plants was analyzed by nucleic acid hybridization using probes complementary to positive-sense viral RNAs (Figure 18). Plant extracts were also probed with an RNA transcript complementary to 18S RNA cloned from _Costaria costata_. Probing the plant extracts for sequence transcribed from the plant genome confirmed that each extract contained approximately the same amount of nucleic acid. For comparison, the replication of CPSMV in Black and Arlington seedlings in the absence of CPMV

was also analyzed (Figure 19). Plants were inoculated with 7.5 ug/ml of CPSMV and individual plants were sampled as outlined above.

In Black and Arlington seedlings singularly inoculated with CPSMV, both viral RNAs were actively replicating by 4 days p.i. In co-inoculated Black cowpeas which had either infected or uninfected secondary leaves, CPSMV replication was detectable by 4 or 6 days p.i., respectively. CPMV replication in both group of plants was similar and reflective of CPMV replication in singularly inoculated Black seedlings (Figure 13). Although symptom expression was delayed in all mixed inoculated Black seedlings, the pattern of CPSMV replication in plants with infected secondary leaves paralleled CPSMV replication in singularly inoculated plants. The onset of active CPSMV replication in plants with uninfected secondary leaves relative to plants with infected secondary leaves was delayed by 4 days. Thus, not only has symptom expression been delayed, but replication of the CPSMV genome has also been delayed by an identical period of time.

Primary leaves of co-inoculated Black and Arlington seedlings were assayed for CPSMV- and CPMV-protein accumulation at various times p.i. (Figure 20). Primary leaves of plants with uninfected or infected secondary leaves were assayed separately by an antibody-trapped ELISA using monoclonal antibodies SB2 and DG11. CPMV capsid protein was detected in neither Black nor Arlington seedlings. In

Figure 18. Comparison of the accumulation of CPMV and CPSMV plus sense ssRNA in the primary co-inoculated leaves of Black cowpeas. Samples A,C,E,G and I, represent plants with uninfected secondary leaves; samples B,D,F,H and J, represent plants with infected secondary leaves. CPSMV RNA1 (A,B), CPSMV RNA2 (C,D), CPMV RNA1 (E,F), CPMV RNA2 (G,H) and 18s rRNA were assayed for with minus sense ssRNA transcribed from pG2DG1, pG4ZDG2, pG2SB1, pG2SB2 and pG4Z18, respectively. The second and third columns are 1/10 and 1/100 dilutions of the first column, respectively.

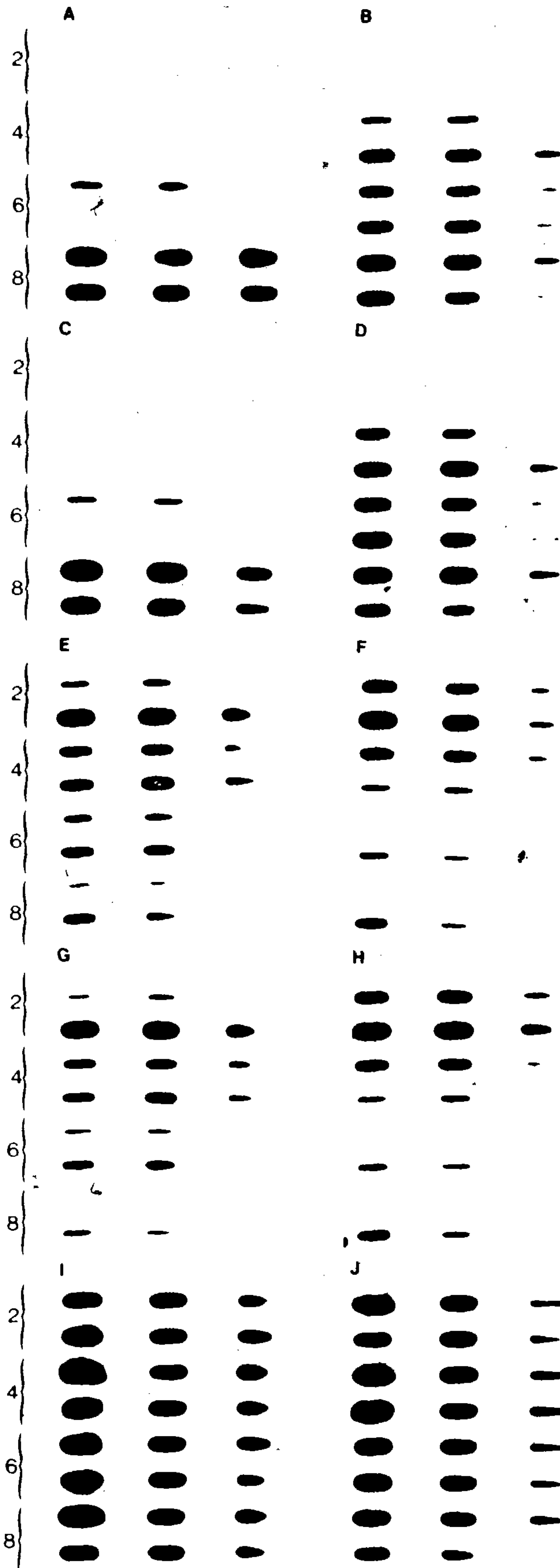
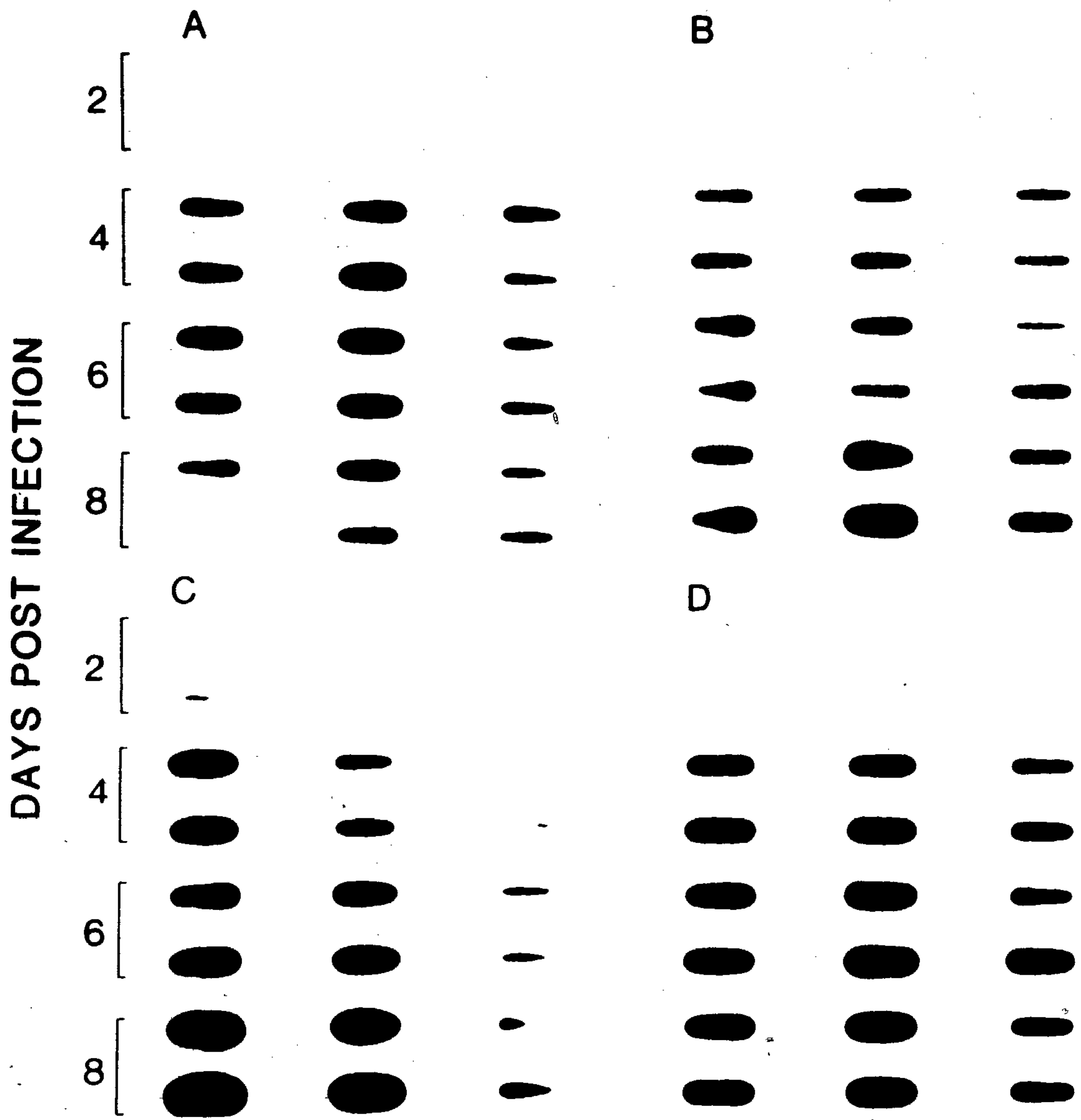

DAYS POST INFECTION
A
B
C
D
E
F
G
H
I
J
2
4
6
8

Figure 19. Accumulation of CPSMV plus sense ssRNA in primary inoculated leaves of Black (A,C) and Arlington (B,D) cowpeas. RNA1 (A,B) and RNA2 (C,D) were assayed with minus sense ssRNA transcripts from pG2DG1 and pG4ZDG2, respectively. The second and third columns are 1/10 and 1/100 dilutions of the first column, respectively.

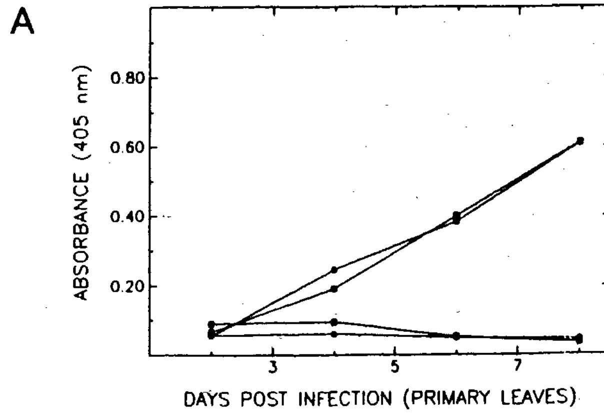

A
B
C
D
DAYS POST INFECTION
2
4
6
8
2
4
6
8

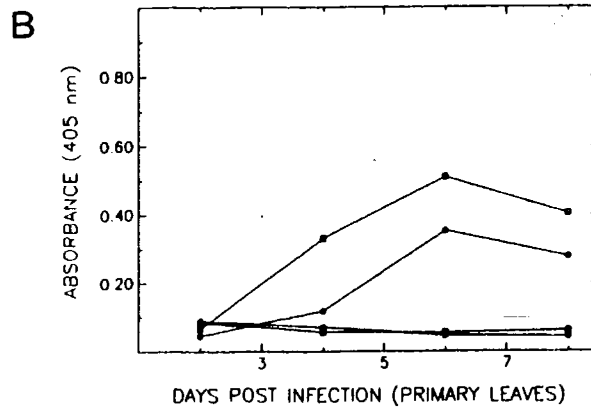
Arlington seedlings with uninfected secondary leaves, CPSMV protein accumulation in the inoculated leaves was depressed and delayed by 2 days p.i. compared to plants that developed infected secondary leaves. However, no difference was observed in the accumulation of CPSMV protein between Black seedlings with infected or uninfected secondary leaves. The accumulation of CPSMV protein in singularly inoculated Black and Arlington cowpeas mirrored the patterns and concentrations of CPSMV protein accumulation in co-inoculated plants with infected secondary leaves.

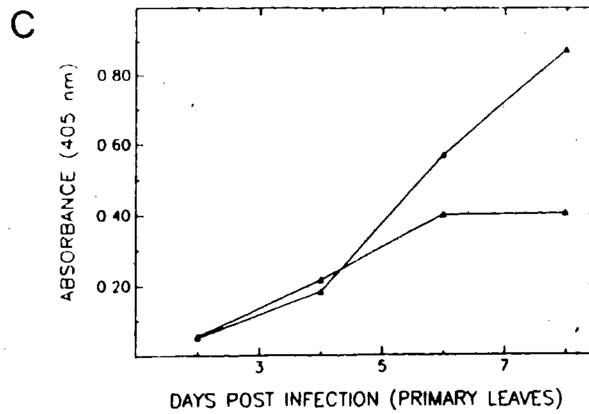
By 14 days p.i. co-inoculated Black seedlings had either collapsed or developed symptomless secondary leaves. .pn126 Samples of secondary leaves from both classes were assayed for the presence of CPMV and CPSMV RNA by hybridization with probes complementary to positive-sense viral RNA (Figure 21). CPMV RNAs were not detected in samples from either collapsed or symptomless leaves. CPSMV RNAs were detected in all the extracts from collapsed leaves, but not in extracts from symptomless secondary leaves. The secondary leaves from both group of plants were similiarly analyzed for the accumulation of virus by ELISA (data not shown). The results were identical to those obtained with nucleic acid hybridization analysis. Therefore, in plants with uninfected trifoliates CPSMV is localized in the primary inoculated leaves.

It was observed that on the primary inoculated leaves of Black and Arlington seedlings which had uninfected secondary

Figure 20. Comparison of virus accumulation in Black (A) and Arlington (B) cowpeas which produced uninfected (U) or infected (SI) secondary leaves. Plants were co-inoculated with 75 ug/ml CPMV and 7.5 ug/ml CPSMV. Virus was assayed for by antibody-trapped ELISA using monoclonal antibodies SB2 (CPMV in U (●) and SI (■) plants) and DG11 (CPSMV in U (○) and SI (□) plants). Black (△) and Arlington (▲) cowpeas were inoculated with 7.5 ug/ml of CPSMV for comparison (C). Each point represents an average of the same five test plants, identified at the time of initial inoculation. Background < 0.100.


A
ABSORBANCE (405 nm)
0.80
0.60
0.40
0.20
3
5
7
DAYS POST INFECTION (PRIMARY LEAVES)

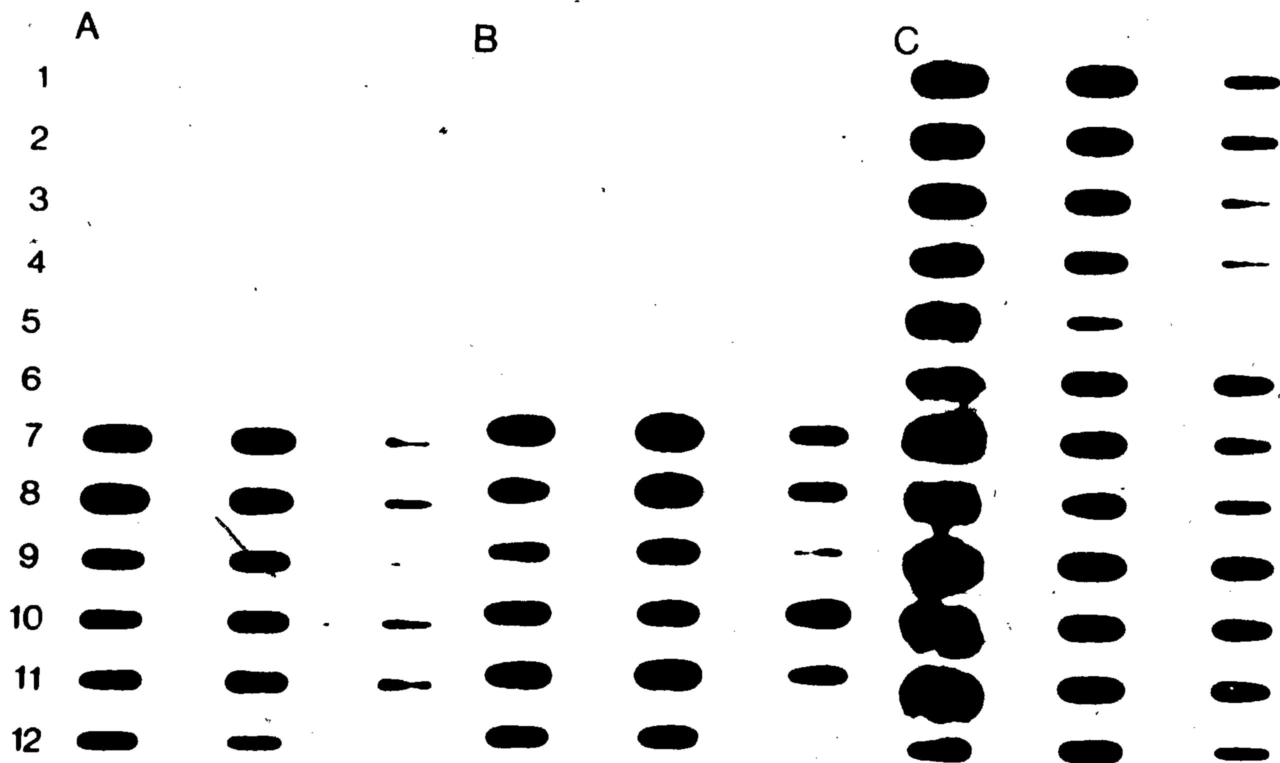

B
ABSORBANCE (405 nm)
0.80
0.60
0.40
0.20
3
5
7
DAYS POST INFECTION (PRIMARY LEAVES)


C
ABSORBANCE (405 nm)
0.80
0.60
0.40
0.20
3
5
7
DAYS POST INFECTION (PRIMARY LEAVES)

leaves distinct necrotic lesions developed while the rest of the leaf was symptomless. This was in contrast to the large, irregularly shaped necrotic areas found on the primary inoculated leaves of plants with infected secondary leaves (Figure 17). Inoculated primary leaves of co-inoculated Black cowpeas were sampled 14 days p.i., and analyzed for viral RNA replication as outlined above (Figure 22). Samples of primary leaves were collected from: A) necrotic tissue of plants with infected secondary leaves; B) necrotic lesions of plants with uninfected secondary leaves; and C) symptomless tissue of plants with uninfected secondary leaves. CPMV RNAs were not detected in any of the plant extracts. CPSMV RNAs were detected in samples A and B, but not in sample C. Similiar samples from co-inoculated Black and Arlington cowpeas were collected and analyzed for viral protein accumulation by an antibody-trapped ELISA. Identical results were obtained (Table 15). Thus, interference by CPMV in the replication of CPSMV confined the latter virus to distinct areas in co-inoculated primary leaves.

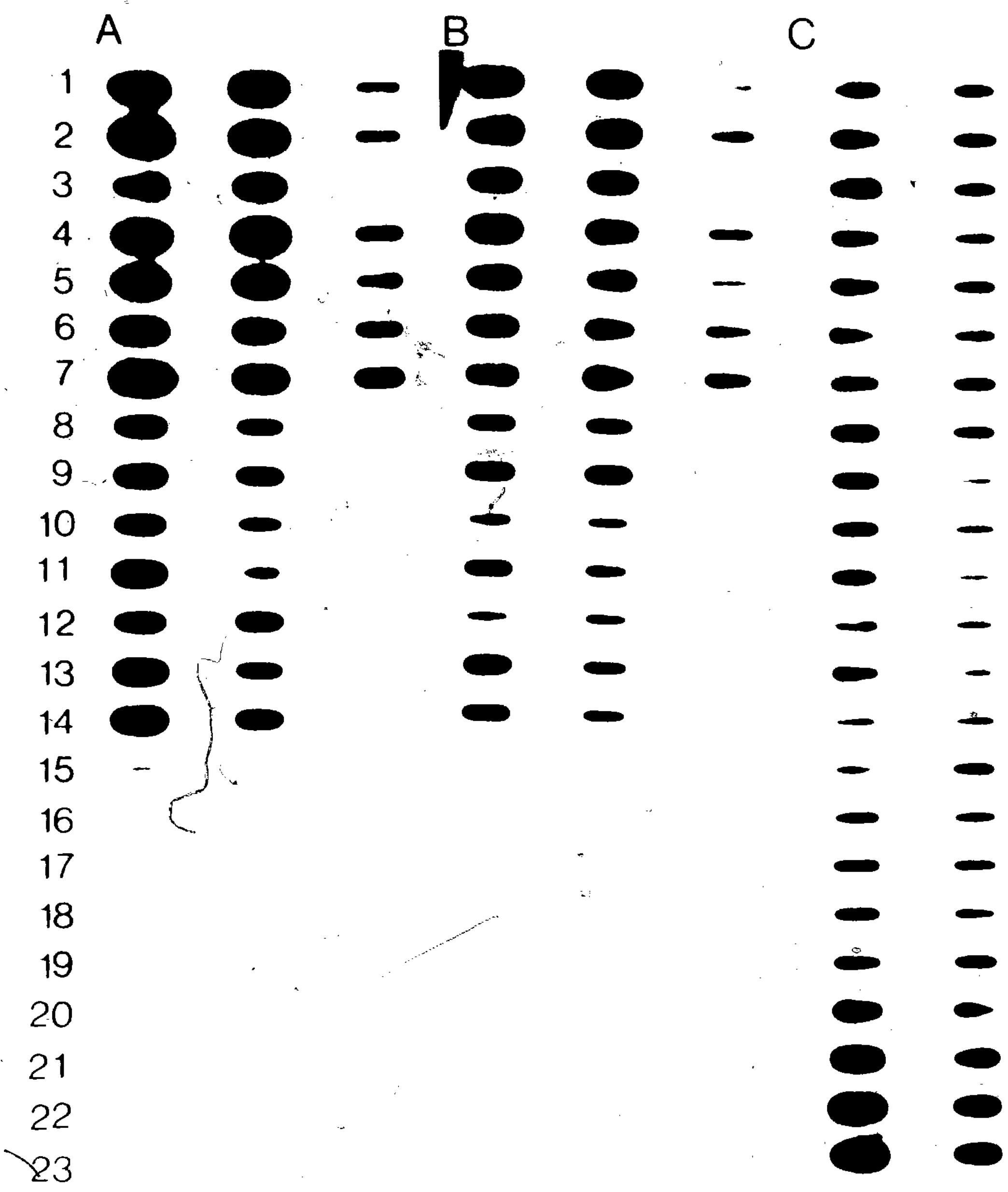
Requirement of infectious CPMV RNA for interference. Purified CPMV RNA was rendered non-infectious by UV irradiation. The irradiated RNA was examined by denaturing agarose gel electrophoresis and found to be degraded to lengths of approximately 1000 to 2000 bases (data not shown). The degraded RNA was assayed for infectivity on Blackeye-5

Figure 21. Comparison of the accumulation of plus sense CPSMV RNA1 (A) and RNA2 (B) between, symptom-free (1-6) and collapsed (7-12) secondary leaves of Black cowpeas co-inoculation with CPMV and CPSMV. RNA1 and RNA2 were assayed for with minus sense ssRNA transcribed from pG2DG1 and pG4ZDG2, respectively. Each plant sample was probed with a minus sense ssRNA transcript of 18s rRNA (C), to ensure a uniform concentration of all samples. Plants were assayed 14 days p.i.. The second and third columns are 1/10 and 1/100 dilutions of the first column, respectively.



A
B
C
1
2
3
4
5
6
7
8
9
10
11
12

Figure 22. Comparison of the accumulation of plus sense CPSMV RNA1 (A) and RNA2 (B) in primary leaves of Black cowpeas co-inoculated with CPMV and CPSMV. Samples 1-7: represent primary leaves of seedlings with infected secondary leaves. Samples 8-14: represent necrotic lesions on primary leaves of seedlings with uninfected secondary leaves. Samples 15-21: represent symptom-free regions on primary leaves of seedlings with uninfected secondary leaves. Samples 22-23: represent healthy plant tissue. RNA1 and RNA2 were assayed for with minus sense ssRNA transcribed from pG2DG1 and pG4ZDG2, respectively. Each plant sample was probed with a minus sense ssRNA transcript of 18s rRNA (C), to ensure a uniform concentration of all samples. Plants were assayed 14 days p.i.. The second and third columns are 1/10 and 1/100 dilutions of the first column, respectively.


A
B
C
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23

cowpeas. Residual biological activity was not observed in the irradiated RNA. Furthermore, interference was not observed when irradiated CPMV RNA was co-inoculated with CPSMV RNA on Black seedlings (Table 16). Other investigators have also reported that the genome of the interfering virus had to be biologically active (Bruening et al., 1979; Saaijer-Riep & de Jager, 1988; Sterk & de Jager, 1987).

Palukaitis and Zaitlin (1984) speculated that replicated positive sense RNA of the interfering virus would hybridize to minus sense RNA of the challenging virus. To investigate further, CPMV RNA was degraded by UV-irradiation, and the RNA was separated into poly(A)-tailed and non-poly(A)-tailed fragments by oligo-dT cellulose chromatography. The fractionated CPMV RNA was mixed with CPSMV RNA and annealed as described above for the annealing of dCTP tailed cDNA with dGTP tailed vector. The mixture was co-inoculated on Black cowpeas (Table 16). Neither the poly(A)-tailed nor the non-poly(A)-tailed fraction of CPMV RNA interferred with the replication of CPSMV RNA. The results indicated that neither small fragments of CPMV RNA nor fragments enriched for 5' or 3' termini could interfere with CPSMV replication in Black cowpeas. The genome of the inducing virus was required to be biologically active (Table 16) in contrast to the observation that CPMV replication was maximal after interference had already been established.

Sequence of the 3'-terminal of CPSMV RNA. If the mechanism of

Table 15. Distribution of CPMV and CPSMV in primary infected leaves of Black and Arlington cowpeas co-inoculated with CPMV and CPSMV.

LEAF SAMPLE[1]	NUMBER OF POSITIVE TEST PLANTS[2]		TOTAL NUMBER OF PLANTS ASSAYED
	CPMV	CPSMV	
Black			
systemically infected leaves	0	42	42
co-inoculated leaves-necrotic lesions	0	34	34
co-inoculated leaves-"healthy" areas	0	7	39
Arlington			
systemically infected leaves	0	22	24
co-inoculated leaves-necrotic lesions	0	7	8
co-inoculated leaves-"healthy" areas	0	2	8

1. Plants were co-inoculated with 7.5 ug/ml CPSMV and 75 ug/ml CPMV. Tissue was sampled 14 days post infection.

2. Plant samples were assayed for CPMV and CPSMV by antibody-trapped ELISA using monoclonal antibodies SB2 and DG11, respectively. A positive result represents a value greater than uninfected controls (< 0.100).

Table 16. Effects of UV-irradiation on the ability of CPMV ssRNA to interfere with the replication of CPSMV ssRNA in Black cowpeas.

INOCULUM[1]	SURVIVING[2] PLANTS	TOTAL NUMBER OF PLANTS INOCULATED	PERCENT SURVIVING
CPSMV	1	11	9
CPSMV/CPMV; UV-	7	11	64
CPSMV/CPMV; UV+ unfractionated	1	10	10
CPSMV/CPMV; UV+ non-binding to oligo-dT-cellulose	2	10	20
CPSMV/CPMV; UV+ bound to oligo-dT-cellulose	2	10	20

1. CPSMV and CPMV ssRNA were inoculated at 0.5 ug/ml and 10 ug/ml, respectively.

2. Plants were assayed 14 days post infection.

interference postulated by Palukaitis and Zaitlin (1984) applied to the present system, regions of homology would be expected between the 3'-termini of CPMV and CPSMV genomic RNAs. The 3'-termini of CPSMV RNA1 and RNA2 were cloned, their nucleotide sequences elucidated and compared with the sequences at the 3'-termini of CPMV RNA1 and RNA2 (Figure 23). To ensure that the 3'-termini of the CPSMV RNAs were represented in the clones, oligo-d(T) primed cDNA was tailed with deoxycytidine using terminal deoxynucleotidyl transferase and ligated to deoxyguanidine tailed vector. Clones specific to either RNA1 or RNA2 were selected by hybridization to electroblots of the viral RNA.

The 3'-terminal 70 nucleotides of CPSMV RNA1 and RNA2 share 67% homology compared to 81% homology between the 3'-terminal 70 nucleotides of CPMV RNA1 and RNA2 (Lomonossoff & Shanks, 1983; Van Wezenbeek et al., 1983) and RCMV RNA1 and RNA2 (Shanks et al., 1986) RNAs. There is a sequence of homology common to both RNAs of all three viral genomes: Ux(U/A)xxxU(U/A)UU (Figure 23).

Interference as a genetic trait of the plant. An experiment was conducted to determine if subsequent generations of Black cowpeas which exhibit interference carry a predisposition to CPMV interference in the replication of CPSMV. Black cowpeas were co-inoculated with 75 ug/ml of CPMV and 7.5 ug/ml of CPSMV. At 21 days p.i., samples from plants with visually

Figure 23. Comparison of the 3'-terminal sequences of RNA1 and RNA2 of CPSMV.

A. 3'-terminal sequences of RNA1 and RNA2 of CPSMV. The sequences have been aligned to maximize regions of homology.

```
RNA1  CAGCUUUAGCUGUGUAACGGGCUAGUUUAGAAUUAAUUUAUAAAAAUGUGC
               |||  ||||  |  | |||      ||||||| ||||| |      51
RNA2  UCCUCAGUCCUGAAUAACCUGGCA UUUUCGCCCAAUUUAU AAAAU UAU

RNA1  AAUGU AAGUGAUUAUGUGACCUUU AAGUGUUCAGGUGUUCUU CUUUUpolyA
      |||||    |  || |||||  |||  ||   | |  |||| || |||||    101
RNA2  AAUGUGUGAUUGUUGUGUGA  UUUCUAG   UAACAUGUUAUUACUUUUpolyA
```

B. 3'-terminal sequences of the genomes of three comoviruses. The sequences have been aligned to maximize regions of homology. The 10 nucleotides situated adjacent to the polyA tract share a region of homology (Ux(UA)xxxU(UA)UU).

```
CPMV RNA1    AACAACAAAAAUAUGUGUUUUUAUU-polyA
              |||  ||||   | |  |||||||
CPMV RNA2    GACACAAAAAGAUUUUAAUUUUAUU-polyA

RCMV RNA1    UAU GUGAUAAAGUUUGUGUUUAUU-polyA
             ||| |   |||||||  ||||||||
RCMV RNA2    UAUUGCUUUAAAGUU AUGUUUAUU-polyA

CPSMV RNA1    AAGUGUUCAGGUGUUCUU CUUUU-polyA
               ||   | |  |||| ||·|||||
CPSMV RNA2   CUAG   UAACAUGUUAUUACUUUU-polyA
```

healthy secondary leaves were analyzed by ELISA for CPSMV. Plants testing negative were repotted, and retested 4 weeks later for CPSMV. Initially, of the 127 plant co-inoculated, 44 plants had uninfected secondary leaves. After the second screening 40 plants were maintained and grown to seed. Sixty-eight Black seedlings which were the first generation from plants that were not of those systemically infected by CPSMV, were co-inoculated with CPMV and CPSMV (as above). Sixteen plants had uninfected secondary leaves 21 days p.i. The first generation seedlings were analyzed for CPMV replication by ELISA 14 days p.i.. CPMV was not found in any of the 68 seedlings. Therefore, a predisposition to interference was not carried through to surviving generations.

Co-inoculation of plants with CPMV, CPSMV and SHMV. Saaijer-Riep and de Jager (1988) demonstrated that CPMV interferes with the replication of SHMV in Arlington seedlings. The two viruses were co-inoculated at approximately the same concentration for optimal interference. An experiment was conducted to determine the effect of SHMV on the interference phenomena between CPMV and CPSMV (Table 17).

Black and Arlington cowpeas were inoculated with three different concentrations of SHMV, 75 ug/ml of CPMV and 7.5 ug/ml of CPSMV. Representative plants from each group were assayed 14 days p.i. for the presence of SHMV and CPMV by electron microscopy and/or by antibody-trapped ELISA using monoclonal antibodies specific for SHMV and CPMV. SHMV was

Table 17. Co-inoculation of Black and Arlington cowpeas with SHMV, CPSMV and CPMV.

INOCULUM[1]	BLACK			ARLINGTON		
	SURVIVING PLANTS[2]	TOTAL NUMBER OF PLANTS INOCULATED	PERCENT SURVIVING	SURVIVING PLANTS[2]	TOTAL NUMBER OF PLANTS INOCULATED	PERCENT SURVIVING
SHMV-1	8[3]	8	100	8[3]	8	89
SHMV-2	7[3]	8	100	7[3]	7	100
SHMV-3	7[3]	7	100	6[3]	6	100
SHMV-1/SB/DG	12	47	26	0	47	0
SHMV-2/SB/DG	16	52	31	0	52	0
SHMV-3/SB/DG	5	47	11	0	49	0

1. Concentrations of SHMV-1, SHMV-2 and SHMV-3 were 5 ug/ml, 50 ug/ml and 200 ug/ml, while the concentrations of CPMV and CPSMV were constant at 75 ug/ml and 7.5 ug/ml, respectively.

2. Plants were assayed 14 days post infection.

3. Plants singularly inoculated with SHMV produced secondary leaves, but they exhibited systemic symptoms characteristic of virus infection. Thus these numbers represent, number of infected plants with systemic symptoms.

detected in all assayed plants; CPMV was not detected in any of the assayed plants. Interference by CPMV in the replication of CPSMV was not affected by SHMV in Black cowpeas, except when a high concentration (200 ug/ml) of SHMV was co-inoculated. In Arlington cowpeas, however, the presence of SHMV prevented CPMV interference of CPSMV replication, even at a low concentration (5 ug/ml) of SHMV.

Acquired systemic resistance induced by CPMV. Historically, research on mixed virus infections has examined the ability of an inducing virus to protect a plant against subsequent infection by a challenge virus (Fraser, 1987; Hamilton, 1980). Experiments were conducted to determine if primary inoculation with CPMV would induce immunity against CPSMV infection of the secondary leaves.

The primary leaves of Black cowpeas were inoculated with 75 ug/ml and 250 ug/ml of CPMV, and the secondary leaves challenged with either 1 ug/ml or 7.5 ug/ml of CPSMV (Table 18). For comparison, CPSMV was inoculated on the secondary leaves of previously uninfected plants. In all examples of sequential co-inoculation, the symptoms induced in secondary leaves by CPSMV infection were noticeably reduced compared to singular infection of secondary leaves by CPSMV.

Interference in BE-5 cowpeas. BE-5 cowpeas were susceptible to infection by both CPMV and CPSMV (Beier et al., 1977) (Figure 24). When BE-5 seedlings were co-inoculated with 200

ug/ml of CPMV and 5.8 ug/ml of CPSMV, both viruses accumulated in primary inoculated tissue and secondary leaves (Figures 24 and 25). However, when plants were co-inoculated with 40 ug/ml of CPMV RNA and 1.2 ug/ml of CPSMV RNA, the replication of CPSMV in primary inoculated leaves was depressed (Figure 24). The primary leaves of some of the plants co-inoculated with purified viral RNAs expressed symptoms characteristic of only CPMV infection. Other co-inoculated plants expressed symptoms typical of infection by both viruses. Samples of primary and secondary leaves were collected from both groups of plants and analyzed by ELISA for CPMV and CPSMV (Figure 25). CPSMV was either not detected or detected at a greatly reduced concentration in both the primary and the secondary leaves of plants which expressed only symptoms characteristic of CPMV infection. Both CPMV and CPSMV were detected in all the leaf samples collected from plants expressing symptoms characteristic of infection by both viruses.

Table 18. Challenge by CPSMV of secondary leaves of plants with primary leaves infected by CPMV.

INOCULUM[1]		NUMBER OF PLANTS EXHIBITING VARIOUS SYSTEMIC SYMPTOMS[2]			
PRIMARY LEAF	SECONDARY LEAVES	A	B	C	D
Experiment #1					
------	1 ug/ml CPSMV	19	0	1	2
75 ug/ml CPMV	1 ug/ml CPSMV	9	3	6	0
250 ug/ml CPMV	1 ug/ml CPSMV	6	3	7	2
------	7.5 ug/ml CPSMV	15	1	3	0
75 ug/ml CPMV	7.5 ug/ml CPSMV	7	7	4	2
250 ug/ml CPMV	7.5 ug/ml CPSMV	8	4	4	3
Experiment #2					
------	1 ug/ml CPSMV	8	2	0	0
------	7.5 ug/ml CPSMV	12	1	0	0
15 ug/ml CPMV ssRNA	1 ug/ml CPSMV	6	3	2	18
15 ug/ml CPMV ssRNA	7.5 ug/ml CPSMV	7	3	8	2

1. The primary leaves were inoculated with CPMV, and the first set of trifoliates, approximately 2-3 cm in length, were challenged with CPSMV.

2. Symptoms induced by challenge of secondary leaves with CPSMV were catagorized: A, total vascular collapse; B, > 5 necrotic local lesions per challenged leaf; C, < 5 necrotic local lesions per challenged leaf; D, no visible sign of necrotic local lesions on challenged leaves.

Figure 24. Comparison of virus accumulation in BE-5 cowpeas singularly and co-inoculated with CPMV and CPSMV virus or RNA. BE-5 was co-inoculated with: (A) 200 ug/ml CPMV virus and 5.8 ug/ml CPSMV virus; (B) 40 ug/ml CPMV ssRNA and 1.2 ug/ml CPSMV ssRNA. Viral capsid proteins of CPMV (●) and CPSMV (■) were estimated by ELISA. For comparison, BE-5 was inoculated with: (C) 200 ug/ml of CPMV virus (▽) or 40 ug/ml of CPMV ssRNA (▼); (D) 5.8 ug/ml of CPSMV virus (△) or 1.2 ug/ml of CPSMV ssRNA (▲). Each point represents an average of six test plants. Background < 0.100.

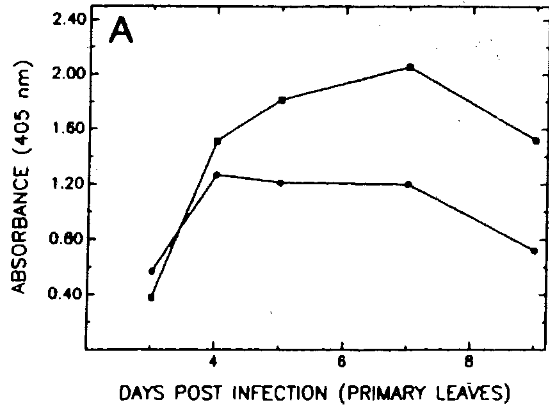
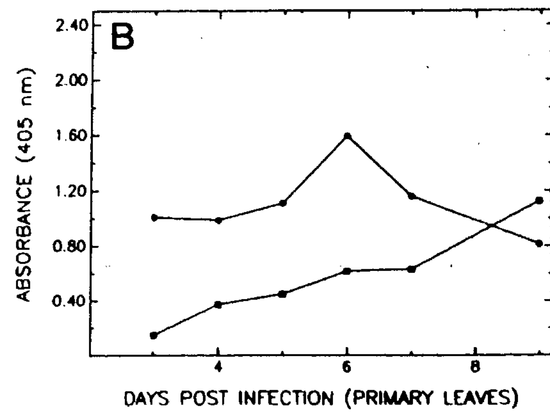
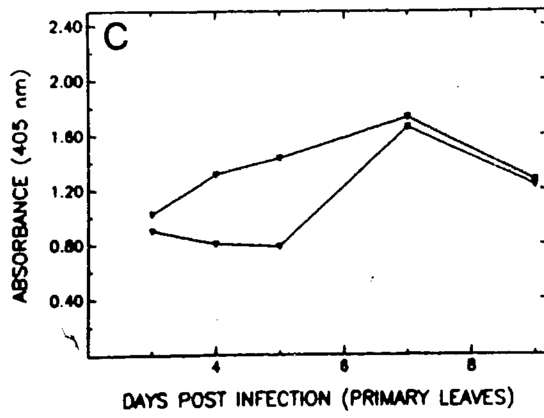
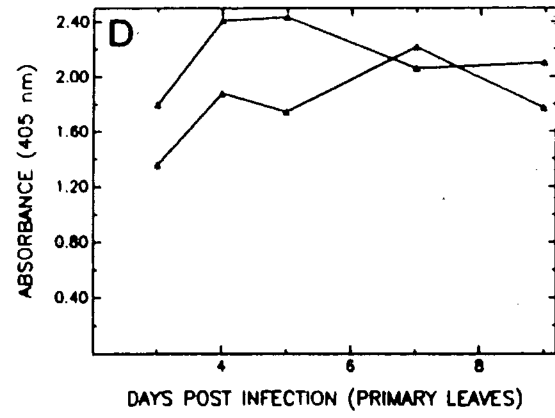
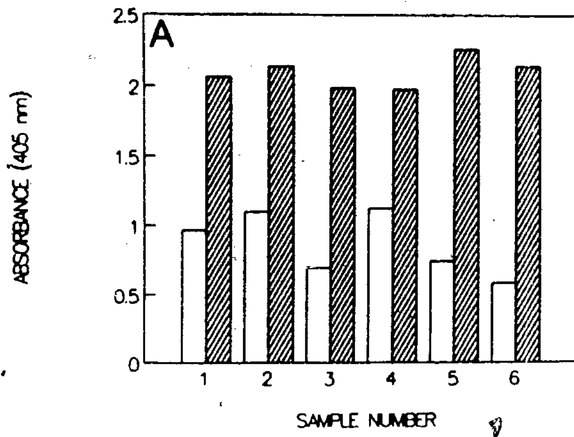
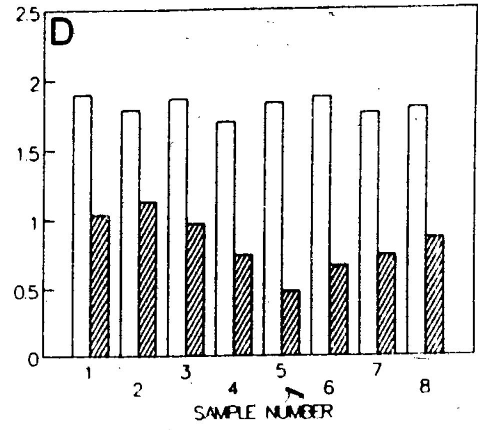
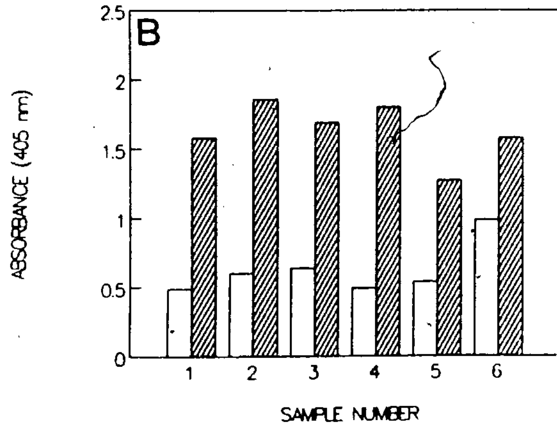
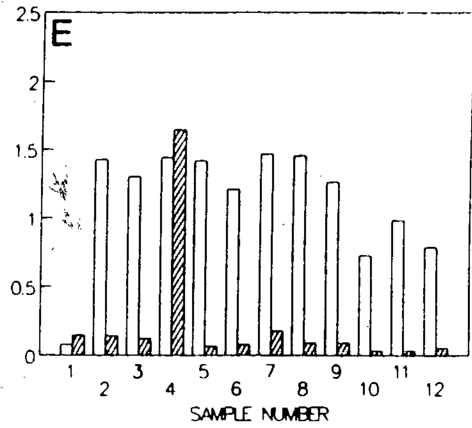

A
ABSORBANCE (405 nm)
2.40
2.00
1.60
1.20
0.80
0.40
4
6
8
DAYS POST INFECTION (PRIMARY LEAVES)

B
ABSORBANCE (405 nm)
2.40
2.00
1.60
1.20
0.80
0.40
4
6
8
DAYS POST INFECTION (PRIMARY LEAVES)

C
ABSORBANCE (405 nm)
2.40
2.00
1.60
1.20
0.80
0.40
4
6
8
DAYS POST INFECTION (PRIMARY LEAVES)

D
ABSORBANCE (405 nm)
2.40
2.00
1.60
1.20
0.80
0.40
4
6
8
DAYS POST INFECTION (PRIMARY LEAVES)

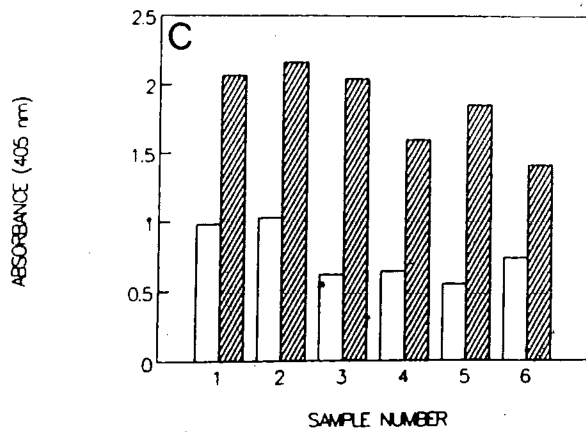
Figure 25. Accumulation of CPSMV and CPMV in secondary leaves of BE-5 cowpeas co-inoculated with CPSMV (▨) and CPMV (□). (A), secondary leaves of plants singularly inoculated with either CPSMV or CPMV ssRNA; (B), secondary leaves of plants co-inoculated with CPSMV and CPMV; (C), secondary leaves of plants co-inoculated with CPSMV and CPMV ssRNA; (D), primary leaves of plants singularly inoculated with either CPSMV or CPMV ssRNA; (E), primary leaves of plants co-inoculated with CPSMV and CPMV ssRNA exhibiting only symptoms characteristic of CPMV infection; (F), secondary leaves of plants co-inoculated with CPSMV and CPMV ssRNA exhibiting only symptoms characteristic of CPMV infection. (Plant sample numbers from (E) and (F) represent the same plant.) Plants were assayed fourteen days post infection by antibody-trapped ELISA using monoclonal antibodies SB2 and DG11. Background < 0.100.

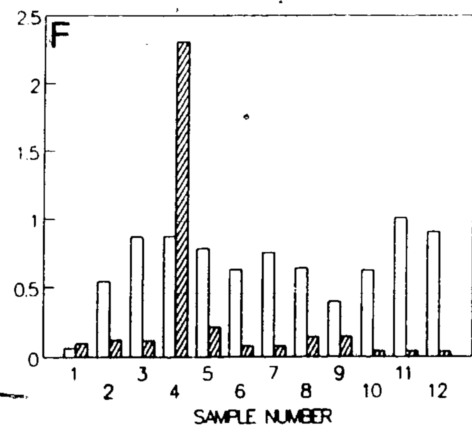

A
ABSORBANCE (405 nm)
0
0.5
1
1.5
2
2.5
1
2
3
4
5
6
SAMPLE NUMBER


D
0
0.5
1
1.5
2
2.5
1
2
3
4
5
6
7
8
SAMPLE NUMBER


B
ABSORBANCE (405 nm)
0
0.5
1
1.5
2
2.5
1
2
3
4
5
6
SAMPLE NUMBER


E
0
0.5
1
1.5
2
2.5
1
2
3
4
5
6
7
8
9
10
11
12
SAMPLE NUMBER


C
ABSORBANCE (405 nm)
0
0.5
1
1.5
2
2.5
1
2
3
4
5
6
SAMPLE NUMBER


F
0
0.5
1
1.5
2
2.5
1
2
3
4
5
6
7
8
9
10
11
12
SAMPLE NUMBER

DISCUSSION:

The replication of CPMV was examined in the "operationally" immune cowpeas Black and Arlington. Low levels of CPMV RNA accumulation were detected in the plants; however, the concomitant accumulation of viral capsid polypeptides was not detected. Beier et al. (1977) identified sixty-five varieties of Vigna unguiculata, including Black and Arlington, as "operationally immune" to infection by CPMV when inoculated with a virus concentration one hundred times that required to uniformly infect a susceptible variety (ie. 250 ug/ml). Plants were assayed on the basis of symptom development and the ability to identify infectious virus in inoculated plants. The definition of "operational immunity" was modified to exclude virus replication at low levels not detectable by these criteria (Ponz et al., 1988a). Eastwell et al., (1983) identified low levels of the double-stranded RNA replicative form of CPMV (Shanks et al., 1985) in infected Black and Arlington seedlings.

Neither infectious virus nor viral capsid polypeptides were detected in either Black or Arlington seedlings inoculated with CPMV. CPMV RNA was translated as a single, large polypeptide precursor, and cleaved into smaller, functional polypeptides by a 24 kDa protease encoded on viral RNA1 (Verver et al ., 1987). The specificity of the 24 kDa protease was modulated by a 32 kDa protein, also encoded on viral RNA1 (Vos et al., 1988). The 24 kDa and 32 kDa

polypeptides together cleaved between Gln and Met amino acid residues; the same cleavage site required to separate 60 kDa capsid polypeptide intermediate from the precursor polypeptide encoded by RNA2 (Vos et al., 1988). Extracts of Arlington protoplasts contain a specific proteinaceous agent which inhibits cleavage of the RNA2 polypeptide precursor at the Gln-Met cleavage site (Sanderson et al., 1985). Therefore, it would have been unexpected to detect CPMV 23 kDa and 37 kDa capsid polypeptides in Arlington seedlings. It was not determined if the 60 kDa polypeptide precursor of the capsid proteins was present in plant extracts. However, the 60 kDa polypeptide could have been unknowingly detected in ELISA of inoculated cowpeas because monoclonal antibody SB2 bound an epitope on the coat protein of CPMV composed of contiguous amino acid residues. SB2 bound the epitope whether the virus was in a native or denatured configuration.

Genetic crosses between Arlington and Blackeye-5 seedlings indicated that the inhibitor of CPMV replication is inherited as a single dominant character (Kiefer et al., 1984). Many traits of the immunity in Black cowpeas behaved in parallel fashion, suggesting that a single dominant character for CPMV resistance may also be present in Black cowpeas. However, it cannot be discounted that there are separate gene functions conferring immunity against CPMV infection in Black seedlings. More detailed analysis revealed a number of additional factors that displayed complex patterns of heredity

that may have contributed to immunity in Arlington seedlings (Ponz et al., 1988a).

Experiments were performed to challenge hypotheses explaining the interference in the replication of CPSMV when CPMV and CPSMV are co-inoculated in cowpeas immune to infection by CPMV. The efficiency of interference declined rapidly as the time between CPMV and CPSMV inoculation was increased. This suggested that CPMV did not induce a persistent defence mechanism in the primary inoculated leaf which laterally diffused in the leaf and interfered with CPSMV replication at an adjacent site. However, Black and Arlington cowpeas inoculated with CPMV exhibited acquired systemic resistance against subsequent secondary infection by CPSMV. Symptoms induced by systemically inoculated CPSMV were attenuated but not delayed, compared to symptom expression in primarily uninfected cowpeas (Table 18). The protective effect of acquired systemic resistance was minor relative to that of interference.

The direct involvement of capsid proteins in the protection of plants by secondary virus infection has been implicated by classical cross-protection experiments (Dodds et al., 1984; Horikoshi et al., 1987). Horikoshi et al. (1987) provided evidence that the coat protein of brome mosaic virus (BMV) inhibited transcription of BMV RNA *in vitro*. Moreover, they were able to demonstrate that the coat protein did not react with the replicase complex directly, but rather formed a

stable complex with the RNA, thus blocking transcription. If this situation reflects interference or cross-protection *in vivo*, accumulation of the coat protein of the inducing virus would be required. This has not been observed in Black or Arlington cowpeas infected with CPMV.

Results involving transgenic plants expressing the coat protein gene have echoed the central role of the coat protein in protecting plants against subsequent virus infection (Hemenway et al., 1988; Loesch-Fries et al., 1987; Nelson et al., 1987; Turner et al., 1987; Van Dun et al., 1987). The presence of actively expressed coat protein gene offered resistance to PVX, AMV and TMV (Hemenway et al., 1988; Loesch-Fries et al., 1987; Nelson et al., 1987, respectively). In systems examining protection against AMV and TMV the protecting effect of the coat protein was nullified if the challenge inoculum consisted of isolated RNA, rather than intact virions. These data suggested that the uncoating of the viral RNA was blocked. Resistance was overcome in transgenic plants expressing the coat protein of PVX when inoculated with isolated RNA. In this case, apparently coat protein was not involved in blocking uncoating of the inoculating virus; however, coat protein may have regulated an early event in virus replication. Furthermore, it was demonstrated that the genetic background of the transgenic plants influenced the efficiency of the protection offered by the expression of the coat protein gene (Nelson et al., 1987). The induced

resistance to virus infection in transgenic systemic hosts for TMV was effectively overcome by inoculation with isolated TMV RNA rather than intact virions. In contrast, TMV RNA was much less efficient in establishing an infection in transgenic plants with a local lesion heritage.

Together, these data suggested that in systems where the coat protein gene is expressed in transgenic plants either uncoating of the viral RNA was blocked (TMV and AMV) or an early event in viral replication was regulated by the coat protein (PVX). As noted above, these hypotheses cannot be extended to CPMV/CPSMV as coat protein did not accumulated in inoculated plants and inoculation with isolated RNA was at least equally as effective as inoculation with intact virions for establishing interference (Table 14). Furthermore, the nucleic acid free top component of CPMV when co-inoculated with CPSMV did not interfere in the replication of CPSMV. Thus CPMV does not follow emerging patterns of cross-protection or interference demonstrated in other plant systems. This conclusion reinforces the unique nature of virus-virus interactions of CPMV/CPSMV compared to other systems.

Two possible mechanisms or a combination thereof are envisioned to explain the effect of CPMV on the replication of CPSMV: 1) CPMV induced a local plant defence mechanism in the area of infection; 2) transcription or translation of CPMV RNA directly affected CPSMV replication.

In co-inoculated plants CPMV may have induced the

formation of the necrotic local lesions associated with interference. In mixed inoculated plants with uninfected secondary leaves, CPSMV was localized in the necrotic local lesions formed on the primary inoculated leaves. More extensively necrosis was observed on primary leaves singularly inoculated with CPSMV, but no symptoms were associated with singular inoculation by CPMV. Therefore, necrosis was apparently induced by CPSMV, but co-infection with CPMV retarded the replication of CPSMV.

Initially it was assumed that CPMV RNA hybridized with a complementary sequence in CPSMV RNA. Recently, it was demonstrated that a satellite RNA of CMV contains anti-sense regions complementary to the viral coat protein gene (Rezaian & Symons, 1986). A role for these anti-sense RNA regions in the regulation of viral coat protein synthesis was hypothesized. In the present system it was demonstrated that interference was established prior to significant replication of positive sense RNA of the inducing virus. However, it could not be discounted with certainty that a region of the genome of the inducing virus in the inoculum did not hybridize to the challenge virus and interfere with its replication. In co-inoculated cowpeas, CPMV RNA had not accumulated to a significant concentration throughout the plant at the time when interference was established. Eggen and Van Kammen (1988) suggested replication of CPMV was concentrated at the membrane bound replicase. A significant concentration of CPMV RNA at

the localized site of viral replication may have precipitated the formation of RNA-RNA hybrids with CPSMV RNA, thus preventing or delaying the replication of CPSMV. Alternatively, an RNA-RNA hybrid could have been formed with the inoculating RNA of CPMV. However, interference was not observed when plants were co-inoculated with CPSMV RNA and either poly(A)+ fragments of CPMV RNA or biologically inactive (ie. UV-irradiated) CPMV RNA. Furthermore, duplex formation between CPMV and CPSMV would likely reduce the amount of CPMV available for replication (as well as reducing CPSMV), but no effect on CPMV replication is observed.

Alternatively, translation product(s) of CPMV may be required to initiate the interference response. It was demonstrated that co-inoculation of CPSMV with CPMV RNA1 but not CPMV RNA2 was sufficient for interference. Eggen and Van Kammen (1988) suggested that the 170 kDa precursor polypeptide translated from RNA1 binds to the cytoplasmic membrane forming a membrane-bound replication complex (replicase). Viral RNA would bind to the replication complex prior to transcription of the RNA. CPSMV RNA may form an abortive complex with the CPMV replicase to sequester the infecting CPSMV RNA and retard the replication of CPSMV. Eventually, in some plants, residual CPSMV replication saturated the CPMV replicase and normal CPSMV replication resumed after a time delay. The plants would exhibit vascular collapse, as did plants singularly inoculated with CPSMV. However, in a minority of the co-inoculated plants

CPSMV replication would be delayed sufficiently to allow the defence response of the plant to confine the virus at the site of inoculation.

For many positive-sense single-stranded RNA viruses, the replicase recognition sequence was located at the 3'-terminal of the viral RNA (Ahlquist et al., 1984; Ali Rezaian et al., 1984). Furthermore, Morch et al. (1977) demonstrated experimentally the competition for specific nucleotide binding sites by the replicase. The replicase of turnip yellow mosaic virus could be effectively inhibited *in vitro* by the addition of RNA transcripts homologous to the 3'-end of the viral genome. Transcripts from other regions of the genome were not inhibitory. The replicase recognition sequence of CPMV and CPSMV RNA is unknown, although a homologous region has been identified at the 3' terminal of the RNAs of three comoviruses (Figure 23).

It has been demonstrated that Arlington extracts contained an inhibitor of the proteolytic processing of the gln-met cleavage site of the CPMV RNA2 polyprotein (Ponz et al., 1988a; Sanderson et al., 1985). The CPMV RNA1 polypeptide contains similar Gln-Met cleavage sites that presumably would also be cleaved. The successful replication of viral RNA required processing of the RNA1 polyprotein, the process would have been aborted because of the protease inhibitor in immune cowpeas (Sanderson et al., 1985).

The level of CPMV replication was not increased in co-

inoculated Black and Arlington cowpeas, thus it was assumed the protease of CPSMV could not be substituted for the inhibited protease of CPMV. It has not been determined whether the 24 kDa protease and the 32 kDa protein of CPSMV can faithfully cleave the precursor polypeptide translated from CPMV RNA2. However, Goldbach and Krjit (1982) demonstrated that the 32 kDa protein and/or the 24 kDa protease of CPMV could not cleave the precursor polypeptide translated from CPSMV RNA2.

An alternative mechanism to explain the interference of CPSMV replication by CPMV is the non-specific cleavage of the CPSMV precursor polypeptide by the 24 kDa protease of CPMV. Goldbach and Krijt (1982) observed that the 24 kDa protease or a combination of the 24 kDa protease and the 32 kDa protein of CPMV caused an "irregular" cleavage in the precursor polypeptide translated from CPSMV RNA2. It was not determined if a similar cleavage sight existed in CPSMV RNA1. Such an "irregular" cleavage of CPSMV precursor polypeptide(s) would certainly have delayed the expression of CPSMV symptoms. However, whether there would be enough CPMV protease to interfere in the replication of CPSMV sufficiently to allow the plant necrosis to limit virus spread, would again be determined by a probability dependent on the environment in the specific infection site.

In conclusion, it has been observed that CPMV interfered in the replication of CPSMV when co-inoculated to cowpeas

resistant to infection by CPMV (Black and Arlington). The interference phenomenon was also observed in BE-5 cowpeas, a cultivar susceptible to CPMV infection, when the ratio of co-inoculated inducing virus (CPMV): challenge virus (CPSMV) was increased eight-fold. By all measures (RNA and protein) CPMV can inhibit CPSMV replication in cultivars susceptible or immune to CPMV, but in either case, the extent of CPMV replication is unaffected by CPSMV. Although, viral coat protein was demonstrated as the central component in establishing cross-protection in other viral systems, the accumulation of CPMV coat protein was not observed in Black and Arlington cowpeas. The precise mechanism responsible for the interference phenomenon could not be elucidated at present. However, it was postulated that CPMV interferred in the early stages of CPSMV replication. Detailed analysis of the interference response required further experiments using transgenic cowpeas expressing either anti-sense transcripts or viral proteins encoded by specific regions of the CPMV genome.

LIST OF REFERENCES

Abel, P.P., Nelson, R.S., De, B., Hoffmann, N., Rogers, S.G., Fraley, R.T. & R.W. Beachy, 1986. Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. Science 232: 738-743.

Agrawal, H.O., 1964. Identification of cowpea mosaic virus isolates. Mededelingen Landbouwhogeschool Wageningen 64: 1-53.

Agrawal, H. & D.Z. Maat, 1964. Serological relationships among polyhedral plant viruses and production of high-titred antisera. Nature 202: 674-675.

Ahlquist, P., Dasgupta, R. & P. Kaesberg, 1984. Nucleotide sequence of the brome mosaic virus genome and its implications for viral replication. J. Mol. Biol. 172: 369-383.

Albersio, J., Lima, A. & M.R. Nelson, 1975. Squash mosaic virus variability: nonreciprocal cross-protection between strains. Phytopathology 65: 837-840.

Ali Rezaian, M., Skene, K.G.M. & J.G. Ellis, 1988. Anti-sense RNAs of cucumber mosaic virus in transgenic plants assessed for control of the virus. Plant Mol. Biol. 11: 463-471.

Ali Rezaian, M. & R.H. Symons, 1986. Anti-sense regions in satellite RNA of cucumber mosaic virus form stable complexes with the viral coat protein gene. Nuc. Acids Res. 14: 3229-3239.

Ali Rezaian, M., Williams, R.H.V., Gordon, K.H.J., Gould, A.R. & R.H. Symons, 1984. Nucleotide sequence of cucumber mosaic virus RNA2 reveals a translation product significantly homologous to corresponding proteins of other viruses. Eur. J. Biochem. 143: 277-284.

Allison, R.F., Johnston, R.E. & W.G. Dougherty, 1986. The nucleotide sequence of tobacco etch virus genomic RNA: evidence for synthesis of a single polypeptide. Virology 154: 9-20.

Al Moudallal, Z., Brianid, J.P. & M.H.V. Van Regenmortel, 1985. A major part of the polypeptide chain of tobacco mosaic virus protein is antigenic. EMBO J. 4:1231-1235.

Altschuh, D., Al Moudallal, Z., Briand, J.P. & M.H.V. Van Regenmortel, 1985. Immunochemical studies of tobacco mosaic virus. VI. Attempts to localize viral epitopes with monoclonal antibodies. Mol. Immunol. 22: 329-337.

Antoniw, J.F. & R.F. White, 1986. Changes with time in the distribution of virus and PR protein around single local leisions of TMV infected tobacco. Plant. Mol. Biol. 6: 145-149.

Argos, P., 1988. A sequence motif in many polymerases. Nuc. Acids Res. 16: 9909-9916.

Assink, A-M., Swaans, H. & A. Van Kammen, 1973. The localization of virus-specific double-stranded RNA of cowpea mosaic virus in subcellular fractions of infected Vigna leaves. Virology 53: 384-391.

Bailey, J.M. & N. Davidson, 1976. Methylmercury as a reversible denaturing agent for agarose gel electrophoresis. Anal. Biochem. 70:75-85.

Barlow, D.J., Edwards, M.S. & J.M. Thornton, 1986. Continuous and discontinuous protein antigenic determinants. Nature 322: 747-748.

Barrett, A.D.T. & N.J. Dimmock, 1986. Defective interfering viruses and infections of animals. Curr. Top. Microbiol. Immunol. 128: 55-84.

Baron, M.H. & D. Baltimore, 1982. Anti-VPg antibody inhibition of the poliovirus replicase reaction and production of covalent complexes of VPg-related proteins and RNA. Cell 30: 745-752.

Beier, H., Bruening, G., Russell, M.L. & C.L. Tucker, 1979. Replication of cowpea mosaic virus in protoplasts isolated from immune lines of cowpea. Virology 95: 165-175.

Beier, H., Siler, D.J., Russell, M.C. & G. Bruening, 1977. Survey of susceptibility to cowpea mosaic virus among protoplasts and intact plants from Vigna sinensis lines. Phytopathology. 67: 917-921.

Benjamin, D.C., Berzofsky, J.A., East, I.J., Gurd, F.R.N., Hannum, C., Leach, S.J., Margolia, E., Michael, J.G., Miller, A., Prager, E.M., Reichlin, M., Sercarz, E.E., Smith-gill, S.J., Todd, P.A. & A.C. Wilson, 1984. The antigenic structure of proteins: a reappraisal. Ann. Rev. Immunol. 2: 67-101.

Birnboim, H.C. & J. Doiley, 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nuc. Acids Res. 7: 1513-1517.

Bittner, M., Kupferer, P. & C.F. Morris, 1980. Electrophoretic transfer of proteins and nucleic acids from slab gels to diazobenzyl-oxymethyl cellulose or nitrocellulose sheets. Anal. Biochem. 102: 459-471.

Branch, A.D., Benenfeld, B.J., Franck, E.R., Shaw, J.F., Varban, M.L., Willis, K.K., Roser, D.L. & H.D. Robertson, 1988. Interference between coinoculated viroids. Virology 163: 538-546.

Bruening, G., 1969. The inheritance of top component formation in cowpea mosaic virus. Virology 37: 577-584.

Bruening, G., 1977. Plant covirus systems: two-component systems. Compreh. Virol. 11: 79-111.

Bruening, G., 1978. Comovirus group. CMI/AAB Description of Plant Viruses no. 199. Commonwealth Mycological Institute, Ferry Lane, Kew, Surrey, England and the Association of Applied Biologists.

Bruening, G. & H.O. Agrawal, 1967. Infectivity of a mixture of cowpea mosaic virus ribonucleoprotein components. Virology 32: 306-320.

Bruening, G., Lee, S.-L. & H. Beier, 1979. Immunity to plant virus infection, pp. 421-440 in: Plant Cell and Tissue Culture: Principles and Applications, Sharp, W.R. & P.O. Larsen, eds.

Burns, D.M. & I.R. Beacham, 1983. A method for the ligation of DNA following isolation from low melting temperature agarose. Anal. Biochem. 135: 48-51.

Carr, J.P., Dixon, D.C., Nikolau, B.J., Voelkerding, K.V. & D.F. Klessig, 1987. Synthesis and localizatioin of pathogenesis-related proteins in tobacco. Mol. Cell. Biol. 7: 1580-1583.

Carrington, J.C., Cary, S.M. & W.G. Dougherty, 1988. Mutational analysis of tobacco etch virus polyprotein processing: cis and trans proteolytic activities of polyproteins containing the 49-kilodalton proteinase. J. Virology 62: 2313-2320.

Church, G.M. & W. Gilbert, 1984. Genomic sequencing. Proc. Natl. Acad. Sci. 81: 1991-1995.

Clark, M.F., Lister, R.M. & M. Bar-joseph, 1986. Elisa Techniques, pp. 742-766 in: Methods in Enzymology, Volume 118, Weissbach, A. & H. Weissbach, eds. Academic Press, New York.

Cockbain, A.J., Bowen R. & S. Vorra-Urai, 1976. Seed transmission of broadbean strain virus and echtes ackerbohnenmosaik-virus in field beans (Vicia faba). Ann. Appl. Biol. 84: 321-332.

Cornelissen, B.J.C., Horowitz, J., Van Kan, J.A.L., Goldberg, R.B. & J.F. Bol, 1987. Structure of tobacco genes encoding pathogenesis-related proteins from the PR-1 group. Nuc. Acids Res. 15:6799-6811.

Cox, R.A., 1968. The use of quanidium chloride in the isolation of nucleic acids, pp. 120-129 in: Methods in Enzymology, Volume 12B, Grossman, L. & K. Moldave, eds. Academic Press, New York.

Crouce, G.F., Frischauf, A. & H. Lehrach, 1983. An integrated and simplified approach to cloning into plasmids and single-stranded phages, pp. 78-85 in: Methods in Enzymology, Volume 101, Grossman, L. & K. Moldave, eds. Academic Press, New York.

Crowther, R.A., Geelen, J.L.M.C. & J.E. Mellama, 1974. A three-dimensional image reconstruction of cowpea mosaic virus. Virology 57: 20-27.

D'Alessio, J.M., Noon, M.C., Ley, H.L. & G.F. Gerard, 1987. One-tube double-stranded cDNA synthesis using cloned M-MLV reverse transcriptase. Focus 9:1-4.

Darvill, A.G. & P. Albersheim, 1984. Phytoalexins and their elicitors- A defense against microbial infection in plants. Ann. Rev. Plant Physiol. 35: 243-275.

Davis, L.S., M.D. Dibner & J.F. Battey, 1986. Basic Methods In Molecular Biology, p. 327. Elsevier, New York

Davis, R.E. & A.F. Ross, 1968. Increased hypersensitivity induced in tobacco by systemic infection by potato virus Y. Virology 34: 509-520.

Daubert, S.D., Bruening, G. & R.C. Najarian, 1978. Protein bound to the genome RNA's of cowpea mosaic virus. Eur. J. Biochem. 92: 45-51.

de Jager, C.P., 1979. Cowpea severe mosaic virus. CMI/AAB Description of Plant Viruses no. 209. Commonwealth Mycological Institute, Ferry Lane, Kew, Surrey, England and the Association of Applied Biologists.

de Jager, C.P. & A. Van Kammen, 1970. The relationship between the components of cowpea mosaic virus. III. Location of genetic information for two biological functions in the middle component of CPMV. Virology 41: 281-287.

de Jager, C.P. & J.B.M. Wesseling, 1981. Spontaneous mutations in cowpea mosaic virus overcoming resistance due to hypersensitivity in cowpea. Physiol. Plant Pathol. 19: 347-348.

Dekker, E.L., Dore, I., Porta, C. & M.H.V. Van Regenmortel, 1987. Conformational specificity of monoclonal antibodies in the diagnosis of tobacco mosaic virus. Arch. Virol. 94: 191-203.

Dekker, E.L., Pinner, M.S., Markham, P.G. & M.H.V. Van Regenmortel, 1988. Characterization of maize streak virus isolates from different plant species by polyclonal and monoclonal antibodies. J. Gen. Virol. 69: 883-900.

de Zoeten, G.A. & R.W. Fulton, 1975. Understanding genetic possibilities. Phytopathology 65: 221-222.

Dodds, J.A. & R.I. Hamilton, 1974. Masking of the RNA genome of tobacco mosaic virus by the protein of barley stripe mosaic virus in doubly infected barley. Virology 59: 418-427.

Dodds, J.A. & R.I. Hamilton, 1976. Structural interactions between viruses as a consequence of mixed virus infections. Adv. Virus Res. 20: 33-86.

Dodds, J.A., Lee, S.Q. & M. Tiffany, 1985. Cross protection between strains of cucumber mosaic virus: effect of host and type of inoculum on accumulation of virions and double-stranded RNA of the challenge strain. Virology 144: 301-309.

Dore, I., Atschuh, D., Al Moudallal, Z. & M.H.V. Van Regenmortel, 1987. Immunochemical studies of tobacco mosaic virus. VII. Use of comparative surface accessibility of residues in antigenically related viruses for delineating epitopes recognized by monoclonal antibodies. Mol. Immunol. 24: 1351-1358.

Dorssers, L., Van Der Krol, S. Van Der Meer, J., Van Kammen, A. & P. Zabel, 1984. Purification of cowpea mosaic virus RNA replication complex: identification of a virus-encoded 110,000-dalton polypeptide responsible for RNA chain elongation. Proc. Natl. Acad. Sci. 81: 1951-1955.

Dorssers, L., Van Der Meer, J., Van Kammen & P. Zabel, 1983. The cowpea mosaic virus RNA replication complex and the host-encoded RNA-dependent RNA-polymerase-template complex are functionally diferent. Virology 125: 155-174.

Dorssers, L., Zabel, P., Van Der Meer, J. & A. Van Kammen, 1982. Purification of a host-encoded RNA-dependent RNA polymerase from cowpea mosaic virus-infected cowpea leaves. Virology 116: 236-249.

Eastwell, K.C., Kiefer, M.C. & G. Bruening, 1983. Immunity of cowpeas to cowpea mosaic virus, pp. 201-211 in: Plant Molecular Biology, UCLA Symposium on Molecular Cell BIology, Volume 12, R.B. Goldberg, ed. Vol. 12. Alan R. Liss, New York.

Ecker, J.R. & R.W. Davis, 1987. Plant defense genes are regulated by ethylene. Proc. Natl. Acad. Sci. 84: 5202-5206.

Eggen, R., Kaan, A., Goldbach, R. & A. Van Kammen, 1988. Cowpea mosaic virus RNA replication in crude membrane fractions from infected Cowpeas and _Chenopodium amaranticolor_. J. Gen. Virol. 69: 2711-2720.

Eggen, R. & A. Van Kammen, 1988. Evolution of RNA viruses, pp 46-69 in: RNA Genetics, Volume I, Domingo, E., Holland, J.J. & P. Ahlquist, eds. CRC Press Inc., Florida.

Esau, K., 1967. Anatomy of plant virus infections. Ann. Rev. Phytopathol. 73: 269-273.

Evans, D., 1985. Isolation of a mutant of cowpea mosaic virus which is unable to grow in cowpeas. J. Gen. Virol. 66: 339-343.

Faulkner, G. & W.C. Kimmins, 1975. Staining reactions of the tissue hardening lesions induced by wounding tobacco mosaic virus and tobacco necrosis virus in bean. Phytopathology 65: 1396-1400.

Feinberg, A.P. & B. Vogelstein, 1983. A technique for radiolabeling DNA restriction endnuclease fragments to high specific activity. Anal. Biochem. 132: 6-13. _Addendum:_ Anal. Biochem. 137: 266-267. 1984.

Fernow, K.H., 1967. Tomato as a test plant for detecting mild strains of potato spindle tuber virus. Phytopathology 57: 1347-1352.

Francki, R.I.B., Milne, R.G. & T. Hatta, 1985. Comovirus group, pp. 1-22 in: Atlas of plant viruses, Volume 2, Davies, J.W., ed. CRC Press Inc., Florida.

Francki, R.I.B., 1985. Plant virus satellites. Ann. Rev. Microbiol. 39: 151-174.

Franssen, H., Goldbach, R., Broekhujsen, M., Moerman, M. & A. Van Kammen, 1982. Expression of middle component RNA of cowpea mosaic virus: *in vitro* generation of a precursor to both capsid proteins by a bottom-component RNA-encoded protease from infected cells. J. Virol. 41: 8-17.

Franssen, H., Leunissen, J., Goldbach, R., Lomonossoff, G. & D. Zimmern, 1984. Homologous sequences in non-structural proteins from cowpea mosaic virus and picornaviruses. EMBO J. 3: 855-861.

Fraser, R.S.S., 1987. Biochemistry of Virus-Infected Plants, pp. 152-170. Research Studies Press Ltd, Letchworth.

Fraser, R.S.S. & S.A.R. Loughlin, 1980. Resistance to tobacco mosaic virus in tomato: effects of the *Tm-1* gene on virus multiplication. J. Gen. Virol. 48: 87-96.

Friquet, B., Djavadi-Ohaniance, L. & M.E. Goldberg, 1984. Some monoclonal antibodies raised with a native protein bind preferentially to the denatured antigen. Mol. Immunol. 21: 673-677.

Fulton, R.W., 1951. Superinfection by strains of tobacco mosaic virus. Phytopathology 41: 579-592.

Fulton, R.W., 1975. Unilateral cross-protection among some NEPO viruses. Acta Hortic. 41: 579-592.

Fulton, R.W., 1978. Superinfection of strains of tobacco streak virus. Virology 85: 1-8.

Fulton, J.P., Gerreich, R.C. & H.A. Scott, 1987. Beetle transmission of plant viruses. Ann. Rev. Phytopathol. 25: 111-123.

Geelen, J.L.M.C., Rezelman, G. & A. Van Kammen, 1973. The infectivity of the two electrophoretic forms of cowpea mosaic virus. Virology 51: 279-286.

Geelen, J.L.M.C., Van Kammen A. & B.J.M. Verduin, 1972. Structure of the capsid of cowpea mosaic virus. The chemical subunit: molecular weigth and number of subunits per particle. Virology 49: 205-213.

Gergerich, R.C. & H.A. Scott, 1988. The enzymatic function of ribonuclease determines plant virus transmission by leaf-feeding beetles. Phytopathology 78: 270-272.

Getzoff, E.D., H.M. Geysen, Rodda, S.I., Alexander, H., Tainer, J.A. & R.A. Lerner, 1987. Mechanism of antibody binding to a protein. Science 235: 1191-1196.

Gianinazzi, S. & B. Kassanis, 1974. Virus resistance induced in plants by polyacrylic acid. J. Gen. Virol. 23: 1-9.

Gibbs, A.J., 1969. Plant virus classification. Adv. Virus Res. 14: 263-328.

Gibbs, A.J., Giussoni-Belli, G. & H.G. Smith, 1968. Broad-bean stain and true broad-bean mosaic virus. Ann. Appl. Biol. 61: 99-107.

Goding, J.W., 1980. Antibody production by hybridomas. J. Immunol. Methods 39: 285-308.

Goldbach, R. & J. Krijt, 1982. Cowpea mosaic virus-encoded protease does not recognize primary translation products of M RNAs from other comoviruses. J. Virol. 43: 1151-1154.

Goldbach, R. & G. Rezelman, 1983. Orientation of the cleavage map of the 200-kilodalton polypeptide encoded by the bottom-component RNA of cowpea mosaic virus. J. Virol. 46: 614-619.

Goldbach, R., Rezelman, G. & A. Van Kammen, 1980. Independent replication and expression of B-component RNA of cowpea mosaic virus. Nature 286: 297-300.

Goldbach, R., Rezelman, G., Zabel, P. & A. Van Kammen, 1982. Expression of the bottom-component RNA of cowpea mosaic virus: evidence that the 60-kilodalton VPg precursor is cleaved into single VPg and a 58-kilodalton polypeptide. J. Virol. 42: 630-635.

Goldbach, R. & A. Van Kammen, 1985. Structure, replication and expression of the bipartite genome of cowpea mosaic virus, pp. 83-120 in: Molecular Plant Virology, Volume 2, Davies, J.W., ed. CRC Press, Inc., Florida.

Goodman, R.M. & A.F. Ross, 1974. Enhancement by potato virus Y of potato virus X synthesis in doubly infected tobacco depends on the timing of invasion by the viruses. Virology 58: 263-271.

Gorbalenya, A.E., Koonin, E.V., Donchenko, A.P. & V.M. Blinko, 1988. A conserved NTP-motif in putative helicases. Nature 333: 22.

Grunstein, M. & D.S. Hogness, 1975. Colony hybridization: A method for the isolation of cloned DNAs that contain a specific gene. Proc. Natl. Acad. Sci. 72: 3961-3966.

Gubler, U.& B.J. Hoffman, 1983. A simple and very efficient method for generating cDNA libraries. Gene 25: 263-269.

Halk, E.L., 1986. Serotyping plant viruses with monoclonal antibodies. Methods in Enzymol. 118: 766-780.

Hamilton, R.I., 1980. Defenses triggered by previous invaders: viruses, pp. 279-303 in: Plant Disease, Volume 5. Academic Press, New York.

Harrison, B.D. & D.J. Robinson, 1978. The tobraviruses. Advan. Virus Res. 23: 25-77.

Hemenway, C., Fong, R-X., Kaniewski, W.K., Chau, N-H. & N.E. Turner, 1988. Analysis of the mechanism of protection in transgenic plants expressing the potato virus X coat protein or its antisense RNA. EMBO J. 7: 1273-1280.

Hillman, B.I., Carrington, J.C. & T.J. Morris, 1987. A defective interfering RNA that contains a mosaic of a plant virus genome. Cell 51: 427-433.

Hodgman, T.C., 1988. A new superfamily of replicative proteins. Nature 333: 22-23.

Hooft Van Huijsduijnen, R.A.M., Cornelissen, B.J.C., Van Loon, L.C., Van Boom, J.H., Tromp, M. & J.F. Bol, 1985. Virus-induced synthesis of messenger RNAs for precursors of pathogenesis-related proteins in tobacco. EMBO J. 4: 2167-2171.

Horikoshi, M., Nakayama, M., Yamaoka, N., Furusawu, I. & J. Shishiyama, 1987. Brome mosaic virus coat protein inhibits viral RNA synthesis in vitro. Virology 158: 15-19.

Houwing, C.J. & E.M.J. Jaspars, 1986. Coat protein blocks the in vitro transcription of the virion RNAs of alfalfa mosaic virus. FEBS Letters 209: 284-288.

Icenogle, J., Gilbert, S.F., Grieves, J., Anderegg, J. & R. Rueckert, 1981. A neutralizing monoclonal antibody against poliovirus and its reaction with related antigens. Virology 115: 211-215.

Karban, R., Adamchak, R. & W.C. Schnathorst, 1987. Induced resistance and interspecific competition between spider mites and a vascular wilt fungus. Science 235: 678-680.

Kassanis, B., Gianinazzi, S. & R.F. White, 1974. A possible explanation of the resistance of virus infected tobacco plants to second virus infection. J. Gen. Virol. 23: 11-16.

Kauffmann, S., Legrand, M., Geoffroy, P. & B. Fritig, 1987. Biological function of "pathogenesis-related" proteins: four PR proteins of tobacco have 1,3-B-glucanase activity. EMBO J. 6: 3209-3212.

Kiefer, M.C., Bruening, G. & M.L. Russell, 1984. RNA and capsid accumulation in cowpea protoplasts that are resistant to cowpea mosaic virus strain SB. Virology 137: 71-81.

Kirkegaard, K. & D. Baltimore, 1986. The mechanism of RNA recombination in poliovirus. Cell 47: 433-443.

Kitamura, N., Semler, B.L., Rothberg, P.G., Larsen, G.R., Adler C.J., Dorner, A.J., Emini, E.A., Hanecak, R., Lee, J.J., Van Der Werf, S., Anderson, C.W. & E. Wimmer, 1981. Primary structure, gene organization and polypeptide expression of poliovirus RNA. Nature 291: 547-553.

Koenig, R. & L. Torrance, 1986. Antigenic analysis of potato virus X by means of monoclonal antibodies. J. Gen. Virol. 67: 2145-2151.

Kridl, J.C. & G. Bruening, 1983. Comparison of capsids and nucleoproteins from cowpea mosaic virus-infected cowpea protoplasts and seedlings. Virology 129: 369-380.

Kuc. J., 1983. Induced systemic resistance in plants to diseases caused by fungi and bacteria, pp. 191-221 in: The Dynamics of Host Response, J.A. Bailey & B.J. Deverall, eds. Academic Press, Sydney.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

Land, H., Grez, M., Hauser, H., Lindenmaier, W. & G. Schultz, 1981. 5'-terminal sequences of eukaryotic mRNA can be cloned with high efficiency. Nuc. Acids Res. 9: 2251-2266.

Lazzarini, R.A., Keene, J.D. & M. Schubert, 1981. The origins of defective interfering particles of the negative-strand RNA viruses. Cell 26: 145-154.

Lin, M.T., Hill, J.H., Kitajima, E.W. & C.L. Costa, 1984. Two new serotypes of cowpea severe mosaic virus. Phytopathology 75: 581-585.

Loebenstein, G., Spiegel, S. & A. Gera, 1982. Localized resistance and barrier substances, pp. 211-230 in: Active Defense Mechanisms In Plants, R.K.S. Wood, ed. Plenum Publishing, London.

Loebenstein, G. & A. Stein, 1985. Plant defense responses to viral infections, pp. 413-433 in: Cellular and Molecular Biology of Plant Stress, Key, J.L. & T. Kosuge, eds. Alan R. Liss, New York.

Loesch-Fries, L.S., Merlo, D., Zinnen, T., Burhop, L., Hill, K., Krahn, K., Jarvis, N., Nelson, S. & E. Halk, 1987. Expression of alfalfa mosaic virus RNA4 in transgenic plants confers virus resistance. EMBO J. 6: 1845-1851.

Lok, S. & M.G. Abou Haidar, 1981. The polar alkaline disassembly of papaya mosaic virus. Virology 113: 637-643.

Lomonossoff, G.P. & M. Shanks, 1983. The nucleotide sequence of cowpea mosaic virus B RNA. EMBO J. 2: 2253-2259.

Malyshenko, S.I., Lapchic, L.G., Kondakova, O.A., Kuznetzova, L.L., Taliansky, M.E. & J.G. Atabekov, 1988. Red clover mottle comovirus B-RNA spreads between cells in tobamovirus-infected tissues. J. Gen Virol. 69: 407-412.

Maniatis, T., Fritsch, E.F. & J. Sambrook, 1982. Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory.

Massalski, P.R. & B.D. Harrison, 1987. Properties of monoclonal antibodies to potato leafroll luteovirus and their use to distinguish virus isolates differing in aphid transmissibility. J. Gen. Virol. 68: 1813-1821.

Maxum, A. & W. Gilbert, 1980. Sequencing end-labeled DNA with base-specific chemical cleavages, pp 499-560 in: Methods in Enzymology, Volume 65, Grossman, L. and K. Moldave, eds. Academic Press, New York.

Mazzone, H.M., Incardona, N.L. & P. Kaesberg, 1962. Biochemical and biophysical properties of squash mosaic virus and related macromolecules. Biochim. Biophys. Acta. 55:164-175.

Melton, D.A., Kriegg, P.A., Rebagliati, M.R., Maniatis, T., Zinn, K. & M.R. Green, 1984. Efficient _in vitro_ synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage Sp6 promoter. Nuc. Acids Res. 12: 7035-7056.

Melton, D.A., 1985. Injected anti-sense RNAs specifically block messenger RNA translation _in vivo_. Proc. Natl. Acad. Sci. 82: 144-148.

Meshi, T., Motoyoshi, F., Adachi, A., Watanabe, Y., Takamatsu, N. & Y. Okada, 1988. Two concomitant base substitutions in the putative replicase genes of tobacco mosaic virus confer the ability to overcome the effects of a tomato resistance gene, Tm-1. EMBO J. 7: 1575-1581.

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

Messing, J., 1983. New M13 vectors for cloning, pp. 20-79 in: Methods in Enzymology, Volume 101, Grossman, L. & K. Moldave, eds. Academic Press, New York.

Meyer, M., Hemmer, O., Mayo M.A. & C. Fritsch, 1986. The nucleotide sequence of tomato black ring virus RNA-2. J. Gen. Virol. 67: 1257-1271.

Morch, M.D., Joshi, R. L., Denial, T.M. & A.L. Haenni, 1987. A new 'sense' RNA approach to block viral RNA replication _in vitro_. Nuc. Acids Res. 15: 4123-4130.

Motte, P., Alberici, G., Ait-Abdellah, M. & D. Bellet, 1987. Monoclonal antibodies distinguish synthetic peptides that differ in one chemical group. J Immunol. 138: 3332-3338.

Mozes, R., Antignus, Y., Sela, I. & I. Harpaz, 1978. The chemical nature of an antiviral factor (AVF) from virus-infected plants. J. Gen. Virol. 38: 241-249.

Muller, S., Plaue, S., Couppez, M. & M.H.V. Van Regenmortel, 1986. Comparison of different methods for localizing antigenic regions in histone H2A. Mol. Immunol. 23: 593-601.

Nachman, I., Loebenstein, G., Van Praugh, T. & A. Zelcer, 1971. Increased multiplication of cucumber mosaic virus in a resistant cucumber caused by actinomycin D. Physiol. Plant Pathol. 1: 67-72.

Negruk, V.I., Grill, L.K. & S. Semancik, 1980. In vitro ^{32}P-labeling of viroid RNA for hybridization studies. J. Virol. Meth. 1: 229-234.

Nelson, R.S., Abel, P. & R.N. Beachy, 1987. Leisions and virus accumulation in inoculated transgenic tobacco plants expressing the coat protein gene of tobacco mosaic virus. Virology 158: 126-132.

Niblett, C.L., Dickson, E., Fernow, K.H., Horst, R.K. & M. Zaitlin, 1978. Cross protection among four viroids. Virology 91: 198-203.

Niblett, C.L. & J.S. Semancik, 1969. Conversion of the electrophoretic forms of cowpea mosaic virus in vivo and in vitro. Virology 38: 685-693.

Niblett, C.L. & J.S. Semancik, 1970. The significance of the coat protein in infection by the electrophoretic forms of cowpea mosaic virus. Virology 41: 201-207.

Nicklin, M.J.H., Toyoda, H., Murray, M.G. & E. Wimmer, 1986. Proteolytic processing in the replication of polio and related viruses. Biotechnology 4: 33-42.

Nomoto, A., Omata, T., Toyopda, H., Kuge, S., Horie, H., Kataoka, Y., Genba, Y., Nakano, Y. & N. Imura, 1981. Complete nucleotide sequence of the attenuated poliovirus Sabin 1 strain genome. Proc. Natl. Acad. Sci. 79: 5793-5797.

Oi, V.T. & L.A. Hezenberg, 1980. Immunoglobulin-producing hybrid cell lines, pp. 351-372 in: Selected Methods in Cellular Immunology, B.R. Michell & S.M. Sheigi, eds. Academic Press, New York.

Owens, R.A. & G. Bruening, 1975. The pattern of amino acid incorporation into two cowpea mosaic virus proteins in the presence of ribosome-specific protein synthesis inhibitors. Virology. 64: 520-530.

Palukaitis, P. & M. Zaitlin, 1984. A model to explain the "cross-protection" phenomenon shown by plant viruses and viroids, pp. 420-429 in: Plant-Microbe Interactions: Molecular and Genetic Perspectives, T. Kosuge & E.W. Nester, eds. Macmillan, New York.

Patel, P.N., 1982. Genetics of cowpea reactions to two strains of cowpea mosaic virus from Tanzania. Phytopathology 72: 460-466.

Peacock, S.I., McIver, C.M. & J.J. Monahan, 1981. Transformation of *E. coli* using homopolymer-linked plasmid chimeras. Biochim. Biophys. Acta. 655: 243-250.

Pelham, H.R.B., 1979. Synthesis and proteolytic processing of cowpea mosaic virus proteins in reticulocyte lysates. Virology 96: 463-477.

Pestka, S., Daugherty, B.L., Jung, V., Hotta, K. & R.K. Pestka, 1984. Anti-mRNA: specific inhibition of translation of single mRNA molecules. Proc. Natl. Acad. Sci. 81: 7525-7528.

Pfitzner, U.M. & H.M. Goodman, 1987. Isolation and characterization of cDNA clones encoding pathogenesis-related proteins from tobacco mosaic virus infected tobacco plants. Nuc. Acids Res. 15: 4449-4465.

Pfitzner, U.M., Pfitzner, A.J.P. & H.M. Goodman, 1988. DNA sequence analysis of a PR-1a gene from tobacco: molecular relationship of heat shock and pathogen responses in plants. Mol. Gen. Genet. 211: 290-295.

Ponz, F. & G. Bruening, 1986. Mechanisms of resistance to plant viruses. Ann. Rev. Phytopathol. 24: 355-381.

Ponz, F., Glascock, G.B. & G. Bruening, 1988a. An inhibitor of polyprotein processing with the characteristics of a natural virus resistance factor. Mol. Plant-Microbe Interactions 1: 25 -31.

Ponz, F., Russell, M.L., Rowhani, A. & G. Bruening, 1988b. A cowpea line has distinct genes for resistance to tobacco ringspot virus and cowpea mosaic virus. Phytopathology 78: 1124-1128.

Putnak, J.R. & B.A. Phillips, 1981. Picornavirus structure and assembly. Microbiol. Rev. 45: 287-310.

Register, J.C. & R.N. Beachy, 1988. Resistance to TMV in transgenic plants results from interference with an early event in infection. Virology 166: 524-532.

Rezelman, G., Franssen, H.J., Goldbach, R.W., Ie, T.S. & A. Van Kammen, 1982. Limits to independence of bottom component RNA of cowpea mosaic virus. J. Gen. Virol. 60: 335-346.

Rezelman, G., Goldbach, R., & A. Van Kammen, 1980. Expression of bottom component RNA of cowpea mosaic virus in cowpea protoplasts. J. Virol. 36: 366-373.

Rios, G. P. & B.P. Das Neves, 1982. resistencia de Linhagens E Cultivares de Caupi (Vigna unguiculata (L.) Walp.) ao Virus do Mosaico Severo (VMSC), Fitopathologia Brasilevia 7: 175-184.

Robertson, D.G., 1965. The local leision reaction for recognizing cowpea varieties immune from and resistant to cowpea yellow mosaic virus. Phytopathology 55: 923-925.

Robinson, D.J., 1977. A varient of tobacco rattle virus: evidence for a second gene in RNA-2. J. Gen. Virol. 35: 37-43.

Rombaut, B., Vrijsen, R., Brioen, P. & A. Boeye, 1982. A pH-dependent antigenic conversion of empty capsids of poliovirus studied with the aid of monoclonal antibodies to N and H antigens. Virology 122: 215-218.

Ross, A.F., 1961a. Localized acquired resistance induced by localized virus infections in plants. Virology 14: 329-339.

Ross, A.F., 1961b. Systemic acquired resistance induced by localized virus-infections in plants. Virology 14: 340-358.

Rott, M.E., Rochon, D.M. & J.H. Tremaine, 1988. A 1.9 kilobase homology in the 3'-terminal regions of RNA-1 and RNA-2 of tomato ringspot virus. J. Gen. Virol. 69: 745-750.

Ruziscka, P., Gombos, Z. & G.L. Farkas, 1983. Modification of the fatty acid composition of phospholipids during the hypersensitive reaction in tobacco. Virology 128: 60-64.

Saaijer-Riep, J.D. & C.P. de Jager, 1988. Inhibition of different cowpea viruses by non-virulent cowpea mosaic virus is dependent on the type of immunity of the plant to the inhibitory virus. Neth. J. Plant Pathol. 94: 253-256.

Sanderson, J.L., Bruening, G. & M.C. Russell, 1985. Possible molecular basis of immunity of cowpeas to cowpea mosaic virus, pp. 401-412 in: Cellular and Molecular Biology of Plant Stress. Alan R. Liss, New York.

Sanger, F., Nicklen, S. & A.R. Coulsen, 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. 74: 5463-5467.

Schmidt, T., Johnson, J.E. & W.E. Phillips, 1983. The spherically averaged structure of cowpea mosaic virus components by X-ray solution scattering. Virology 127: 65-73.

Schnathorst, W.C. & D.E. Mathre, 1966. Cross-protection in cotton with strains of Verticillium albo-atrum. Phytopathology 56: 1204-1209.

Sedmak, J.J. & S.E. Grossberg, 1977. A rapid, sensitive, and versatile assay for protein using coomassie brilliant blue G250. Anal. Biochem. 79: 544-552.

Sela, I., Harpaz. I. & Y. Birk, 1966. Identification of the active component of an antiviral factor isolated from virus-infected plants. Virology 28:71-78.

Sela, I., Hauschner, A. & R. Mozes, 1978. The mechanisms of stimulation of the antiviral factor (AVF) in Nicotiana leaves: the involvement of phosphoacylation and the role of the N-gene. Virology 89: 1-6.

Semler, B.L., Kuhn R.J. & E. Wimmer, 1988. Replication of the poliovirus genome, pp. 23-48 in: RNA genetics, Volume 1, E. Domingo, J.J. Holland & P. Ahlquist, eds. CRC Press, Boca Raton, Florida.

Shanks, M., Stanley, J. & G.P. Lomonossoff, 1986. The primary structure of red clover mottle virus middle component RNA. Virology 155: 697-706.

Shaw, J.G., Plaskitt, K.A. & T.M.A. Wilson, 1986. Evidence that tobacco mosaic virus particles disassemble cotranslationally in vivo. Virology 148: 326-336.

Sherwood, J.L. & R.W. Fulton, 1982. The specific involvement of coat protein in tobacco mosaic virus cross protection. Virology 119: 150-158.

Sheshberadaran, H., Chen, S.N. & E. Norrby, 1983. Monoclonal antibodies against five structural components of measles virus: characterization of antigenic determinants on nine strains of measles virus. Virology 128: 341-353.

Siler, D.J., Babcock, J. & G. Bruening, 1976. Electrophoretic mobility and enhanced infectivity of a mutant of cowpea mosaic virus. Virology 71: 560-567.

Simpson, R.B., 1964. Association constants of methylmercuric and mercuric ions with nucleosides. J. Amer. Chem. Soc. 86: 2059-2065.

Sober, J., Jarvekulg, L., Toots, I., Radavsky, J., Villens, R. & M. Saarma, 1988. Antigenic characterization of potato virus X with monoclonal antibodies. J. Gen. Virol. 69: 1799-1807.

Stace-Smith, R., 1981. Comoviruses, pp. 171-194 in: Handbook of plant virus infections and comparative diagnosis, E. Kurstak, ed. Elsevier/North-Holland Biomedical Press.

Stanley, J., Rottier, P., Davies, J.W., Zabel, P. & A. Van Kammen, 1978. A protein linked to the 5'-terminal of both RNA components of the cowpea mosaic virus genome. Nuc. Acids Res. 5: 4505-4522.

Sterk, P. & C.P. de Jager, 1987. Interference between cowpea mosaic virus and cowpea severe mosaic virus in a cowpea host immune to cowpea mosaic virus. J. Gen. Virol. 68: 2751-2758.

Stollar, D. & L. Levine, 1963. Two-dimensional immuno-diffusion, pp. 848-854 in: Methods in Enzymology, Volume 6, Colowick, G. & A. Kaplan. Academic Press, New York.

Swaans, H. & A. Van Kammen, 1973. Reconsideration of the distinction between the severe and yellow strains of cowpea mosaic virus. Neth. J. of Plant Pathol. 79: 257-265.

Tainer, J.A., Getzoff, E.D., Alexander, H., Houghten, R.A., Olson, A.J. & R.A. Lerner, 1984. The reactivity of antipeptide antibodies is a function of the atomic mobility of sites in a protein. Nature 312: 127-134.

Tamburro, A.M., Guantieri, V., Piazzolla, P. & D. Gallitelli, 1978. Conformational studies on particles of turnip yellow mosaic virus. J. Gen Virol., 40: 337-344.

Thomson, A.D., 1958. Interference between plant viruses. Nature 181: 1547-1548.

Thongmeearkom, P. & R.M. Goodman, 1978. Complementation and pseudrecombination between RNAs from two natural isolates of cowpea mosaic virus (severe subgroup). Virology 85: 75-83.

Thung, T.H., 1931. Smetstof en plantencel by enkele viruszieкten van de tabaksplant. abstr. Rev Appl. Mycol. 11: 750-751.

Torrance, L., Larkins, A.P. & G.W. Butcher, 1986. Characterization of monoclonal antibodies against potato virus X and comparison of serotypes with resistance groups. J. Gen. Virol. 67: 57-67.

Tremaine, J.H., Mackenzie, D.J. & W.P. Ronald, 1985a. Monoclonal antibodies as structural probes of southern bean mosaic virus. Virology 144: 80-87.

Tremaine, J.H., Ronald, W.P. & D.J. Mackenzie, 1985b. Southern bean mosaic virus monoclonal antibodies: reactivity with virus strains and with the virus antigen in different conformations. Phytopathology 75: 1208-1212.

Tumer, N.E., O'Connell, K.M., Nelson, R.S., Sanders, P.R., Beachy, R.N., Fraley, R.T. & D.M. Shah, 1987. Expression of alfalfa mosaic virus coat protein gene confers cross-protection in transgenic tobacco and tomato plants. EMBO J. 6: 1181-1188.

Van Der Meer, J., Dorssers, L., Van Kammen, A. & P. Zabel, 1984. The RNA-dependent RNA polymerase of cowpeas is not involved in cowpea mosaic virus RNA replication: immunological evidence. Virology 132:413-425.

Van Der Sheer, C. & J. Groenewegan, 1971. Structures in cells of Vigna unguiculata infected with cowpea mosaic virus. Virology 46: 493-497.

Van Dun, C.M.P., Overduin, B., Van Vloten-Doting, L. & J.F. Bol, 1988. Transgenic tobacco expressing tobacco streak virus or mutated alfalfa mosaic virus coat protein does not cross-protect against alfalfa mosaic virus infection. Virology 164: 383-389.

Van Kammen, A., 1968. The relationship between the components of cowpea mosaic virus. I. Two ribonucleotide particles necessary for the infectivity of CPMV. Virology 34: 312-318.

Van Kammen, A., 1972. Plant viruses with a divided genome. Ann. Rev. of Phytopathol. 10: 125-150.

Van Kammen, A. & C.P. de Jager, 1978. Cowpea mosaic virus. CMI\AAB Description of Plant Viruses no. 197. Commonwealth Mycological Institute, Ferry Lane, Kew, Surrey, England and the Association of Applied Biologists.

Van Loon, L.C., 1977. Induction by 2-chloroethylphosphonic acid of viral-like leisions, associated proteins, and systemic resistance in tobacco. Virology 80: 417-420.

Van Loon, L.C., 1983. Mechanisms of resistance in virus-infected plants, pp. 123-190 in: The Dynamics of Host Defense, J.A. Bailey & B.J. Deverall, eds. Academic Press, Sydney.

Van Loon, L.C., 1985. Pathogenesis-related proteins. Plant Mol. Biol. 4: 111-116.

Van Loon, L.C. & A. Van Kammen, 1970. Polyacrylamide disk electrophoresis of the soluble leaf proteins from *Nicotiana tabacum* var. "Samsun" and "Samsun NN". II. Changes in protein constitution after infection with tobacco mosaic virus. Virology 40: 199-211.

Van Regenmortel, M.H.V., 1982. Serology and Immunochemistry of Plant Viruses, pp 39-42. Academic Press, Inc., New York.

Van Regenmortel, M.H.V. & W. Lelarge, 1973. The antigenic specificity of different states of aggregation of tobacco mosaic virus protein. Virology 52: 89-104.

Van Wezenbeek, P., Verver, J., Harmsen, J., Vos, P. & A. Van Kammen, 1983. Primary structure and gene organization of the middle-component RNA of cowpea mosaic virus. EMBO J. 2: 941-946.

Van Wyke, K.L., Yewdell, J.W., Reck, L.J. & B.R. Murphy, 1984. Antigenic characterization of influenza A virus matrix protein with monoclonal antibodies. J. Virol. 49: 248-252.

Verver, J., Goldbach, R., Garcia, J.A. & P. Vos, 1987. *In vitro* expression of a full-length DNA copy of cowpea mosaic virus B RNA: identification of the B RNA encoded 24-Kd protein as a viral protease. EMBO J. 6: 549-554.

Vos, P., Verver, J., Jaegle, M., Wellink, J., Van Kammen A. & R. Goldbach, 1988. Two viral proteins involved in the proteolytic processing of the cowpea mosaic virus polypeptides. Nuc. Acids Res. 16: 1967-1985.

Vos, P., Verver, J., Van Wezenbeek, P., Van Kammen A. & R. Goldbach, 1984. Study of the genetic organization of a plant viral RNA genome by *in vitro* expression of a full-length DNA copy. EMBO J. 3: 3049-3053.

Wagih, E.E. & R.H.A. Coutts, 1982. Peroxidases, polyphenoloxidase and ribonuclease in tobacco necrosis virus-infected or mannitol osmotically-stressed cowpea and cucumber tissue. I. Quantitative alterations. Phytopathol. Z. 104: 1-12.

Wellink, J., Jaegle, M., Prinz, H., Van Kammen A. & R. Goldbach, 1987. Expression of the middle component RNA of cowpea mosaic virus *in vivo*. J. Gen. Virol. 68: 2577-2585.

Wellink, J., Rezelman, G., Goldbach, R. & K. Beyreather, 1986. Determination of the proteolytic processing sites in the polypeptide encoded by the bottom-component RNA of cowpea mosaic virus. J. Virol. 59: 50-58.

Wellink, J., Van Lent, J. & R. Goldbach, 1988. Detection of viral proteins in cytoplasmic structures in cowpea protoplasts infected with cowpea mosaic virus. J. Gen. Virol. 69: 751-755.

Weststeijen, E.A., 1984. Evidence for a necrosis-inducing factor in tobacco mosaic virus-infected Nicotiana tabacum cv. Xanthi nc. grown at 22 C but not at 32 C. Physiol. Plant Pathol. 25: 83-91.

Whitaker-Dowling, P. & J.S. Younger, 1987. Viral interference-dominance of mutant viruses over wild-type virus in mixed infections. Microbiol. Rev. 51: 179-191.

Wood, H.A., 1972. Genetic complementation between the two nucleoprotein components of cowpea mosaic virus. Virology 49: 592-598.

Wu, J.-H., 1973. Wound-healing as a factor in limiting the size of lesions in Nicotiana glutinosa leaves infected by the very mild strain of tobacco mosaic virus (TMV-VM). Virology 51: 474-484.

Wu, J.H. & J.E. Dimitman, 1970. Leaf structure and callose formation as determinants of TMV movement in bean leaves as revealed by UV irradiation studies. Virology 40: 820-827.

Wu, J-H., & I. Rappaport, 1961. An analysis of the interference between two strains of tobacco mosaic virus on Phaseolus vulgaris L. Virology 14: 259-263.

Wu, G-J. & G. Bruening, 1971. Two proteins from cowpea mosaic virus. Virology 46: 596-612.

Yanisch-Perron, C., Vieira, J. & J. Messing, 1985. Improved M13 phage cloning vectors and host strains: Nucleotide sequences of the M13 mp18 and pUC19 vectors. Gene 33: 103-119.

Ye, X.S. & J. Kuc, 1988. Systemic resistance to Peronospora tabacina induced by tobacco mosaic virus on tobacco cv. KY. (Abstr.) Phytopathology 78: 1547.

Zabel, P., Moerman, M., Van Straaten, F., Goldbach, R. & A. Van Kammen, 1982. Antibodies against the genome-linked protein VPg of cowpea mosaic virus recognize a 60,000-dalton precursor polypeptide. J. Virol. 41: 1083-1088.

Zaitlin, M., 1976. Viral cross-protection: more understanding is needed. Phytopathology 66: 382-383.

Zaumeyer, W.S. & J.P. Meiners, 1975. Disease resistance in beans. Ann. Rev. Phytopathol. 13: 313-334.

Zimmern, D., 1988. Evolution of RNA viruses, pp. 211-240 in: RNA genetics, Volume 2, Domingo, E., Holland, J.J. & J.W. Davies, eds. CRC Press Inc., Florida.

Zimmern, D. & T. Hunter, 1983. Point mutation in the 30-K open reading frame of TMV implicated in temperature-sensitive assembly and local leision spreading of mutant Ni2519. EMBO J. 2: 1893-1892.

Zinnen, T.M. & R.W. Fulton, 1986. Cross-protection between sunn-hemp mosaic and tobacco mosaic viruses. J. Gen. Virol. 67: 1679-1687.

APPENDIX A:

The results from Tables 1, 2, 4, 5 and 6 presented as numerical values with standard deviations.

Outline of Statistical Methods.

Assays involving monoclonal antibodies: all points were replicated four times per experiment, and each experiment was repeated a minimum of three times. The mean value of the results for each point was determined per experiment. A mean was calculated from the mean values determined from homologous experiments, and the standard deviation was calculated for the mean values obtained from individual experiments.

TABLE 1. Characterization of monoclonal antibodies produced against CPMV-SB.

MONOCLONAL ANTIBODY	ANTIBODY-TRAPPED[1]		PLATE-TRAPPED[2]	
	CPMV	CPSMV	CPMV	CPSMV
SB1	0.128	0.105	0.311	0.114
SB2	2.722	0.191	2.293	0.163
SB3	0.091	0.101	0.302	0.222
SB4	1.087	0.294	0.382	0.309
SB5	1.901	0.095	0.209	0.095
SB7	0.217	0.235	0.187	0.239
SB8	0.149	0.131	0.159	0.131
SB9	0.074	0.109	0.208	0.207

1. Plates were coated with homologous rabbit immunoglobulins. Background = 0.080. Standard deviation = +/- 8.5 %

2. Background = 0.095. Standard deviation = +/- 9.0 %.

TABLE 2. Characterization of monoclonal antibodies produced against CPSMV-DG.

MONOCLONAL ANTIBODY	ANTIBODY-TRAPPED[1]		PLATE-TRAPPED[2]	
	CPMV	CPSMV	CPMV	CPSMV
DG3	0.168	1.527	0.686	0.718
DG4	0.069	0.524	0.081	0.532
DG5	0.886	1.638	0.153	1.460
DG7	0.079	1.260	0.192	0.352
DG8	0.096	0.181	0.109	0.109
DG9	0.052	0.745	0.074	0.318
DG11	0.059	2.914	0.094	0.378

1. Plates were coated with homologous rabbit immunoglobulins. Background = 0.050. Standard deviation = +/- 6.5 %

2. Background = 0.080. Standard deviation = +/- 9.0 %.

TABLE 4. Monoclonal antibody binding to altered virus conformations in plate-trapped ELISAs.

MONOCLONAL ANTIBODY	ABSORBANCE[1] DENATURANT			
	SDS+UREA		GUANIDINE	
	CPMV-SB	CPSMV-DG	CPMV-SB	CPSMV-DG
SB1	0.198	0.090	0.211	0.197
SB2	2.052	0.183	3.000	0.134
SB3	0.128	0.183	0.168	0.109
SB4	0.187	0.175	0.116	0.108
SB5	0.099	0.096	0.119	0.106
SB7	0.203	0.243	0.112	0.107
SB8	0.225	0.238	0.132	0.126
SB9	0.419	0.552	0.120	0.103

1. Backgroud = 0.095. Standard deviation = +/- 10 %

TABLE 5. Monoclonal antibody binding to altered virus conformations in plate-trapped ELISAs.

MONOCLONAL ANTIBODY	ABSORBANCE[1]			
	DENATURANT			
	SDS+UREA		GUANIDINE	
	CPMV-SB	CPSMV-DG	CPMV-SB	CPSMV-DG
DG3	0.853	0.848	0.301	0.140
DG4	0.086	0.978	0.187	0.735
DG5	0.353	1.197	0.122	0.114
DG7	0.129	0.130	0.139	0.115
DG8	0.087	0.089	0.120	0.096
DG9	0.061	0.064	0.108	0.094
DG11	0.071	0.075	0.108	0.091

1. Backgroud = 0.085. Standard deviation = +/- 10 %

TABLE 6. Reactivity of monoclonal antibody SB2 in plate-trapped ELISAs with virus proteins disrupted with guanidine-LiCl.

PROTEIN SAMPLE (0.3 ug/ml)		ABSORBANCE[1]
CPMV-SB	- DISRUPTED VIRUS	3.000
	- LARGE SUBUNIT (VP37)	0.897
	- SMALL SUBUNIT (VP23)	0.183
CPSMV-DG	- DISRUPTED VIRUS	0.103
	- LARGE SUBUNIT	0.124
	- SMALL SUBUNIT	0.132
BUFFERED BLANK		0.132

1. Standard deviation = +/- 12 %.