IDENTIFICATION OF TWO POTYVIRUSES OF *PHASEOLUS VULGARIS* IN SOUTH AFRICA

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ABSTRACT

A survey was conducted by researchers at ARC-PPRI on dry beans (*Phaseolus vulgaris*) during 1993. All the viruses known to occur on dry beans in South Africa were found, as well as a few unidentified viruses. Of these, samples 93/1 and 93/65 form the basis of this thesis.

Electron microscopy (EM) indicated that these viruses could be potyviruses, as they were flexuous particles of approximately 700 to 800 nm. Observation of pinwheels in ultrathin sections of *Nicotiana benthamiana* infected with isolate 93/1 and *Phaseolus vulgaris* infected with isolate 93/65, confirmed that the viruses probably belonged to the Potyvirus genus, family Potyviridae. Further serological tests indicated that the viruses were related but not homologous to strains of clover yellow vein (ClYVV) and blackeye cowpea mosaic (BlCMV) viruses respectively. None of these viruses have previously been described as occurring in South Africa.

As we were unable to positively identify the viruses with serological methods, we needed to characterise these viruses on a molecular level. Potyvirus specific oligonucleotide primers were used for PCR amplification of viral cDNA. The primers amplify an approximately 700 bp fragment of the virus genome, spanning the 3' non-coding region as well as a part of the coat protein gene: one primer is complementary to the poly(A) tail, and the other to a sequence coding for a conserved block of amino acid sequences (also known as the WCIEN block) in the mid-region of the coat protein. The nucleic acid sequences of the PCR products were compared to that of other potyviruses to positively identify these isolates.

Isolate 93/1 was identified as a strain of clover yellow vein virus, while isolate 93/65 is a strain of bean common mosaic virus. Isolate 93/65 is probably a pathogroup VII strain of BCMV. This strain has not been previously reported in South Africa. As most dry bean cultivars in South Africa contain only the *I*-gene and none of the recessive genes to obtain complete resistance to BCMV (A. Liebenberg, pers. comm.), this strain could add to the incidence of the “black root” symptom observed in bean fields. Clover yellow vein virus is not seed transmitted in *P. vulgaris*, but it does cause severe yield loss as almost no seed is produced by infected plants. Both viruses could therefore have an adverse effect on the dry bean industry in South Africa under the appropriate conditions for virus spread.

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LIST OF ABBREVIATIONS

A	Adenine / adenosine
AAAA	poly(A)
AMV	Alfalfa mosaic virus
APS	Ammonium persulphate
ARC	Agricultural Research Council
ARC-PPRI	ARC Plant Protection Research Institute
ARC-OPS	ARC Oil and Protein Seed Centre
ATP	Adenosine 5'-triphosphate
AzMV	Azuki bean mosaic virus
BCMV	Bean common mosaic virus
BCMNV	Bean common mosaic necrosis virus
bp	Base pair
BLAST	Basic local alignment search tool
BlCMV	Blackeye cowpea mosaic virus
BYMV	Bean yellow mosaic virus
C	Cytosine / cytidine
CABMV	Cowpea aphid-borne mosaic virus
CABMV-SAP	Cowpea aphid-borne mosaic virus, South African passiflora strain
cDNA	Complementary DNA
CI	Cellular inclusion / cytoplasmic inclusion
CMV	Cucumber mosaic virus
CP	Coat protein
ClYVV	Clover yellow vein virus
cv	Cultivar
cvs	Cultivars
DEMV	Dendrobium mosaic virus
DIECA	Diethyldithiocarbamate (sodium salt)
DIG	Digoxigonin
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
ds	Double stranded
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscope
$F(ab')_2$	Fragment of IgG that has the antigen binding site
Fc	Fragment of IgG that crystallizes
G	Guanine / guanosine
GAR-Fc	Goat anti-rabbit Fc fragment
HC	Helper component
HC-Pro	Helper component-proteinase
HPLC	High performance liquid chromatography
HPRI	Human placental ribonuclease inhibitor
IC-RT-PCR	Immunocapture reverse transcription PCR

IgG	γ-immunoglobulin
IPTG	Isopropyl-β-D-thiogalactoside
ISEM	Immunosorbent electron microscopy
kb	Kilobase
kDa	Kilo Dalton
LB	Luria Bertani broth
M	Molar
MAb	Monoclonal antibody
ME	Mercapto ethanol
Mr	Relative molecular weight
mRNA	Messenger RNA
NCR	Non-coding region
NI	Nuclear inclusion
nm	nanometre
nt	Nucleotide(s)
OD	Optical density
oligo(dT)	Oligodeoxythymidylic acid
OMV	Ornithogolum mosaic virus
ORF	Open reading frame
P1	Protein 1
P3	Protein 3
PCR	Polymerase chain reaction
PBS	Phosphate-buffered saline
PEG	Polyethylene glycol
PeMoV	Peanut mottle virus
PMV	Pepper mottle virus
Poly(A)	Polyadenylic acid
PStV	Peanut stripe virus
PVP	Polyvinylpyrrolidone
PVY	Potato virus Y
RNA	Ribonucleic acid
RT-PCR	Reverse transcription PCR
SbMV	Soybean mosaic virus
SCMV	Sugarcane mosaic virus
SDS	sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
T	Thymine / thymidine
TAE	Tris acetic acid EDTA buffer
TBE	Tris boric acid EDTA buffer
TEMED	N,N,N',N'-tetramethyl-ethylenediamine
TEV	Tobacco etch virus
TGA	Thioglycolic acid
Tris	Tris(hydroxymethyl)aminomethane
TVMV	Tobacco vein mottling virus
ss	Single stranded
U	Uracil

u	unit(s)
UCT	University of Cape Town
UV	Ultraviolet
VPg	Virus coded protein
WMV2	Watermelon mosaic virus strain 2
w/v	weight per volume
w/w	weight per weight
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactosidase
ZYMV	Zucchini yellow mosaic virus

CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

Information on the viruses occurring in dry beans (*Phaseolus vulgaris*) in South Africa is not complete as very few surveys have been conducted. Information obtained during a survey performed by Klesser in 1961 is difficult to interpret as the virological techniques used at that time differ from those used today, and none of her isolates are still available. Information about the viruses occurring on beans is important for both plant breeders and quarantine purposes.

Of the 38 viruses known to occur naturally on beans, only five are known to occur on beans in South Africa. They are bean common mosaic virus (BCMV), bean yellow mosaic virus (BYMV), cucumber mosaic virus (CMV), peanut mottle virus (PeMoV), and alfalfa mosaic virus (AMV) (M. Koch, unpublished; Klesser, 1961; Edington and Whitlock, 1988; G. Pietersen, personal communication).

In an attempt to obtain more information about the viruses of dry beans, a survey was conducted by researchers at ARC-PPRI during 1993 in the Gauteng, North West and Mpumalanga Provinces of South Africa. All the previously described viruses were found, as well as a few hitherto unidentified viruses, of which samples 93/1 and 93/65 form the basis of this thesis. Sample 93/1 was obtained at Rietondale, Pretoria (Gauteng), while sample 93/65 was obtained at Potchefstroom (North West Province) on *Phaseolus vulgaris* cv Bonus. Sample 93/1 occurred on a seed lot that originated from a commercial farmer, while 93/65 occurred on a seed lot that formed part of the cultivar trials of the Oil and Protein Seed Centre (ARC-OPS) in Potchefstroom.

Sample 93/1 showed rugosity and a backward curling of the older leaves. The younger leaves showed mosaic and vein clearing, with the areas between the small veins standing out. The veins of the plant infected with sample 93/65 remained dark, while the rest of the leaf showed yellowing. Symptoms of plants inoculated with sample 93/65 were similar to that caused by bean common mosaic virus, but were very mild and masked.

Negative staining and cytopathology showed that both viruses probably belong to the potyvirus group, but neither virus could be positively identified using serological methods.

1.2 AIM

In the light of the fact that the two potyviruses described above were apparently unique, the aim of this project was to identify and to characterise isolates 93/1 and 93/65, and to develop detection systems for these viruses that could be used in future field surveys.

1.3 PROPOSED WORKING PLAN AND METHODS

Specific research plan:

1. Virus isolation
2. Identification of the virus family
3. Identification of the isolates:
    - 3.1 ELISA
    - 3.2 ISEM
    - 3.3 Limited host range determination
    - 3.4 Nucleic acid sequence determination
4. Development of a purification technique
5. Development of a detection technique ($F(ab')_2$ ELISA)
6. Investigation of aphid and seed transmission

CHAPTER 2

LITERATURE REVIEW

2.1 VIRUSES OCCURRING NATURALLY ON *PHASEOLUS VULGARIS*

Currently 38 viruses are known to naturally infect *Phaseolus vulgaris* (Hull, 1991). These include members of the Potyviridae, Geminiviridae, Bromoviridae, Comoviridae, and Tombusviridae families, as well as members of several other genera (Table 2.1). Of these viruses, only five are known to occur on *P. vulgaris* in South Africa. They are alfalfa mosaic virus, bean common mosaic virus, bean yellow mosaic virus, cucumber mosaic virus, and peanut mottle virus (Klesser, 1961; Edington and Whitlock, 1988; Koch, unpublished results). Although soybean mosaic virus and tomato spotted wilt virus occur in South Africa, they have not yet been found on dry beans (Pietersen, 1987, 1988; Pietersen, personal communication).

Table 2.1 Viruses known to occur naturally on *Phaseolus vulgaris*.

Virus name	Group	In South Africa
Alfalfa mosaic	alfalfa mosaic virus	yes
Cowpea chlorotic mottle	bromovirus	
Cowpea mild mottle	carlavirus	
Bean mild mosaic	carmovirus	
Soybean chlorotic mottle	caulimovirus	
Bean pod mottle	comovirus	
Bean rugose mosaic	comovirus	
Cowpea severe mosaic	comovirus	
Quail pea mosaic	comovirus	
Peanut stunt	cucumovirus	
Cucumber mosaic	cucumovirus	yes
Bean calico mosaic	geminivirus	
Bean dwarf mosaic	geminivirus	
Bean golden mosaic	geminivirus	
Beet curly top	geminivirus	
Cotton leaf crumple	geminivirus	
Horsegram yellow mosaic	geminivirus	
Jatropha mosaic	geminivirus	

Table 2.1 Viruses known to occur naturally on *Phaseolus vulgaris*.

Virus name	Group	In South Africa
Squash leaf curl	geminivirus	
Tobacco yellow dwarf	geminivirus	
Tobacco streak	ilarvirus	
Bean leaf roll	luteovirus	
Milk vetch dwarf	luteovirus	
Soybean dwarf	luteovirus	
Tobacco necrosis	necrovirus	
Tobacco black ring	nepovirus	
Tobacco ringspot	nepovirus	
Bean common mosaic	potyvirus	yes
Blackeye cowpea mosaic[1]	potyvirus	
Bean yellow mosaic	potyvirus	yes
Clover yellow vein	potyvirus	
Passionfruit woodiness	potyvirus	
Peanut mottle	potyvirus	yes
Soybean mosaic	potyvirus	yes[2]
Southern bean mosaic	sobemovirus	
Pea early browning	tobravirus	
Tomato spotted wilt	tospovirus	yes[2]

1. Now considered to be a strain of BCMV
2. Not found on dry beans in South Africa

R. Hull, 1991; Brunt *et al.* 1990; Klesser, 1961; Edington and Whitlock, 1988; Koch, unpublished results

2.2 THE POTYVIRIDAE

The Potyviridae is the largest and one of the economically most important plant virus families. The family is currently divided into three genera, *i.e.* Potyvirus, Rymovirus, and Bymovirus (Shukla *et al.*, 1994). The viruses in the genera are transmitted by aphids, eriophyid mites, and a fungus (*Polymyxa graminis*) respectively. Two more taxa, tentatively named the Ipomovirus and Macluravirus genera, are also being proposed by some researchers. The Ipomoviruses are transmitted by whiteflies, while the Macluraviruses are transmitted in a non-persistent manner by aphids (Shukla *et al.*, 1994; Brunt *et al.*, 1996).

Members of the family Potyviridae have flexuous filamentous particles of 600-900 nm in length. Potyviruses are approximately 750 nm in length; Rymoviruses, 700 nm; ipomoviruses, 900 nm; macluraviruses, 650 nm. Bymovirus particles are however 275 nm and 550 nm long.

The presence of a large number of cellular inclusions in the host cells that appear as pinwheel-shaped inclusions in transverse sections and as bundles in longitudinal sections, is a diagnostic characteristic of the Potyviridae. Some viruses also form nuclear and/or amorphous inclusions (Dougherty and Carrington, 1988; Dougherty and Hiebert, 1980b).

Potyviruses have sedimentation coefficients ($s_{20}o_w$) of 150-160S, and buoyant densities in CsCl of 1.325-1.335 gcm^{-3} at 25°C. Bymoviruses have buoyant densities of 1.29 gcm^{-3}. The extinction coefficient for the Potyviridae is 2.4-2.9 $mgml^{-1}cm^{-1}$ at 260 nm (Hollings and Brunt, 1981).

2.2.1 The Potyvirus genus

The Potyvirus genus has at least 180 definite and possible members, making it the largest plant virus genus (Ward and Shukla, 1991). These viruses are responsible for at least 40% of all plant diseases of known viral origin (Langeveld *et al.*, 1991).

2.2.1.1 Host range and symptoms

Potyviruses have a narrow, often very restricted host range. Symptoms in systemically infected leaves of dicotyledonous plants include vein clearing, mosaic, mottling, puckering, and rugosity. Leaf distortion and local or systemic necrosis may also occur. Fruits of infected plants are mottled or misshapen and the yield greatly reduced. Potyviruses also cause a number of metabolic disturbances in the host plant, such as a decrease in photosynthesis, an increase in respiration and a decrease in growth-regulating substances. The severity of disease symptoms caused by potyviruses are often temperature dependent (Hollings and Brunt, 1981).

2.2.1.2 Particle morphology

Potyviruses have flexuous filamentous particles of 11 x 750 nm with helical symmetry and a pitch of approximately 3.4 nm (Hollings and Brunt, 1981). In the sap of some plants and in the presence of divalent metallic ions, some of these viruses appear to be 850 nm long (Shukla *et al.*, 1994). Discrete membrane-associated aggregates of virus particles are frequently found in negatively stained plant sap (Hollings and Brunt, 1981).

2.2.1.3 Transmission

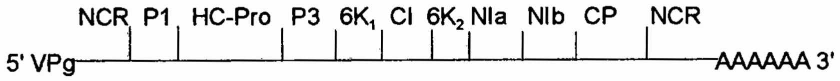
Aphids transmit members of the potyvirus genus in a non-persistent manner with differing degrees of efficiency. The virus is acquired in probing feeds of less than one minute, and no latent period before transmission of the virus is observed. Viruliferous aphids remain infective for a relatively short period - seldom longer than 4 hours in a non-feeding aphid, and usually less than one hour in a feeding aphid. A specific potyvirus can be transmitted by several different aphid species, while other aphids are unable to transmit the virus. The transmission efficiency can also vary between different aphid colonies of a specific species (Hollings and Brunt, 1981; Brunt *et al.*, 1996)

Some potyviruses are also transmitted by seed and/or pollen. Seed transmission of the virus is host plant species and cultivar specific (Brunt *et al.*, 1996).

2.2.1.4 The genome organisation

Potyviruses have a single stranded positive sense RNA genome with a molecular weight of approximately 3.0-3.5 x 10^6 (8.8 to 10 kb) (Carrington *et al.*, 1988; Domier *et al.*, 1986; Langeveld *et al.*, 1991). The RNA has a poly(A) tract of variable length at the 3' end (Allison *et al.*, 1985a; Hari *et al.*, 1979; Hari, 1981) and a VPg protein covalently linked to the 5' terminal end (Hari, 1981; Siaw *et al.*, 1985) The RNA has a non-coding region of variable length at the 5' and 3' ends. No subgenomic mRNAs are observed. The full-length RNA transcripts are observed only in the polyribosomes (Vance and Beachy, 1984b)

The genome has a single open reading frame coding for a single polyprotein. This protein is cleaved into at least eight functional proteins by proteolysis. Of these proteins, only the VPg and CP are observed in the intact virion. All the other proteins, except the $6K_1$ and $6K_2$ proteins, are observed in infected cells (Carrington *et al.*, 1988; Carrington and Dougherty, 1987b; Vance and Beachy, 1984a).



P1	first protein
HC-Pro	helper component
P3	third protein
CI	cellular inclusion protein
NIa	nuclear inclusion protein a
NIb	nuclear inclusion protein b
CP	coat protein
NCR	non-coding region
$6K_1$	6 kDa protein 1
$6K_2$	6 kDa protein 2
Vpg	genome-inked viral coded protein
AAA	poly(A) tail

Fig 2.1 Schematic representation of the potyvirus genome organisation (Adapted from Riechmann *et al.*, 1992). (Not drawn to scale.)

2.2.1.4.1 The 3' non-coding region

The 3' non-coding region of most potyviruses varies in length from 186 nt for TEV (Allison *et al.*, 1986) to 333 for PMV (Allison *et al.*, 1985b). Garlic virus 2 has however a 3' non-coding region of 604 nt (Nagakubu *et al.*, 1994). Computer analysis indicate that the region can be folded into a stable secondary structure with several hairpin loops (Turpen, 1989).

The 3' non-coding regions are AU-rich, and many potyviruses have an AUAUAUA sequence found within 60 nucleotides following the stop codon of the open reading frame. This sequence is not observed in BYMV or SMV-N (Hammond and Hammond, 1989; Eggenberger *et al.*, 1989). Inverted repeats (GAGGN/N'CCUC) are also observed (Shukla *et al.*, 1994).

An apparent polyadenylation sequence (AAUAAA) is observed in some potyviruses such as TVMV, PMV, PVY-RR and SMV-N, but it is not observed in PPV-D, TEV, SCMV-JG, BYMV. The polyadenylation signal is found significantly further upstream than is typical for a functional signal, and it is therefore unlikely that the poly(A) tract is added in response to this signal (Hammond and Hammond, 1989). Bryan *et al.* (1992) observed that 14 potyviruses and their strains contain consensus sequences in their 3' terminal regions. These sequences are 5'AU$^{U}/_{C}$GAGGN3' (Motif A) and 5'N'CCUC3' (Motif B), and are separated by 3 nucleotides. This region may form a stem-loop structure. Some viruses have a substitution in the GAGGN and N'CCUC motifs, but still form the proposed stem-loop through complementary changes in each motif, or the formation of G-U bands.

The function of the 3' non-coding region is interaction with the virus replicase during initiation of minus-strand RNA synthesis as well as prevention of degradation by exonucleases (Shukla *et al.*, 1994). The conserved stem-loop structure described before could be the recognition site for the viral replicase (Bryan *et al.* 1992).This region also effects the severity of symptom expression (Shukla *et al.*, 1994).

2.2.1.4.2 The 5' non-coding region

The length of the 5' non-coding region varies from as little as 85 nucleotides to more than 200 nucleotides. The region contains two blocks of conserved sequences. One of these blocks is known as the "potybox". In some viruses this region also contains a copy of the "potybox". Secondary structures in the 5' non-coding region may play a role in encapsidation, translation or replication of the virus (Shukla *et al.*, 1994)

2.2.1.5 Potyviral proteins

2.2.1.5.1 The coat protein

The coat protein (CP) is approximately 95%(w/w) of the total virion composition. It has a molecular weight of approximately 32 to 36 kDa and consists of approximately 2 000 copies of a single polypeptide (Gough and Shukla, 1981). Each polypeptide consists of approximately 300 amino acids.

The polypeptide is folded in such a way that the N- and C-terminal regions are located on the particle surface (Allison *et al.*, 1985b). These regions are easily removed by mild enzyme treatment with enzymes such as trypsin or lysyl endopeptidase. Treatment with trypsin will cause the removal of thirty to sixty-seven amino acids from the N-terminal and eighteen to twenty amino acids from the C-terminal regions of the CP. Lysyl endopeptidase treatment of the CP will result in the removal of the N-terminal region (Werkmeister and Shukla, 1991; Shukla *et al.*, 1988c). Removal of the termini does not affect the infectivity or the morphology of the virus. The coat protein often migrates as two bands during SDS-PAGE electrophoresis due to partial degradation by proteolytic enzymes of host or microbial origin resulting in the removal of the N- and C-termini of the CP (Moghal and Francki, 1976). The N-termini of the CP subunits of different viral strains vary in length and sequence while the C-terminal ends vary in length by only one or two residues (Shukla and Ward, 1989b).

The conserved domains of the CP may be involved in protein-protein and/or protein-RNA interactions (Dougherty *et al.*, 1985). The N-terminal portion may be involved in aphid transmission (Allison *et al.*, 1985b) and differences in this part could be associated with specific vector-HC interactions (Hammond and Hammond, 1989).

2.2.1.5.2 Other proteins

Cellular inclusion protein (CI) has ATPase (adenosine triphosphatase) and helicase activities and is associated with virus replication (Laín *et al.*, 1989, 1991)

The small nuclear inclusion protein, NIa, is a two-domain protein. The N-terminal part of the NIa protein is the VPg, and the C-terminal part is the proteinase (Shahabuddin *et al.*, 1988; Shukla *et al.*, 1994). NIa is the major proteinase of potyviruses, and is involved in the processing of the C-terminal two-thirds of the polyprotein (García *et al.*, 1989).

The large nuclear inclusion protein, NIb, is a RNA-dependent RNA polymerase (Carrington and Dougherty, 1987a). Nuclear inclusions consist of equimolar amounts of NIa and NIb. The CI, VPg and NIb proteins form a membrane-associated replication complex.

It has been suggested that the $6K_1$ and $6K_2$ proteins could also be involved in RNA replication (Shukla *et al.*, 1994).

The helper component (HC) protein is involved in aphid transmission and is also a proteinase. The N-terminal third of the polyprotein is processed by the HC proteinase and the P1 proteinase (Carrington *et al.*, 1989; Shukla *et al.*, 1994).

2.2.1.6 Nucleic acid and amino acid sequence homology between potyviruses

Nucleic acid sequence homology of the coding and non-coding regions of distinct viruses vary between 30-60%, while the homology between strains is greater than 95% (Frenkel *et al.*, 1992). The same trend is observed when looking at amino acid sequence identities between different potyviruses (Ward *et al.*, 1992).

Amino acid sequence homology of the coat protein between distinct members of the potyvirus group varies between 38-71% (average 54%) and within a strain between 90-99% (average 95%). The N-terminal region is highly variable

(Hammond and Hammond, 1989; Shukla and Ward, 1988; Ward *et al.*, 1992). The coat protein has a unique amino acid composition characteristic of the group and has very little sequence identity with similar proteins in other virus groups (Fauquet *et al.*, 1986a,b; Domier *et al.*, 1987).

The 3' non-coding region can serve as a basis for identification and classification of potyviruses. In strains of a virus this region is similar in length and sequence, but it differs greatly between distinct members of the group. Strains of a virus have more than 83% sequence identity in this region, while distinct potyviruses have only between 39 and 53% sequence identity (Frenkel *et al.*, 1989; Hu *et al.*, 1995). cDNA probes involving this region should be able to detect all members of a specific potyvirus species (Frenkel *et al.*, 1992).

P1 is the most variable protein with only 18-19% homology between distinct viruses. P3 has 30-33% homology between distinct viruses. NIb is the most conserved protein of potyviruses with an amino acid homology of 61-64% between distinct viruses, and more than 98% between strains of a virus (Shukla *et al.*, 1994).

2.2.1.7 Cytopathology

The formation of pinwheel-shaped cellular inclusions is characteristic of the potyvirus group. These inclusions are formed by the CI protein. The pinwheels consist of central tubules with attached lamellae or plates. If the lamellae are rolled, the pinwheels appear as scrolls. Stacked lamellae cause the inclusions to appear as laminated aggregates (Christie and Edwardson, 1977).

Potyviruses can be divided into three groups according to the type of inclusions they produce: group 1 produces pinwheels and scrolls; group 2, pinwheels and laminated aggregates; group 3, pinwheels, scrolls and laminated aggregates (Christie and Edwardson, 1977).

Inclusions have been detected in the light microscope as early as 8 hours after infection. The CI first appear at the cell periphery where they increase in size and number. As infection progresses, the number of peripheral inclusions decreases while large numbers of CI appear in the central regions of the cell. (Christie and Edwardson, 1977).

Virus particles are sometimes observed distributed randomly in the cytoplasm of infected cells or as bunch-like aggregates, but are not as easily observed as the cellular inclusions. The virus particles are more commonly found as monolayers closely associated with the membranes of mitochondria, the tonoplast or cytoplasmic lamellae, or in cytoplasmic strands projecting into or bridging the cell vacuole (Hollings and Brunt, 1981; Lesemann, 1988).

Potyviruses also induce the formation of nuclear and amorphous inclusions. Nuclear inclusions consist of equimolar amounts of NIa and NIb proteins. These inclusions can be crystalline, globular, fibrous or fimbriate. The formation of these inclusions can vary even between strains of a virus (Shukla *et al.*, 1994).

2.2.2 The BCMV subgroup

A BCMV subgroup of the genus Potyvirus has been proposed (Dijkstra and Khan, 1992). This subgroup consists of a number of closely related yet distinct potyviruses, such as BCMV, BCMNV, SMV, PWV, ZYMV, and CABMV, and their strains. These viruses have close serological and biological characteristics, and it is often difficult to unambiguously identify individual viruses (Mink *et al.*, 1994; Mink and Silbernagel, 1992)

2.2.2.1 Bean common mosaic virus (BCMV)

Symptoms caused by BCMV include systemic mosaic, systemic necrosis (black root), and leaf malformation. BCMV belongs to subdivision I of the potyvirus group according to the type of CI formed as it produces pinwheels and scrolls (McKern *et al.*, 1992). BCMV is of economic importance as it can cause severe crop loss. The percentage crop loss caused by BCMV varies as it is determined by the virus strain as well as the host genotype. Crop losses between 35% and 98% have been documented for *P. vulgaris.* A moderate outbreak of BCMV can cause a 53% crop loss (Van Rheenen and Muigai, 1984).

BCMV is transmitted in a non-persistent manner by various aphids such as *Aphis fabae*, *A. gossypii*, *A. craccivora*, *Myzus persicae* and *Acyrthosiphon pisum*. Most of these aphids do not colonise *P. vulgaris* but transmit the virus efficiently as winged migrants. The virus can be acquired in probes of 15-60 seconds and transmitted within one minute.

BCMV can also be transmitted by seed and pollen. Depending on the virus strain and host genotype, up to 83% of seed produced by infected *P. vulgaris* plants may give rise to infected seedlings (Brunt *et al.*, 1996).

The natural host range of BCMV include leguminous weeds and wild legumes, *Macroptilium lathyroides*, *Phaseolus acutifolius*, *P. coccineus*, *Vigna radiata* etc. BCMV infects 100 spp of 44 genera of nine plant families (McKern *et al.*, 1992). The virus spreads wherever beans are grown.

BCMV strains are divided according to serological, biochemical and biological data into two serogroups, *i.e.* serogroup A and B. These serogroups are now considered to be two distinct potyviruses and are called bean common mosaic necrosis virus (formerly serogroup A) and bean common mosaic virus (formerly serogroup B) (Brunt *et al.*, 1996; McKern *et al.*, 1992a, b; Vetten *et al.*, 1992). BCMNV (bean common mosaic necrosis virus) induces temperature-insensitive necrosis in bean cultivars that carry the I gene (McKern *et al.*, 1992a), and has a particle length of 810-818 nm. BCMV induces temperature dependent necrosis in plants with the I-gene, and has a particle length of 847-886 nm (Vetten *et al.*, 1992). BCMV and BCMNV can also be separated on the basis of their nucleic acid and amino acid sequence data. When drawing a phylogenetic tree based on the amino acid sequence data of the coat proteins of potyviruses, the BCMNV strains form a separate group within the bean common mosaic subgroup (Mink *et al.* , 1994; Collmer *et al.*, 1996). The two viruses have sequence identity of 56 to 63% in their 3' non-coding regions, and 87 to 89% identity in their coat proteins (Khan *et al.*, 1993).

BCMV and BCMNV can be further classified into pathogroups according to the reactions on a range of differential *P. vulgaris* cultivars (Drijfhout *et al.*, 1978; Spence and Walkey, 1995). The division of the strains into pathogroups can be seen in Table 2.2. Isolates of BICMV, PStV (peanut stripe virus), AzMV (azuki bean mosaic virus) and three isolates of soybean mosaic virus are now recognised as strains of BCMV (McKern *et al.*, 1992).

In South Africa, BCMV has been found in all the main bean growing areas in the highveld areas of Gauteng and Mpumalanga, the North West Province, Kwazulu-Natal, and the Free State Province. It is regarded as the economically most important viral disease on dry beans in the country. In 1986 the Dry Bean Board decided that the I-gene should be bred into all susceptible cultivars within a five year period, or be removed from the cultivar list. This has however not been realised and at least ten highly susceptible cultivars are still being planted commercially. Seventeen local isolates of BCMV have been tested to establish to which one of the pathogenicity groups they belong. All isolates except one, which conformed with group VI, conformed with pathogenicity group V (Drijfhout *et al.*, 1978; Edington and Whitlock, 1988).

Table 2.2 Division of BCMV and BCMNV strains.[1]

Virus	Pathogroup	Strain code
BCMNV	III	NL-8
	VI	NL-3, NL-5, TN-1
BCMV	I	US-1, NL-1, PR-1
	II	NL-7, US-7
	IV	NL-6, US-3, US-4, US-5, CH-2, FLA, NVRS
	V	NL-2, US-2 (Z, P, D), NY-15
	VII	NL-4, US-6, US-10, RU-1
	PStV	Stripe
	BICMV	Type
	AzMV	
	SMV	PM, PN, 74

[1] Data obtained from McKern *et al.*, 1992a, and Spence and Walkey, 1995.

2.2.2.2 Blackeye cowpea mosaic virus (BlCMV)

BlCMV has similar biological and physical properties to CABMV (cowpea aphid borne mosaic virus) and BCMV. BlCMV and CABMV produce similar symptoms in their plant host range and have a close serological relationship, causing many isolates to be misidentified. BlCMV and CABMV viruses can be distinguished by symptoms on certain cultivars of *V. unguiculata* and *P. vulgaris*. The viruses can also be distinguished using monoclonal antibodies specific to strains of the viruses (Huguenot *et al.*, 1993).

BlCMV is transmitted by *Aphis craccivora* and *Myzus persicae*. The rate of transmission by *A. craccivora* is 59% (Purcifull and Gonsalves, 1985).

BlCMV is assigned to subdivision I by Edwardson as it produces pinwheels and scrolls. Nuclear inclusions have been reported for some isolates (Purcifull and Gonsalves, 1985).

High performance liquid chromatography (HPLC) peptide profiles indicate a profile similarity with strains of BCMV which is within the range observed among strains of a potyvirus. The only substantial differences observed occurred within the N-terminal peptides (McKern *et al.*, 1992). Although BlCMV was originally described as a distinct virus, it is therefore now considered to be a strain of BCMV. In the past, BlCMV was mainly considered a distinct virus because of differences in their pathogenicity to beans and cowpeas (Khan *et al.*, 1993).

BlCMV is closest in pathogenicity to pathogroup I BCMV viruses. In *Vigna*, symptoms of BlCMV-W has a closer resemblance to that of BCMV strain NY15 (pathogroup Va). Clear differences exist in pathogenicity of BCMV and BlCMV to cowpea and bean (Lana *et al.*, 1988).

There is no difference in CP size between BCMV and BlCMV (Lana *et al.*, 1988). These viruses have 3' non-coding regions of similar length (253-256 nt), with 93-96% nucleic acid sequence similarity. In their coat proteins they have similarity of 94-97%, indicating that BlCMV is a strain of BCMV (Khan *et al.*, 1993).

2.2.3 The BYMV subgroup

The BYMV subgroup of the Potyvirus genus includes bean yellow mosaic virus (BYMV), clover yellow vein virus (ClYVV), pea mosaic virus (PMV), white lupin mosaic virus (WLMV), and their strains. These viruses have many properties in common, and it is difficult to unambiguously identify strains by host range and serological tests ((Fortass *et al.*, 1991; Hewish *et al.*, 1993). Besides the close serological relationship and overlapping host ranges, they also have high sequence identity, and group together when a phylogenetic tree of potyviruses is drawn. Nucleic acid sequence data of the coat protein and 3' non-coding region can be used to distinguish between these viruses (Fortass *et al.*, 1991; Tracy *et al.*, 1992)

2.2.3.1 Clover yellow vein virus (ClYVV)

ClYVV was first isolated from clover (*Trifolium repens*) in 1965. Natural hosts include bean, broad bean, lupins, peas, sweatpeas, squash, coriander, and clover. The virus occurs in Australia, Canada, Japan, Europe, the UK, the USA, and New Zealand. Symptoms caused by ClYVV in infected plants include mosaic, vein clearing, and chlorosis.

ClYVV is transmitted by *Myzus persicae*, *Acyrthosiphon pisum*, *A. solani*, *Macrosiphum euphorbiae*, and *Aphis craccivora*, but not by *Aphis fabae* (Shukla *et al.*, 1994; Brunt *et al.*, 1996). The virus is not seed-transmitted in *P. vulgaris* cv The Prince (Hollings and Stone, 1974).

ClYVV belongs to subdivision 2 of the potyvirus group according to the divisions of Christie and Edwardson (1977) as it produces pinwheels and laminated aggregates in infected plants. It also causes crystalline cytoplasmic inclusions, needle-like crystalline nuclear inclusions as well as nuclear enlargements. Abnormal enlargement of structurally normal mitochondria also occur.

Strains of ClYVV have a close serological relationship, and can be differentiated by monoclonal antibodies and peptide profiling of the coat protein. Strains of the virus have between 88-93% amino acid sequence identity in the coat protein and between 92-98% nucleotide sequence identity in the 3' non-coding region of strains. Short conserved regions of nearly 100% similarity exist between BYMV and ClYVV coat protein amino acid sequences. The 3' non-coding region shows 71-77% homology with BYMV and virtually none with other potyviruses. In ClYVV this region is 177-178 nucleotides long (Uyeda, 1992).

The coat protein of ClYVV consists of 271-273 amino acid residues, which corresponds to a molecular weight of approximately 31 kDa (Shukla *et al.*, 1994).

2.3 IDENTIFICATION OF POTYVIRUSES

Various techniques such as cytopathology, serology, cross-protection, vector transmission, symptomatology, host range, high performance liquid chromatography (HPLC) peptide profiling, nucleic acid hybridisation, polymerase chain reaction (PCR), and nucleic acid sequence comparisons can be used to identify a potyvirus. Many of these techniques have limitations and should be used in conjunction with other techniques. Some techniques such as cytopathology and vector transmission studies give an indication of the group to which the virus belongs, but cannot be used for identification of a specific virus (Shukla *et al.*, 1994).

2.3.1 Host range and symptomatology

Host range and symptomatology are the easiest and cheapest techniques available. The majority of potyviruses do not induce unique symptoms in their hosts, and have overlapping host ranges. Repeated mechanical inoculation in some hosts may lead to changes in host range and symptomatology. In spite of these problems, these techniques still play a significant role in the identification of strains and pathotypes of a given virus. In many cases the reaction of differential cultivars is the only way to distinguish between strains and pathotypes of a virus (Shukla *et al.*, 1994).

2.3.2 Serology

Serology is one of the most efficient methods for potyvirus identification and is widely used. Low quantities of virus can be detected with techniques such as ELISA or ISEM. ELISA is also useful for the screening of a large number of samples. Some ELISA techniques such as $F(ab')_2$ ELISA with polyclonal antisera can be used to detect several strains of a virus. ISEM is often useful for differentiation between strains (Shukla *et al.*, 1994; Derrick, 1975).

Antisera directed against the CP are the easiest to prepare and very convenient to use as the viral particles consist of 95% CP. Virus-specific epitopes are located in the N-terminus while group-specific epitopes are located in the core of the CP. The N-terminus is the most immunodominant part of the CP. (Shukla *et al.*, 1988). An antiserum directed against intact virus particles will be able to detect closely related strains of the specific virus as it is directed against the virus-specific epitopes. An antiserum directed against the core protein is directed against the group-specific epitopes, and should be able to detect distantly related viruses. During purification of potyviruses, the N- and C-termini are often degraded, causing cross-reactivity between different viruses. This can lead to inaccurate identifications (Shukla *et al.*, 1992; 1994).

Cross-reactivity of potyviral antisera is often variable, some strains do not show cross-reactions, and the paired relationships between potyviruses are often inconsistent (Shukla *et al.*, 1994). These factors can often lead to incorrect identification if based on serology alone.

2.3.3 Nucleic acid hybridization

Nucleic acid hybridization has little value as a group-specific technology. Successful cDNA hybridization has not been reported between distinct potyviruses, but only between strains of some potyviruses. Very few sequence matches of 15 or more consecutive residues are present in the potyvirus group (Ward and Shukla, 1991). Hybridisation of the 3' non-coding region of the potyviral genome has been used to successfully discriminate between different potyviruses and their strains (Frenkel *et al.*, 1992).

2.3.4 Polymerase chain reaction (PCR)

Because of the sensitivity and specificity of PCR, this technique is being used more often in the identification of viruses. As potyviruses have a single stranded RNA genome, reverse transcription PCR (RT-PCR) is used for detection and identification of viruses. Group-specific degenerate primers which should be able to amplify all potyviruses, have been developed (Langeveld *et al.*, 1991; Pappu *et al.*, 1993). Primers able to distinguish closely related potyviruses, *e.g.* BYMV and ClYVV, or BCMV and BCMNV, have also been developed (Bariana *et al.*, 1994; Saiz *et al.*, 1994a).

Immunocapture RT-PCR (IC-RT-PCR) is a combination of serology and RT-PCR enabling detection of even lower quantities of virus in infected material. In IC-RT-PCR, virus is trapped with antiserum in a PCR tube. The virus particles are denatured by the addition of Triton X-100 to the RT-PCR mix. The advantages of IC-PCR is that no nucleic acid extractions are necessary, the virus is concentrated, all inhibiting substances in the plant material removed, and all the steps can be performed in one tube (Roussell, pers comm., Wetzel *et al.*,1992).

2.3.5 Sequence comparisons

Amino acid and nucleic acid sequence determinations and comparisons are the most accurate ways of identifying a virus (Rybicki and Shukla, 1992; Ward *et al.*, 1992). When the entire genomes of potyviruses are compared, the average identity between distinct viruses is 54%, while that between strains is approximately 97% (Frenkel *et al.*, 1992). The 3' non-coding region has little homology when distinct viruses are compared, but is more conserved within a strain of a virus. If the amino acid sequences of the coat proteins of different potyviruses are compared, the identity between distinct viruses is 38-71%, while it is 90-99% for strains of a virus. It has also been observed that the same type of distribution is obtained regardless of which of the potyvirus proteins are being compared (Shukla *et al.*, 1994). As not all the different viruses have been sequenced, sequence comparisons cannot be used in isolation, but must be combined with serology and host range studies.

2.3.6 High performance liquid chromatography (HPLC) peptide profiling

HPLC peptide profiling of the coat protein of potyviruses is also a reliable procedure for the identification of potyviruses. It is a measure of the amino acid sequence relationship between potyviruses, and similar variation is observed when this technique is compared to sequence comparisons. HPLC peptide profiles of strains of a virus have more than 90% identity, while distinct viruses have identities of 40-75% (Shukla *et al.*, 1994).

CHAPTER 3

SEROLOGY

3.1 INTRODUCTION

Some of the most important and widely used methods for assay, detection, and diagnosis of viruses depend on the surface properties of viral proteins, especially the coat protein. Serological methods are rapid, convenient, sensitive and specific. Viruses that are not sap transmissible can also be detected by serological methods. The results are directly proportional to the virus concentration, and these methods can therefore be used to determine the level of infection. Antisera can be stored and comparable tests can be made over a number of years and in different laboratories (Matthews, 1991). It is also relatively easy to prepare antisera in large quantities.

Several serological methods can be used to detect virus particles: precipitation methods, radioimmune assay, DAS ELISA, indirect ELISA, F(ab')$_2$ ELISA, dot ELISA, ISEM, *etc.* For potyvirus detection, we have found the DAS or F(ab')$_2$ ELISA systems the most convenient and consistent to use. The different conformation of the viral particles in the indirect ELISA system often lead to false negative results. Precipitation tests also have varied results depending on the virus tested. ELISA has the added advantages that it can be used for mass screening of plants, and that it is sensitive enough to detect virus particles in seed (Hollings and Brunt, 1981).

Both polyclonal and monoclonal antibodies can be used in the serological tests. If care is not taken in the preparation of polyclonal antisera against potyviruses, cross reaction between distantly related viruses can occur due to the loss of the virus-specific epitopes during purification and storage of virus particles. Monoclonal antibodies are very specific and can be used to distinguish between strains of a virus (Huguenot *et al.*, 1993). Monoclonal antibodies able to detect most of the members of the potyvirus group, have also been developed (Jordan and Hammond, 1991).

3.2 MATERIALS AND METHODS

3.2.1 Origin of antisera

The details of antisera used in serological studies are contained in Table 3.1. All antisera were stored in 50% glycerol at 4°C. IgG and $F(ab')_2$ were also stored at 4°C in the presence of sodium azide.

Table 3.1 Origin and type of antisera used in serological studies.

Virus isolate or strain	Type	Origin
BCMV isolate 92/750	PAb[1]	PPRI
BYMV isolate 92/751	PAb	PPRI
SMV isolate 86/20	PAb	PPRI
PeMoV isolate 88/63	PAb	PPRI
CABMV-SAP isolate 92/749	PAb	PPRI
CABMV isolate 87/14	PAb	PPRI
CABMV serotypes C, D, E, F, and G	MAb[2]	C. Huguenot[3]
CABMV serotype E	MAb	C. Huguenot
CABMV serotype C	MAb	C. Huguenot
Potyvirus common epitope	MAb	R. Jordan[4]
ClYVV strain Pratt	PAb	Barnett, USA
BlCMV strain NR	PAb	
BlCMV Georgia strain	PAb	
BlCMV serotype A	MAb	C. Huguenot
BlCMV serotypes A and B	MAb	C. Huguenot

1 Polyclonal antibody (PAb)
2 Monoclonal antibody (MAb)
3 C. Huguenot, Agriculture Canada Research Station, Vancouver, Canada
4 R. Jordan, USDA-ARS, Beltsville

3.2.2 ELISA

The original field material was tested in a $F(ab')_2$ ELISA system for the presence of BCMV, BYMV, PeMoV, SMV, and CABMV-SAP. Infected plant material, freeze-dried and stored at -20°C, was used as positive controls. Healthy *P. vulgaris* cv Bonus maintained in a glasshouse was used as negative control. IgG and $F(ab')_2$ were incubated for four hours at 37°C, while the plant material and goat anti-rabbit-Fc (GAR-Fc) alkaline phosphatase conjugate were incubated overnight at 4°C. p-Nitrophenyl phosphate (Sigma) was used as substrate and the reaction measured at 405 nm with a Titertek Multiscan after a 30 minute incubation at 30°C.

The $F(ab')_2$ ELISA procedure as well as preparation of the IgG, $F(ab')_2$, and conjugate, are described in Appendix A.

The isolates were tested against the potyvirus common epitope monoclonal antibody (Jordan and Hammond, 1991) in an indirect ELISA (see Appendix A).

3.2.3 ISEM

The viruses were initially tested in ISEM with antisera against ClYVV-Pratt, and BlCMV strains NR and Georgia as described in Appendix A. No positive controls were available, but potyvirus isolate 93/632 and its homologous antiserum were included as a control for the ISEM. Isolate 93/65 was subsequently tested with monoclonal antibodies specific for BlCMV serotypes A and B, and CABMV serotypes C, D, E, F, G, as well as the homologous polyclonal antiserum. A positive control for BlCMV was unavailable. CABMV-SAP was used as positive control for the Mab specific for CABMV serotypes C, D, E, F, G.

3.3 RESULTS AND DISCUSSION

3.3.1 ELISA

Both viruses tested negative in F(ab')$_2$ ELISA for SMV 86/20, PeMoV 88/63, BCMV 92/750, BYMV 92/751, CABMV-SAP 92/749, and CABMV 87/14. It was subsequently found that when testing for BCMV, plant material must be macerated in extraction buffer without PVP. When isolate 93/65 was treated in this way, it showed a slight reaction to BCMV 92/750.

In ELISA with potyvirus common epitope antiserum, both viruses tested positive, confirming that they are members of the potyvirus group (results not shown).

3.3.2 ISEM

Isolate 93/1 reacted only with antibodies to ClYVV-Pratt. The reaction was however not very strong. Isolate 93/65 showed a slight reaction when using antiserum to BlCMV strains NR and Georgia, and a stronger reaction when tested with the monoclonal antibody against BlCMV serotypes A and B. Isolate 93/65 tested negative with CABMV monoclonal and polyclonal antibodies (Fig 3.1). Isolate 93/65 is therefore a member of the BCMV group of viruses, and isolate 93/1 probably a strain of ClYVV.

Fig. 3.1. ISEM of isolate 93/65 trapped with homologous antiserum and decorated with (from left to right) homologous antiserum, a Mab against BlCMV serotypes A and B, and a Mab against CABMV. No decoration is observed when using a Mab directed to CABMV. Decoration with the homologous antiserum show a strong reaction, and with a Mab to BlCMV serotypes A and B a slightly weaker reaction. Bar represents 122 nm.

3.4 CONCLUSION

Both viruses were tested with antisera against all the potyviruses known to occur naturally on beans. Isolate 93/1 showed a reaction only with antibodies to ClYVV-Pratt. As the observed decoration was not as strong as that expected for a homologous reaction, isolate 93/1 is probably a different strain of ClYVV. Isolate 93/65 reacted slightly with antiserum to BCMV 92/750, BlCMV strains NR and Georgia, and a Mab to BlCMV serotypes A and B. As the reactions were weak, isolate 93/65 is probably a different strain of BCMV than those tested for in ISEM. As serological data is not enough to confirm whether a virus is a new strain or not, the nucleic or amino acid sequences of part of the virus will have to be determined and compared to that of related viruses.

CHAPTER 4

BIOLOGICAL PROPERTIES

4.1 INTRODUCTION

Symptoms of diseased plants in the field are usually inadequate on their own to give a positive identification of the virus involved. Some viruses, such as cucumber mosaic virus (CMV), are variable in the symptoms they cause. In some crops, a single symptom can be caused by one of several viruses. Inoculation of infected material onto a specific indicator host range, using plants that will give clear, characteristic and consistent symptoms, is one of the basic tools for routine diagnosis. It is important to use the same line of a named variety in order to compare results obtained in different laboratories. Factors such as temperature and light intensity often play a role in symptom expression, and it is therefore important to combine indicator host studies with other identification methods (Matthews, 1991).

Cytological effects induced by a virus can often be used to assist in diagnosis. Plant viruses can often be allocated to a group or family according to the type of inclusions they produce in infected plants (Christie and Edwardson, 1977). Members of the *Potyviridae* always induce the formation of pinwheels in infected cells. The *Potyviridae* are subdivided into three groups according to the type of cellular inclusions observed in infected plants. Group 1 viruses induce the formation of pinwheels and scrolls; group 2, pinwheels and laminated aggregates; group 3, pinwheels, scrolls, and laminated aggregates. Although knowledge of the inclusions formed cannot be used to identify a specific virus, a combination of host range and inclusion data can give a fair indication of the identity of the virus.

Knowledge of the size, shape, and surface features of the virus particle is a basic requirement for virus identification. These results are best obtained using a negative-staining procedure. Three different stains, sodium phosphotungstate, uranyl acetate or ammonium molybdate, are used as the stability of viruses in each of the stains vary (Matthews, 1991).

Coat protein size determination can also be used to confirm the identity of the virus group, although it cannot be used to specifically identify a virus. Potyvirus coat proteins can vary in size between 28 and 40 kDa, with most viruses having a CP in the 33-34 kDa range (Shukla *et al.*, 1994).

Aphid transmission (species, persistency, etc) can also give an indication of the virus group, as the method of transmission is specific for a virus group. Potyviruses for instance are transmitted by a variety of aphids in a non-persistent manner, while geminiviruses are not transmitted by aphids. Aphid transmission can also, in combination with other techniques, give an idea of the identity of the virus, as transmission is virus- and aphid-specific.

Knowledge of seed and aphid transmission is important for the development of control strategies.

Before any of the biological properties of a virus can be determined, the virus must be isolated to ensure that the characteristics of a homologous population is determined. Three methods are generally used to isolate viruses, *i.e.* two consecutive local lesion transfers, two consecutive limiting dilution transfers or two consecutive transmissions using single aphids. Local lesion transfers are usually preferred, as it is the fastest and most convenient method.

4.2 METHODS AND MATERIALS

4.2.1 Virus source and maintenance

Sample 93/1 was obtained from *Phaseolus vulgaris* cv Bonus grown in field trials at Rietondale Research station, ARC-PPRI, Pretoria. The seed used in these trials originated from a commercial dry bean farmer in the Komatiepoort area. The virus was maintained in *Nicotiana benthamiana* grown at 20-30°C in a glasshouse.

Sample 93/65 was obtained from *P. vulgaris* cv Bonus grown as part of the cultivar trials at ARC-OPS, Potchefstroom. The virus was maintained in *P. vulgaris* cv Bonus grown at 20-30°C in a glasshouse.

Original samples and isolates were stored at -80°C, desiccated over calcium chloride and stored at 4°C, and freeze-dried and stored at 4°C (Appendix A).

4.2.2 Host range studies

Both samples were inoculated mechanically onto a limited host range. Field-collected plant material was macerated in a ratio of 1:10 w/v in 0.01 M sodium phosphate pH 7.1 with a mortar and pestle. Celite was added to the extract before rubbing the mixture onto the leaves of one to two week old healthy seedlings of *Phaseolus vulgaris* cv Bonus, *P. vulgaris* cv Bountiful, *Glycine max* cv B66S10, *Vigna unguiculata*, *Nicotiana benthamiana*, *N. tabacum* cv Samsun, *N. glutinosa*, *Chenopodium quinoa*, and *C. amaranticolor*. The seedlings were grown from virus-free seed propagated at ARC-PPRI, Rietondale. Inoculated plants were grown in the glasshouse at 20-30°C and symptom development recorded for eight weeks before the plants were discarded.

Isolate 93/65 was also inoculated onto differential *P. vulgaris* cultivars. The symptoms were recorded for eight weeks and all plants tested in $F(ab')_2$ ELISA with the homologous antisera for the presence of virus.

One plant of each cultivar was inoculated with the 0.01M sodium phosphate buffer pH 7.1 containing celite to serve as a negative control.

4.2.3 Negative staining

Virus-infected plant material was macerated in 0.01 M sodium phosphate pH 7.1 buffer. Negative staining of plant extracts was with 2% uranyl acetate pH 4.3, 2% ammonium molybdate pH 5.3, and 2% potassium phosphotungstate pH 6 or 7. The staining methods are described in Appendix A. Virus preparations were examined in a ABT 002A electron microscope.

4.2.4 Cytopathology

Isolate 93/1 was inoculated onto *N. benthamiana*, while isolate 93/65 was inoculated onto *P. vulgaris* cvs Bonus, Redlands Greenleaf C, and Redlands Greenleaf B. Ultra thin sections of infected plant material was prepared three weeks after inoculation as described in Appendix A. The sections were examined in a JEOL 100C transmission electron microscope for the presence of cellular inclusions and other abnormalities. A healthy plant of each cultivar was treated in the same way to serve as a negative control.

4.2.5 Aphid maintenance and transmission studies

Virus-free aphids were maintained in gauze-covered wooden cages under Gro-Lux lamps. Transmission with *Aphis craccivora*, *Aphis gossypii*, and *Myzus persicae* was studied. The aphids were allowed to feed for 1 to 2 minutes on infected plant material after starving them for at least one hour. Ten aphids were transferred to each healthy plant and a transmission feed of 16 to 20 hours was allowed. The aphids were killed by spraying them with an insecticide. The plants were grown in the glasshouse for eight weeks and symptom development observed.

4.2.6 Virus isolation

Viruses are normally isolated by two successive local lesion transfers. As no local lesion host was found for either sample, other methods had to be used. The limiting dilution transmission method to isolate a virus was attempted with sample 93/1 (Noordam, 1973). A dilution series of infected *N. benthamiana* material was made in inoculation buffer, and twenty plants were inoculated with each dilution. The transmission was considered to be successful if no more than two plants showed symptoms.

An attempt was made to isolate both viruses by two successive single aphid transmissions. *M. persicae* individuals were allowed to starve for two to three hours before an acquisition feeding time of one to two minutes. A single aphid was transferred to each healthy plant and a two-hour transmission feeding time was allowed. After killing the aphids, the plants were incubated in the glasshouse for eight weeks and symptom development observed.

4.2.7 Coat protein size determination

The CP size of isolates 93/1 and 93/65 was determined by SDS-PAGE. Virus was purified according to the methods described in Chapter 5. The purity of the preparations was verified with the EM, and by determining the A_{260nm}:A_{280nm} and A_{260nm}:A_{245nm} ratios (see Chapter 5). Purified virus was mixed with an equal volume of sample buffer. 10-15 µg of the virus preparate was loaded onto four gels. A low molecular weight protein standard (BioRad) was used as a marker to calculate the CP size. The gels were subjected to electrophoresis for 20 hours at 60 V, stained with Coomassie Brilliant Blue and destained in a 45% (v/v) methanol/ 10% (v/v) acetic acid mixture (See Appendix A). The coat protein sizes were calculated using the Fragment Sizer part of the Seqaid II programme (Rhoads and Roufa, 1989).

4.2.8 Seed transmission

Five *P. vulgaris* cv Bonus seedlings were inoculated with isolate 93/65 and grown in the glasshouse until seed was produced. One hundred and thirty-five of the resulting seeds were planted in the glasshouse and grown for four weeks. The seedlings were examined for systemic symptoms and tested with $F(ab')_2$ ELISA (Appendix A) for the presence of virus. Seedlings with absorbance values close to the cut-off value of the ELISA were tested with ISEM (Appendix A) to confirm the presence of virus.

4.3 RESULTS AND DISCUSSION

4.3.1 Host range

Sample 93/1 appears to have a very limited host range (Table 4.1). No local lesion assay host was found that could be used for isolation of the virus. The reactions of the cultivars tested were similar to that expected for ClYVV (Brunt *et al.*, 1996; Hollings and Stone, 1974).

Isolate 93/1 caused severe systemic necrosis and plant death if two-week old beans were inoculated. When four week old seedlings were inoculated, isolate 93/1 induced tip necrosis, but plants were able to recover. These plants showed systemic mosaic and leaf dwarfing similar to that of the original plant. Symptoms on all plants showing systemic symptoms, persisted (Fig 4.1).

Table 4.1 Reactions of various cultivars to Isolate 93/1 compared to that reported for CIYVV

Cultivar	93/0001	CIYVV
P. vulgaris cvs Bonus and Bountiful	severe necrosis, vein clearing, leaf curl	chlorotic local lesions, systemic chlorotic spots, mottle [1]
Glycine max cv B66S10	negative or small necrotic lesions	mosaic or symptomless [2]
Vigna unguiculata	negative	negative
Nicotiana glutinosa	mosaic or negative	negative
N. tabacum cv Samsun	negative	chlorotic local lesions
N. benthamiana	mosaic, leaf curl	not tested
Chenopodium quinoa	local and systemic necrotic lesions	local lesions, systemic wilt, yellowing
C. amaranticolor	chlorotic lesions, systemic necrosis	necrotic local lesions vein banding

1 *P. vulgaris* cv Bonus not tested for CIYVV

2 *G. max* cv B66S10 not tested for CIYVV

Fig. 4.1 Systemic symptoms induced by isolate 93/1 on *P. vulgaris* cv Bonus. Top necrosis is induced when the primary leaves of seedlings are inoculated (plant on left). Some of these plants recover and show mosaic symptoms (plant on right).

Sample 93/65 was inoculated onto various indicator plants to determine the host range and to obtain a local lesion host for isolation of the virus. The host range of the virus is extremely narrow, with only *P. vulgaris* cultivars showing symptoms. No local lesion assay host was found. Symptoms on beans were similar to that of the original plant, but could only be seen clearly if the light intensity in the glasshouse was fairly low (Fig. 4.2). Symptoms were mild and masked, and may disappear at high light intensity or at temperatures above 30°C. Results of the host range study as well as reported reactions of BlCMV and BCMV to the various cultivars, are recorded in Table 4.2. Isolate 93/65 had a narrower host range than expected, and no identification of the virus using this data could be made. Differences of the reaction of the host plants compared to that reported for similar viruses could also be due to differences in the plant cultivars used in the tests.

Fig. 4.2 Systemic symptoms induced by isolate 93/65 on various *P. vulgaris* cultivars. From left to right, healthy leaf; *P. vulgaris* cv Bonus; *P. vulgaris* cv Redlands Greenleaf B; *P. vulgaris* cv Redlands Greenleaf C.

Isolate 93/65 was also inoculated onto differential *P. vulgaris* cultivars to determine the BCMV pathogroup to which it belongs. Plants of cultivar groups 1,2,3, and 6 were infected, indicating that isolate 93/65 could belong to pathogroup VII (see Table 4.3) (Spence and Walkey, 1995). Cultivars in group 4 had atypical infections as only two of the five plants tested for each cultivar, showed symptoms. All *P. vulgaris* cv Sanilac plants tested positive in ELISA, while cv Red Mexican UI 34 had only two positive plants. If group 4 is considered positive, the grouping is still closest to that of group VII. No type strains were available for a comparative study. This is the first report of a BCMV pathogenicity group VII-type virus found in South Africa. This result indicates that isolate 93/65 is probably not a BlCMV strain of BCMV, as BlCMV falls in pathogenicity group I (Lana *et al.*, 1988).

Table 4.2 Reaction of various host plants to isolate 93/65 in comparison to that reported for BICMV and BCMV

Cultivar	93/0065	BICMV	BCMV
Phaseolus vulgaris cv Bonus	Chlorotic to necrotic local lesions, vein banding (mosaic), slight leaf curl	mosaic, mottle, streak, distortion [1]	mosaic, leaf roll, leaf malformation
Phaseolus vulgaris cv Bountiful	Chlorotic to necrotic local lesions, vein banding (mosaic), slight leaf curl	chlorotic local lesions	yellow dots, vascular necrosis, growth reduction
N. benthamiana	negative	mosaic	positive
G. max cv B66S10	negative	mosaic, mottle streak, distortion [2]	necrotic or chlorotic local lesions [2]
C. quinoa	negative or local rings	necrotic local lesions	chlorotic local lesions
V. unguiculata	negative	stunting, mosaic mottle, streak	positive or negative, symptomless local infection
N. glutinosa	negative	negative	negative or chlorotic local lesions
N. tabacum cv Samsun	negative	negative	not tested
N. tabacum cv Rustica	negative	negative	chlorotic local lesions

1 Symptoms not described for *P. vulgaris* cv Bonus, but for other cultivars

2 Symptoms not described on *G. max* cv B66S10

Table 4.3 Reaction of differential cultivars reported for the different BCMV pathogenicity groups compared to that of isolate 93/65.

BCMV differential cultivars		BCMV pathogenicity group										Isolate 93/65	
Group	Cultivar	I B	II B	III A	IV B	IV B	Va B	Vb B	VIa A	Vi A	VII B	Symptoms	ELISA
1	Dubbele Witte	+	+	+	+	+	+	+	+	+	+	+	+
	Stringless Green Refuge	+	+	+	+	+	+	+	+	+	+	+	+
	Bountiful	+	+	+	+	+	+	+	+	+	+	+	+
	Sutter Pink	+	+	+	+	+	+	+	+	+	+	+	+
2	Red Mexican UI 36	-	+t	-	+	+	+t	+	t	+	+t	+	+
	Puregold wax	-	+t	-	+	+	+t	+	t	+	+t	+	+
	Redlands Greenleaf C	-	+t	-	+	+	+t	+	t	+	+t	+	+
3	Great Northern UI 123	-	-	-	+	+	-	-	t	t	+	+	+
	Redlands Greenleaf B	-	-	-	+	+	-	-	t	t	+	+	+
4	Red Mexican UI 34	-	-	+	-	-	+	+	+	+		t	+
	Sanilac	-	-	+	-	-	+	+	+	+	-	t	+
5	Pinto 114	-	-	-	-	-	+	+	+	+	-	-	-
6	Great Northern UI 31	-	-	-	-	-	-	-	-	-	+	-	+
	Monroe	-	-	-	-	-	-	-	-	-	+	+	+
	Red Mexican UI 35											t	+
7	IVT 7214	-	-	-	-	-	-	-	-	-	-	+?	-
8	Black Turtle Soup	-	-	+n	-	-n	-	-	+n	+n	-	+?	-
	Widusa	-	-	+n	-	-n	-	-	+n	+n	-	t	+?
9a	Jubila	-	-	-l	-	+n	-	-n	+n	+n	-	-	-
9b	Topcrop	-	-	-l	-	-n	-	-n	+n	+n	-	-	-
10	Amanda	-	-	-l	-	-n	-	-	-n	+n	-	nd	nd
11	IVT 7233	-	-	-l	-	-	-	-	-l	-l	-	+	-

t = tolerant. Systemic symptoms may be mild, atypical, delayed or absent

-l = resistant, no systemic necrosis, pinpoint local lesions

+n = susceptible, necrosis not temperature dependant, i.e. at 26°C and 32°C

-n = susceptible, necrosis temperature dependant, i.e. at 32°C and not 26°C

-- = negative

A = bean common mosaic necrosis virus (BCMNV)

B = bean common mosaic virus (BCMV)

? = only one or two out of five plants tested showed these results

\+ = positive

4.3.2 Negative staining

Flexuous particles similar in length to those of potyviruses were visible with negative staining with all three stains used when infected plant material of both virus isolates were examined (Fig. 4.3 and Fig. 4.4). Virus particles were best observed when using uranyl acetate. Particle lengths were not determined. Isolate 93/65 often occurred in membrane-bound packs (results not shown).

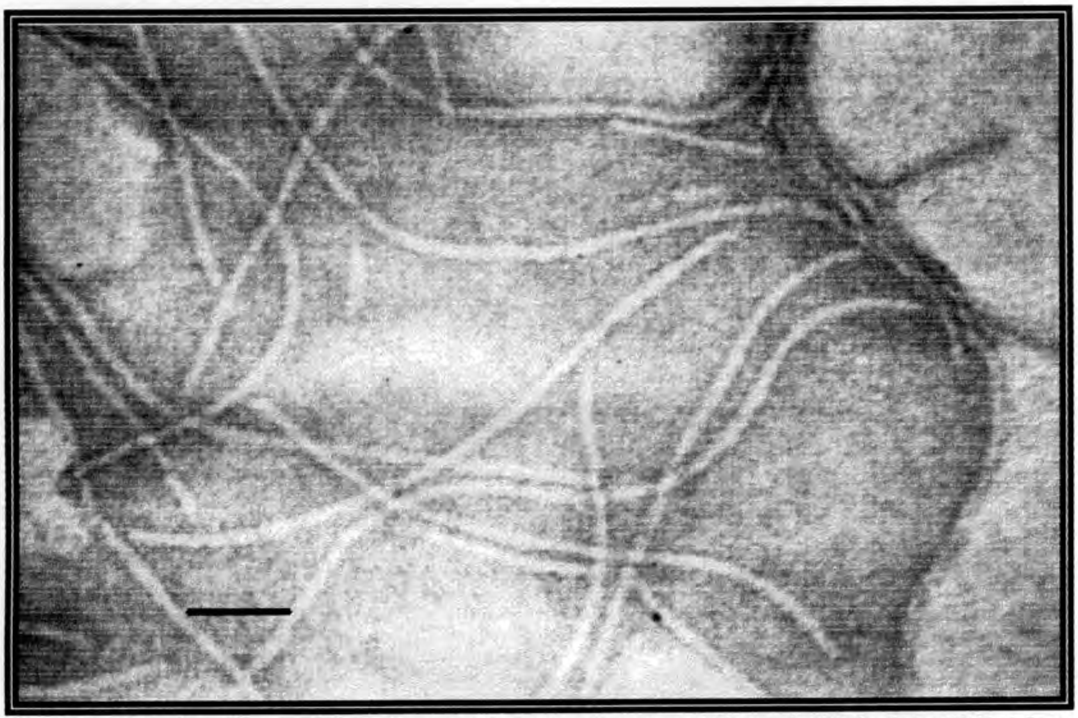


Fig 4.3 Potyvirus-like particles observed in *N. benthamiana* infected with isolate 93/1. Virus particles were stained with 2% (w/v) uranyl acetate pH 4.3. Bar represents 115 nm.

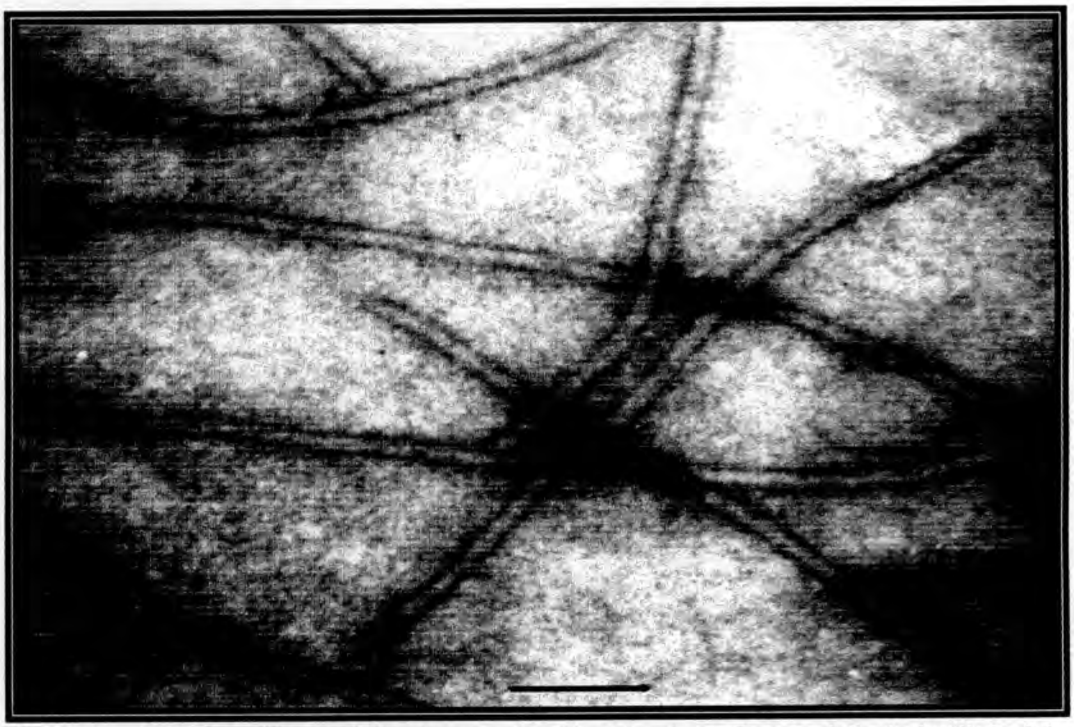


Fig 4.4 Potyvirus-like particle observed in *P. vulgaris* cv Bonus infected with isolate 93/65. Grids were strained with 2% ammonium molybdate pH 5.3. Bar represents 60 nm.

4.3.3 Cytopathology

Isolate 93/1 produced pinwheels and laminated aggregates in infected *N. benthamiana* (Fig 4.5). No scrolls were observed. The virus can therefore be included in group II according to the division of Christie and Edwardson (1977), as is ClYVV. Crystalline cytoplasmic inclusions similar to that described for ClYVV were also observed (Fig 4.5).

Isolate 93/65 produced no pinwheels in *P. vulgaris* cv Bonus three weeks after inoculation. Malformation and disintegration of chromosomes and mitochondria were observed in this cultivar. Pinwheels were observed in *P. vulgaris* cv Redlands Greenleaf B and C three weeks after inoculation (Fig 4.6). These pinwheels were in extremely low concentration. No scrolls or laminated aggregates were observed, and isolate 93/65 could therefore not be assigned to one of the subgroups as proposed by Christie and Edwardson. In Redlands Greenleaf B, monolayers of virus particles enclosed in a membrane, were observed (Fig 4.7). Amorphous cytoplasmic inclusions were observed in Redlands Greenleaf C (not shown).

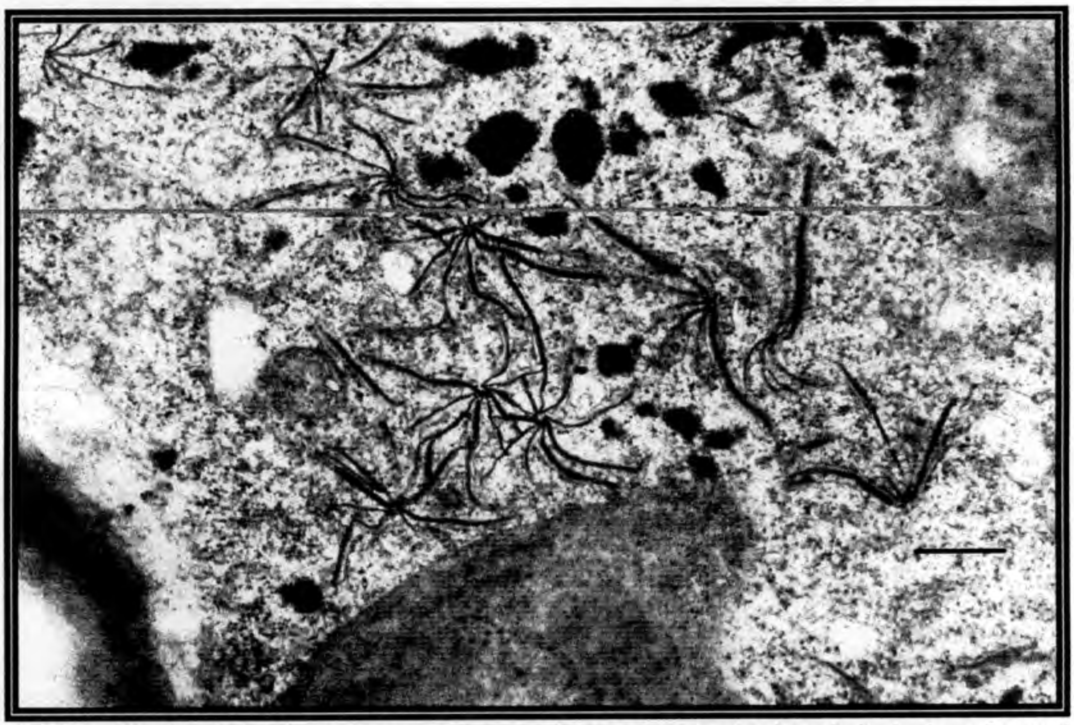


Fig 4.5 Pinwheels, laminated aggregates, and amorphous cytoplasmic inclusions formed by isolate 93/1 in *N. benthamiana*. Bar represents 330 nm.

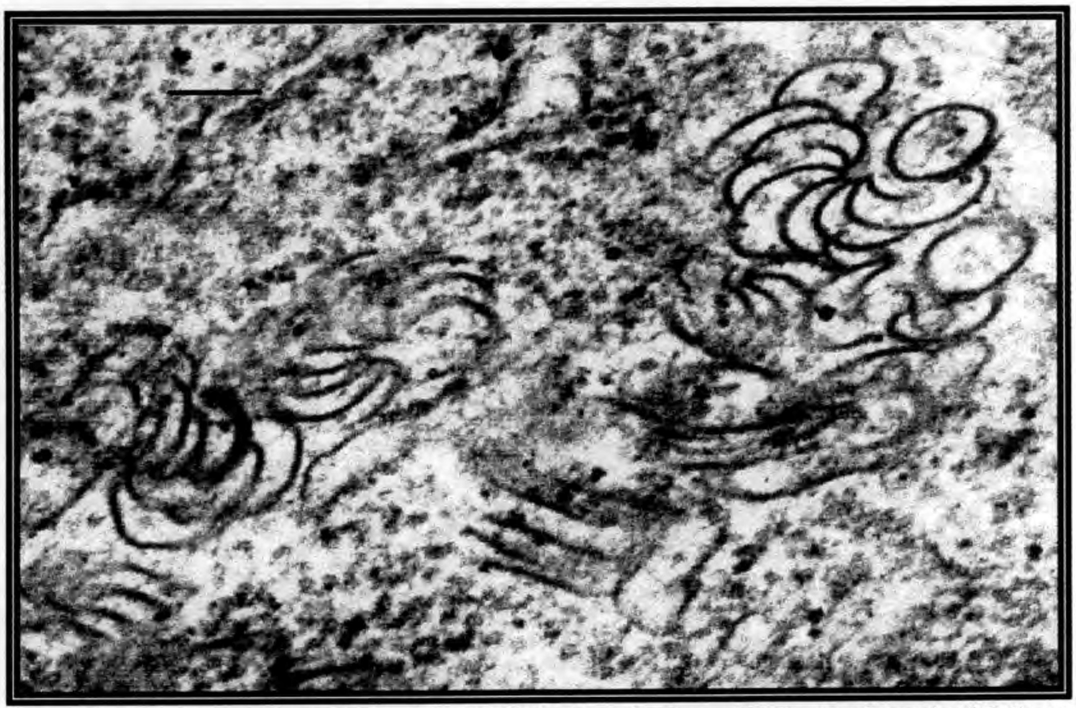


Fig. 4.6 Pinwheels induced by isolate 93/65 in *P. vulgaris* cv Redlands Greenleaf C three weeks after inoculation. Thin sections were observed in a Jeol 100C electron microscope. Bar represents 100 nm.

Fig 4.7 Monolayers of virus particles as observed in *P. vulgaris* cv Redlands Greenleaf B infected with isolate 93/65. Bar represents 125 nm.

4.3.4 Aphid transmission

Isolate 93/1 was transmitted by *Myzus persicae* in a non-persistent manner. The transmission was not very effective as only between 20 and 50% of the test plants showed symptoms. The virus was not transmitted by *Aphis craccivora* or *A. gossypii*. ClYVV has however been reported to be transmitted by both *Aphis craccivora* and *Myzus persicae* (Brunt *et al.*, 1996).

Isolate 93/65 was transmitted by *M. persicae*, *A. craccivora* and *A. gossypii* in a non-persistent manner. All three aphids transmitted isolate 93/65 to approximately 60% of test plants. BCMV is also transmitted by all three aphids.

4.3.5 Virus isolation

As no local lesion host was found for either virus, they could not be isolated by the two successive local lesion transfer method. The limiting dilution transmission method to isolate a virus was attempted with sample 93/1 without success as the virus is very easily transferred to *N. benthamiana*. Only the first dilution series gave an infection rate of less than 10%. All attempts for the second transmission gave infection rates of more than 10% and the virus could therefore not be isolated in this way.

Both viruses were isolated with two successive single aphid transmissions using *Myzus persicae*.

4.3.6 Seed transmission

Symptoms consistent with those caused by isolate 93/65 appeared on the *P. vulgaris* cv Bonus seedlings approximately two weeks after germination. As the virus causes very mild symptoms on *P. vulgaris*, very few seedlings showed clear visual symptoms. ELISA results showed that isolate 93/0065 is seed transmitted in *P. vulgaris* cv Bonus at a rate of 14%. ISEM confirmed that seedlings giving absorbance values close to the ELISA cut-off level of twice the value of the healthy controls, were infected with virus.

P. vulgaris cv Bonus infected with isolate 93/1 produced no seed in the glasshouse. Pods were seldom formed, and usually fell off before viable seed were produced. It is therefore unlikely that isolate 93/1 is seed transmitted in *P. vulgaris* cv Bonus. This conforms with the lack of seed transmission of ClYVV as reported in the literature (Hollings and Stone, 1974).

4.3.7 Coat protein size determination

The coat protein of isolate 93/1 is approximately 32 kDa in size (Fig 4.8) . This is slightly larger than the expected value of 31 kDa as reported for ClYVV by Gough and Shukla (1981), but it is smaller than the value of 36kDa as reported by Fortass *et al.*, 1991.

The coat protein of isolate 93/65 is 34 kDa in size, which corresponds to that reported for BCMV (Shukla *et al.*, 1994). Smaller break-down products of 32 kDa and 29 kDa were also observed (fig 4.8). The 32 kDa band were not observed on all gels. It has been reported that the coat protein of BCMV consistently migrates as two bands of 32-34 kDa and 26-28 kDa (Shukla *et al.*, 1994). These are probably products of the degradation of the coat protein by proteolytic enzymes during purification as reported by Moghal and Francki (1976). It has also been suggested that these bands could be due to differential cleavage of the polyprotein between the Nib and CP. The VFHQ/SKNE site is the predicted one, but cleavage could also occur at a VYHE/SRGT site (Shukla *et al.*, 1994).

The slight difference in the apparent molecular weight reported for the CP of ClYVV and that reported in this study for isolate 93/1, could be due to differences in gel concentration used or to differences in their amino acid compositions. It is known that proteins can give different apparent molecular weights in SDS-PAGE depending on the gel concentration. The CP of TuMV should have a molecular weight of approximately 33.2 kDa based on its CP sequence. Molecular weights of 38 kDa and 32 kDa have been reported in 5% and 15% SDS-PAGE gels respectively (Shukla *et al.*, 1994). Single amino acid substitutions can also change the apparent molecular weight.

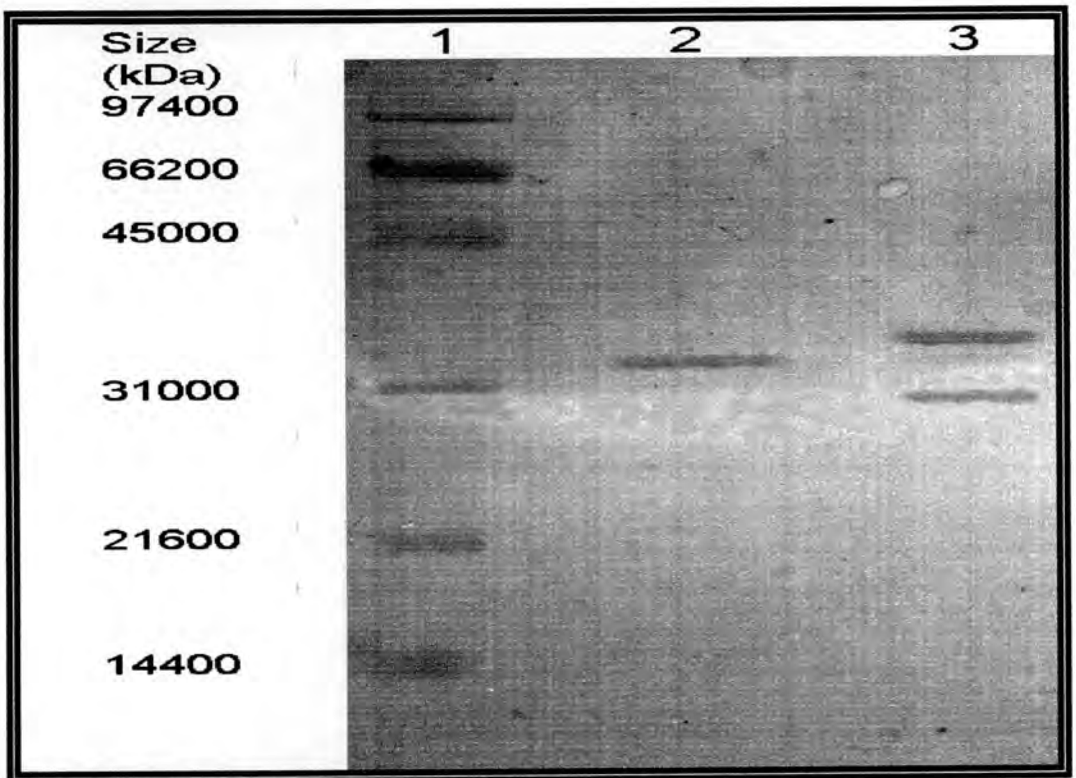


Fig. 4.8 SDS-PAGE of the coat proteins of isolates 93/1 and 93/65. Lane 1, low molecular weight protein marker (BioRad); lane 2, isolate 93/1; lane 3, isolate 93/65. Electrophoresis was for 20 hours at 60 V. The gel was stained with Coomassie Brilliant blue at 4°C, and destained with methanol/acetic acid at room temperature.

CHAPTER 5

VIRUS PURIFICATION AND ANTISERUM PRODUCTION

5.1 INTRODUCTION

It is important to use a very pure virus preparate for immunisation in order to obtain a sensitive and specific antiserum. It is often necessary to optimise the purification method to obtain a high concentration of intact virus particles free from contaminating plant material. The host plant used is also important as it influences the virus concentration. In our experience *N. benthamiana* usually gives a very high yield of virus particles with few contaminating host components remaining.

Most viruses are precipitated near their isoelectric point, and a buffer with a pH higher or lower than the isoelectric point, must be used during purification. Plant proteins and ribosomes are most soluble at pH 7, and are precipitated at around pH 4.5. By lowering the pH of the buffer during the purification procedure, these components can be removed. Many potyviruses are however unstable at a low pH, and other methods must be used to remove the plant proteins and ribosomes. Plant components are also precipitated when stored at pH 7 in high molarity buffers such as citrate, phosphate or borate. As some potyviruses are disrupted by high molarity buffers, this method must also be tested. The type of buffer, *e.g.* phosphate, borate or citrate is also important and must be determined for each virus. Citrate chelates Mg^{2+} and therefore degrades ribosomes. Phosphate tends to aggregate elongated viruses.

Stabilising agents such as TGA (thioglycollic acid), sodium sulphite, or β-mercaptoethanol (ME) at low concentrations of around 0.1 % prevent virus inactivation by oxidation, and may also prevent absorption of host constituents to virus particles. Each of the stabilisers have a different mechanism to prevent oxidation. Sodium sulphite and cysteine reduce the action of phenol oxidase, while hide powder, PVP (polyvinylpyrrolidone), and PEG (polyethylene glycol) bind with phenols. Other chemicals such as Triton X-100, urea, and Tween-80 help with the release of virus particles from host components and reduce particle aggregation. Nicotine, sulphite, TGA, DTT (dithiotreitol), and ME inhibit poly-anionic tannins and polyphenol oxidation of virus particles. DIECA (diethyldithiocarbamate) removes copper ions essential for oxidases, but may inactivate some viruses. Hide or milk powder can also be used as they compete with virus particles for oxidized polyphenols.

Chelating agents such as EDTA remove ribosomes and polyribosomes, and prevent virus aggregation by chelating bivalent cations. They also prevents oxidation of polyphenols. Some viruses need Mg^{2+} and Ca^{2+} for stability and EDTA can then not be used. Bentonite clay absorbs ribosomes, fraction 1 protein, fragmented chloroplasts and ribonucleases, but it may absorb virus particles when saturated with Mg^{2+}. Activated charcoal removes pigments and other host material.

Clarification of the virus extract is obtained by the addition of ethanol, butanol, chloroform, ether, and carbon tetrachloride, single or in combination. Chloroform and carbon tetrachloride are the least hydrophilic and will not precipitate virus particles. They also remove less plant protein. All these chemicals cause the larger host components to coagulate. Vigorous stirring for 10 to 30 minutes enhances the process.

Alternate high and low speed centrifugation is also used to separate host components and virus particles. Chloroplasts, mitochondria, starch grains and fragments of cell walls are removed by low speed centrifugation of 1000 to 10000 g for 5 to 15 minutes. Virus particles are usually subjected to a final high speed centrifugation on a Caesium or sucrose gradient to separate them from remaining host components. Caesium gradients separate different buoyant densities, while sucrose gradients separate components according to their particle weight.

The most abundant soluble plant components are phytoferritin, fraction 1 protein, fraction 2 protein, and ribosomes. Most of these components are smaller than the potyvirus particles and can be removed by differential centrifugation (H. O. Agrawal, 1972).

Many different immunising schedules have been used for antiserum production against potyviruses, but few controlled comparisons have been made. In our experience four intramuscular injections at three to four week intervals give satisfactory results. To prevent a high degree of cross reaction between distantly related potyviruses, freshly prepared virus extractions are injected (Shukla *et al.*, 1994.

Different ELISA systems (DAS ELISA, indirect ELISA, $F(ab')_2$ ELISA, *etc.*) can be used for the detection of potyviruses. In our experience the indirect ELISA system where the virus particles are directly coated onto the ELISA plate, doesn't work for all potyviruses. This is probably due to the change in particle conformation which leads to the necessary epitopes not being available. As we test for a wide variety of plant viruses in our laboratory, we have found the $F(ab')_2$ ELISA system the most convenient to use as a single conjugate (goat anti-rabbit Fc) is used for all viruses.

5.2 MATERIAL AND METHODS

5.2.1 Development of a purification procedure

Different buffers, stabilising additives, clarifying agents, and density gradients were compared to determine the optimum conditions for virus purification.

The samples were examined under the EM at each step and the number of intact and broken virus particles determined. The combination yielding the highest number of intact virus particles was then selected.

The following buffers were tested: 0.5 M borate pH 8, 0.5 M borate pH 7.5, 0.1 M borate pH 8, 0.1 M citrate pH 7.2, 0.5 M citrate pH 8, 0.5 M citrate pH 7.5, 0.2 M phosphate pH 7.5, 0.5 M phosphate pH 7.5, 0.5 M phosphate pH 8, and 0.1 M Tris pH 8.9. Infected plant material was macerated at a ratio of 1:10 in each of the buffers, and examined for the presence of virus with the EM.

The two buffers yielding the highest number of particles were selected and tested with the following stabilisers: 0.5% Na_2SO_3 (0.04 M), 0.25% $Na2SO_3$ (0.02 M), 0.01 M DIECA, 0.14% ME + 1.86% EDTA, 0.2% ME + 0.37% EDTA, and 0.1% TGA.

Plant material was macerated in the two best buffer and additive combinations. Different clarifying agents were added to the extractions after a low speed centrifugation for 10 minutes at 9 000 rpm in a Beckman JA 20 rotor. The clarifying agents tested were 8% (v/v) n-butanol, 8.5% (v/v) n-butanol, 1:1 (v/v) chloroform, 50% (v/v) chloroform, and a combination of 25% (v/v) chloroform and 25% (v/v) CCl_4. The mixtures were stirred for 30 minutes at 4°C when butanol was used, and 10 minutes at 4°C if chloroform was used. The clarifying agents were removed by ultracentrifugation for 60 minutes at 35 000 rpm in a Beckman TY 65 rotor. The pellets were resuspended in the original buffer and the samples subjected to EM analysis.

The best combination of purification conditions was selected, and the virus tested for PEG tolerance. Concentrations of 0%, 4%, 5%, and 8% PEG were tested. After the PEG precipitation, the virus was resuspended in the original buffer and stabiliser, and concentrated by centrifugation through a 5% (w/v) sucrose cushion at 38 000 rpm in a T865 rotor for 2.5 hours. The virus preparate was subjected to EM analysis.

The virus preparate was finally loaded onto a CsCl or Cs_2SO_4 gradient. The salts were removed by dialysis against the original buffer, and the final preparation examined with the EM.

5.2.2 Determination of virus purity

The concentration of the purified virus was determined by determining the absorbance value at 260 nm and using the extinction coefficient value of 2.4 (Purcifull, 1966). Purity of the sample was verified under the electron microscope (EM) as well as by determining the A_{260nm}:A_{280nm} and A_{260nm}:A_{245nm} ratios. These ratios should be 1.14-1.25 and 1.11-1.27 respectively if the virus is free from contaminating proteins, and is typical of nucleoprotein with 5-6% nucleic acid (Purcifull, 1966).

5.2.3 Development of ELISA detection systems

5.2.3.1 Immunisation

Rabbits were immunized with between 0.1 and 1 mg virus per injection, as described in Appendix A. The titre of each bleed was determined with $F(ab')_2$ ELISA.

5.2.3.2 Purification of IgG and $F(ab')_2$

IgG was purified from the serum as described in Appendix A. Fractions of the IgG with absorbance values higher than 1.4 were pooled and the concentration adjusted to 1 mg/ml with buffer ($A_{280nm} = 1.4$).

The $F(ab')_2$ fragments were prepared by a modification of the procedure of Campbell *et al.*, 1970, as described in Appendix A.

The optimum working dilutions of IgG and $F(ab')_2$ were determined with an optimising $F(ab')_2$ ELISA (Appendix A).

5.2.3.3 Titre determination

The titres of all the bleeds were determined with $F(ab')_2$ ELISA as described in Appendix A. Each antiserum dilution was tested against virus-infected and healthy plant material. The pre-immune serum was included.

IgG and $F(ab')_2$ for use in future ELISA were then purified from the best bleed as described in Appendix A, and an optimising $F(ab')_2$ ELISA performed to determine the optimum working dilutions.

5.2.3.4 Determination of sensitivity and specificity of the ELISA systems

For the sensitivity tests, purified virus was diluted in the ELISA extraction buffer. The $F(ab)_2$ ELISA was performed as described in Appendix A, using ELISA extraction buffer as negative control.

For the specificity tests, the ELISA systems were tested against various potyviruses. Virus infected material used was either fresh or desiccated (See Appendix B). Healthy plant material of each of the cultivars tested were used as negative controls.

5.3 RESULTS AND DISCUSSION

5.3.1 Purification of viruses

Both viruses were initially purified using the method described by Hagood (Hagood, 1978). As this method yielded a high number of broken virus particles which would be unsuitable for use in an immunisation schedule, a purification method suitable for each virus had to be developed, as described below.

5.3.1.1 Purification schedule for 93/1

Virus was purified from *N. benthamiana* plants two or three weeks after inoculation. The leaves were macerated in 4x v/w of 0.5 M sodium citrate pH 8.0 containing 0.1% TGA. The sap was expressed through cheesecloth, and centrifuged at 8 000 rpm for 10 minutes in a Beckman JA 14 rotor. N-butanol (8% v/v) was added slowly to the supernatant. The mixture was stirred for 30 minutes, and allowed to stand for 30 minutes at 4°C before centrifugation at 8 000 rpm for 10 minutes in a JA 14 rotor. PEG 6000 (8% w/v) was added to the supernatant, and the mixture stirred for 30 minutes at 4°C. The virus was precipitated with centrifugation for 30 minutes at 8 000 rpm in Beckman JA 14 rotor. The pellets were resuspended in 1/10th the initial buffer volume of 0.5 M sodium citrate, pH 8.0, containing 0.1% TGA. The sample was subjected to another low speed centrifugation at 8 000 rpm for 10 minutes in Beckman JA 20 rotor. The supernatant was layered onto a 5 ml cushion of 30% (w/v) sucrose, and centrifuged at 38 000 rpm for 2.5 hours in T 865 rotor. The pellets were resuspended in 1/10th the buffer volume before the sucrose cushion of 0.5 M citrate buffer pH 8.0 containing 0.1% TGA or 0.05 M sodium phosphate pH 7.5. The suspension was centrifuged at 8 000 rpm for 10 minutes in a Beckman JA 20 rotor before determining the virus concentration. The preparation was fractionated by centrifugation on a 1.3 mg/4 ml Cs_2SO_4 gradient for 4 hours in a Beckman TY 65 rotor at 45 000 rpm. The gradient was made up in 0.05 M sodium phosphate buffer pH 7.5. Not more than 2 mg of virus was loaded per gradient. The bands were visualised under normal white light and removed with a bent needle. The Cs_2SO_4 was removed by dialysis against 0.05 M sodium phosphate pH 7.5.

Isolate 93/1 was easily purified from *N. benthamiana*. Yields as high as 20 mg virus/ 100 g leaf material have been obtained. The virus tends to form head to head aggregates. This aggregation was not lessened by the addition of urea or EDTA to the extraction buffer. The aggregation was only lessened if larger buffer volumes were used during the purification

5.3.1.2 Purification schedule for 93/65

Isolate 93/65 was purified from *P. vulgaris* cv Bonus leaves three weeks after inoculation. The leaves were macerated in 4x v/w of 0.5 M phosphate pH 7.5 containing 1.86% EDTA and 0.14% ME. The sap was expressed through cheesecloth and 8% (v/v) n-butanol added while stirring at room temperature. The mixture was stirred for 40 minutes before centrifugation for 10 minutes at 8 000 rpm in a Beckman JA 14 rotor. The supernatant was loaded onto a 5 ml 30% sucrose cushion and centrifuged for 2.5 h at 38 000 rpm in a T865 rotor. The pellet was resuspended in 1/30th the original buffer volume of 0.1 M sodium phosphate buffer pH 7.5 containing 1.86% EDTA and 0.14% mercaptoethanol. Amounts of 2 mg virus were loaded onto Cs_2SO_4 gradients (1.3 mg Cs_2SO_4/4 ml buffer) and centrifuged for 5 h at 45 000 rpm in a TY 65 rotor. The virus bands were visualised under white light and removed with a needle and syringe. The Cs_2SO_4 was removed by centrifugation through a 30% sucrose cushion at 38 000 rpm in a T 865 rotor, or dialysis against 0.1 M phosphate buffer pH 7.5 containing 1.86% EDTA and 0.14% mercaptoethanol.

Amounts of 2 mg virus/ 100 g of leaf material were obtained when isolate 93/65 was purified.

5.3.2 Development of ELISA detection systems

5.3.2.1 Titre determination and optimising of the $F(ab)_2$ ELISA systems

$F(ab)_2$ fragments were prepared from the third bleed for use in the titre determinations. Neither of the pre-immune sera reacted against virus-infected or healthy material.

Bleeds 7 and 8 had the strongest reaction against isolate 93/65, with both having titres of higher than 1:4096 (fig. 5.1). $F(ab)_2$ and IgG were purified from bleed 7. The optimum dilutions for use in future ELISA tests were 1:500 for the $F(ab)_2$ and 1:200 for the IgG (results not shown).

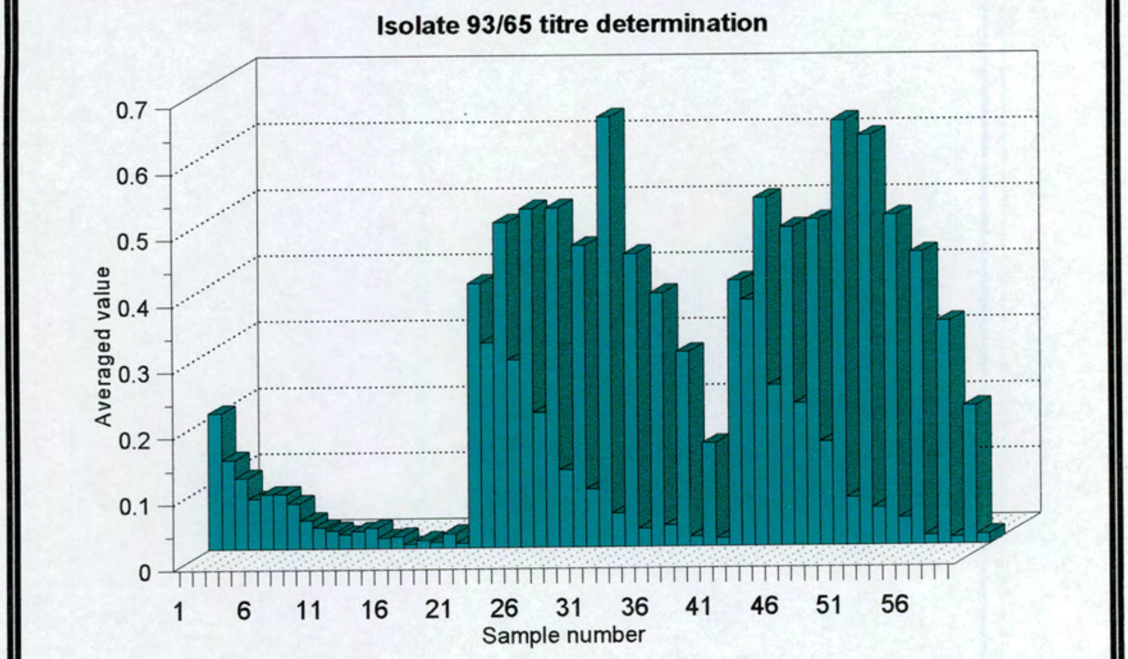


Fig. 5.1 Titre determination for antisera to isolate 93/65. Samples 1-20, pre-immune antiserum; samples 21-40, bleed 7; samples 41-60, bleed 8. Infected and healthy material were alternated. Dilutions of antisera were 1/8, 1/16, 1/32, 1/64, 1/128......1/4096.

Bleed 5 had the highest titre against isolate 93/1 (results not shown). The optimum dilutions of the seroreagents for use in future ELISA tests, were 1:2000 for the $F(ab)_2$, and 1:100 for the IgG (fig. 5.2).

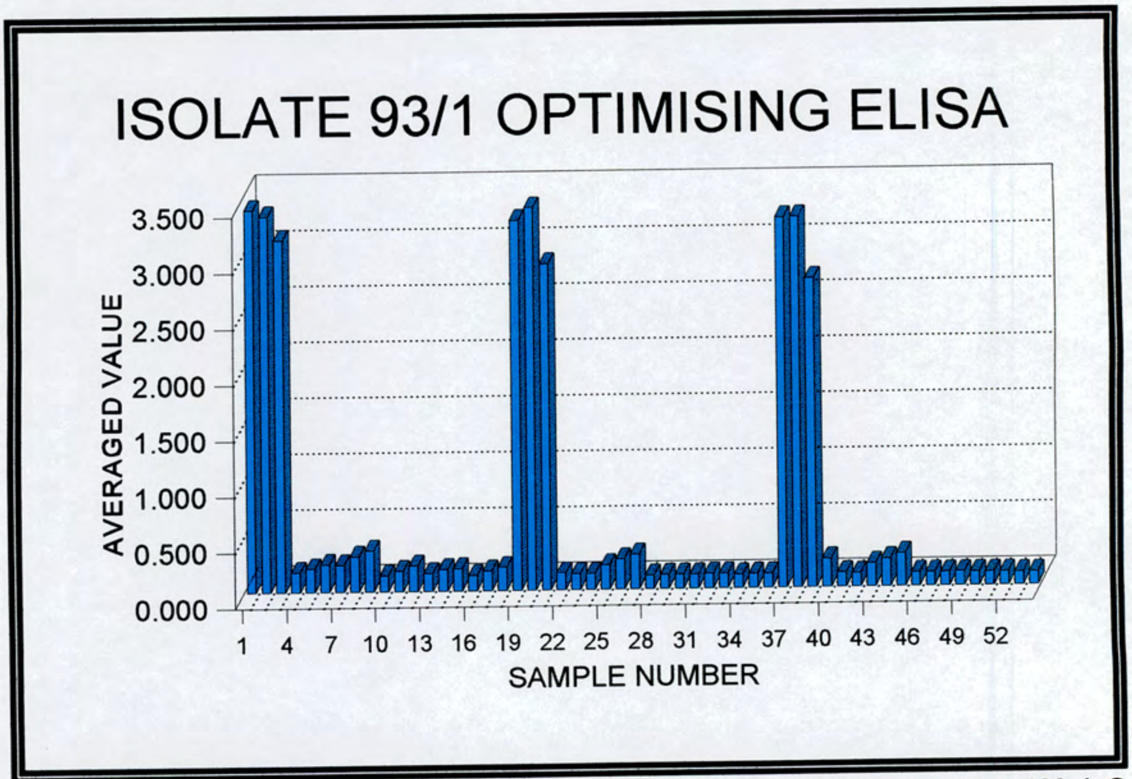


Fig. 5.2 Optimising F(ab')$_2$ ELISA for detection of isolate 93/1. Lanes 1-6, F(ab')$_2$ 1:100, IgG 1:100; lanes 7-12, F(ab')$_2$ 1:100, IgG 1:1000; lanes 13-18, F(ab')$_2$ 1:100, IgG 1:2000; lanes 19-24, F(ab')$_2$ 1:1000, IgG 1:100; lanes 25-30, F(ab')$_2$ 1:1000, IgG 1:1000; lanes 31-36, F(ab')$_2$ 1:1000, IgG 1:1000; lanes 37-42, F(ab')$_2$ 1:2000, IgG 1:100; lanes 43-48, F(ab')$_2$ 1:2000, IgG 1:1000; lanes 49-52, F(ab')$_2$ 1:2000, IgG 1:2000. The sequence in which the samples were added were the same for each group of six lanes: infected material diluted 1:10, 1:100, and 1:1000; healthy material diluted 1:10 and 1:100, and a buffer control.

5.3.2.2 Determination of the sensitivity and the specificity of the ELISA systems

The F(ab)$_2$ ELISA systems were evaluated for sensitivity and specificity. The 93/65 ELISA system could detect 166 ng of virus per well (Fig. 5.3) using as a cut-off value twice the absorbance value of the negative controls. The 93/1 ELISA system could detect 2.6 ng of virus per well (Fig 5.4).

Both systems were therefore sensitive enough to use for the detection of virus in naturally infected material. The 93/65 ELISA system could however give false negative results as the reaction was not very strong. Test plants giving an absorbance value close to the cut-off value of twice the absorbance of the healthy controls, should be inoculated onto a sensitive indicator plant such as *P. vulgaris* cv Stringless Green Refugee, and retested.

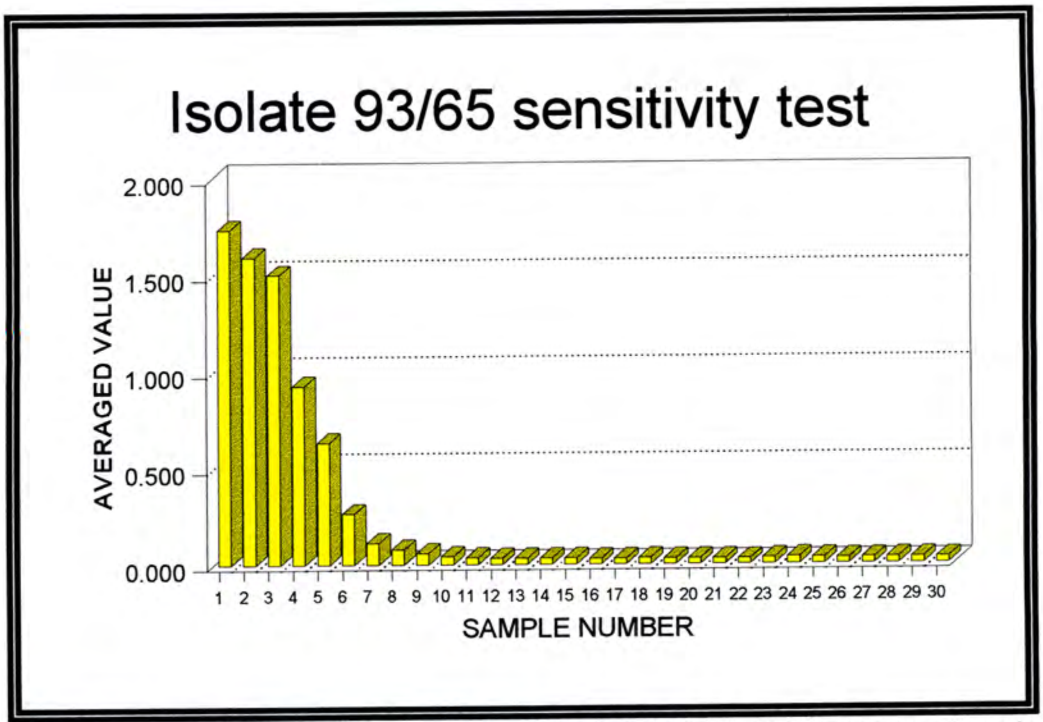


Fig. 5.3 Determination of the sensitivity of the 93/65 $F(ab')_2$ ELISA system. Sample 1, 43 µg purified virus; 2, 21 µg; 3, 11 µg; 4, 2.7 µg; 5, 1.3 µg; 6, 666 ng; 7, 333 ng; 8, 166 ng; 9, 83 ng; 10, 42 ng; 11, 21 ng; 12, 10 ng; 13, 2.6 ng; 14, 1.3 ng; 15, 655 ng; 16, 164 pg; 17, 82 pg; 18, 20 pg; 19, 10 pg; 20, 5 pg; 21, 2.56 pg; 22, 1.28 pg; 23, 640 fg; 24, 320 fg; 25, 80 fg; 26, 40 fg; 27, 20 fg; 28, 10 fg, 29 and 30, ELISA extraction buffer.

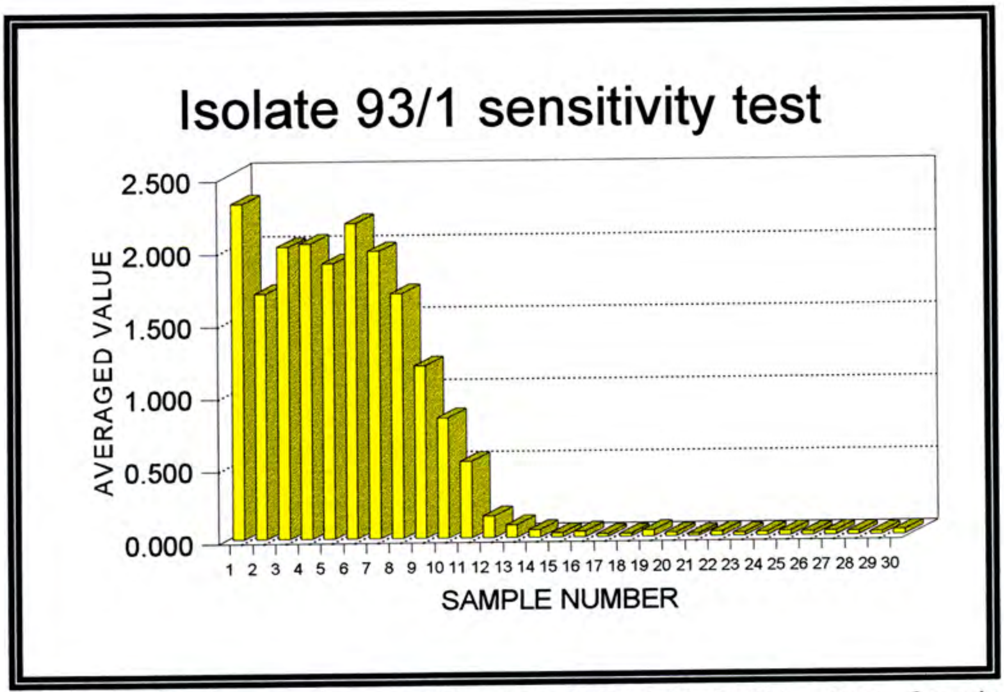


Fig. 5.4 Determination of the sensitivity of the 93/1 $F(ab')_2$ ELISA system. Sample 1, 43 µg purified virus; 2, 21 µg; 3, 11 µg; 4, 2.7 µg; 5, 1.3 µg; 6, 666 ng; 7, 333 ng; 8, 166 ng; 9, 83 ng; 10, 42 ng; 11, 21 ng; 12, 10 ng; 13, 2.6 ng; 14, 1.3 ng; 15, 655 ng; 16, 164 pg; 17, 82 pg; 18, 20 pg; 19, 10 pg; 20, 5 pg; 21, 2.56 pg; 22, 1.28 pg; 23, 640 fg; 24, 320 fg; 25, 80 fg; 26, 40 fg; 27, 20 fg; 28, 10 fg, 29 and 30, ELISA extraction buffer.

The 93/1 ELISA system could detect BYMV 92/751, CABMV-SAP 92/749, PMV 95/931, and isolate 93/65 (Fig 5.3). None of these reactions were as strong as the homologous reaction. These reactions were as expected as ClYVV is serologically related to BYMV, PMV, and BCMV (Brunt *et al.*, 1996; Fortass *et al.*, 1991; Shukla *et al.*, 1994). As the reaction to other viruses were very weak, this system can be used in future field surveys for isolate 93/1-related viruses.

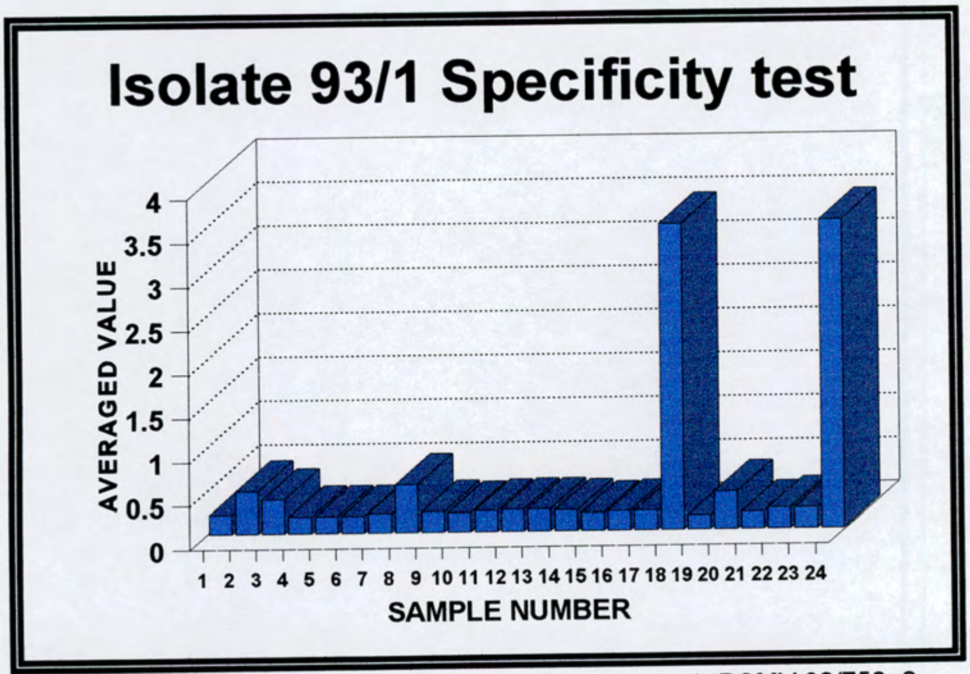


Fig. 5.5 Specificity of the 93/1 ELISA system. Sample 1, BCMV 92/750; 2, BYMV 92/751; 3, isolate 93/65; 4, healthy *P. vulgaris* cv Stringless Green Refugee; 5, healthy *P. vulgaris* cv Bonus; 6, SMV 86/20; 7, PeMoV 88/63; 8, CABMV-SAP 92/749; 9, isolate 93/632; 10, CABMV 87/14; 11, CABMV 90/57; 12, CABMV 93/1510; 13, isolate 90/169; 14, WMV-2 90/106; 15, WMV-Merocco 90/107; 16, PStV 95/399; 17, PVY 92/21; 18, isolate 93/1; 19, healthy *N. benthamiana*; 20, PMV 95/931; 21, healthy *Pisum sativum*; 22 and 23, extraction buffer; 24, positive control (isolate 93/1).

The reaction of the 93/65 ELISA system to the various viruses tested can be seen in Fig 5.4. The 93/65 ELISA system could detect isolate 93/1 very weakly and had a strong reaction against PStV 95/399. The strong reaction against PStV 95/399 was expected as both PStV and BlCMV are now considered to be strains of BCMV, and all strains of BCMV have a close serological relationship. BCMV is also known to be serologically related to ClYVV and BYMV (Brunt *et al.*, 1996; Shukla *et al.*, 1994). BCMV 92/750 was not detected with this ELISA system, as the plant material was macerated in extraction buffer containing PVP,

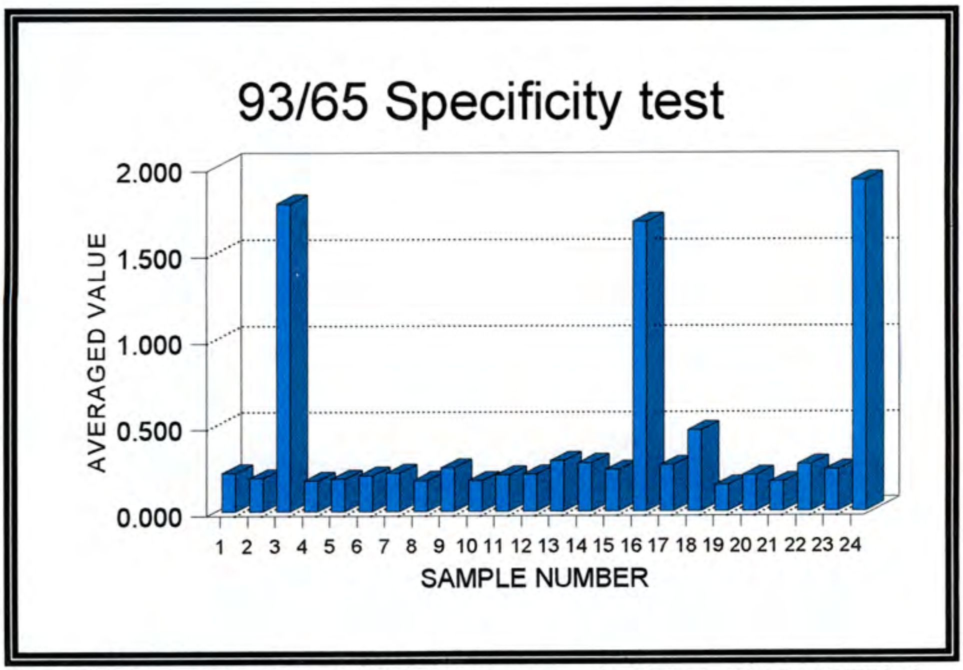


Fig. 5.6 Specificity of the 93/65 ELISA system. Sample 1, BCMV 92/750; 2, BYMV 92/751; 3, isolate 93/65; 4, healthy *P. vulgaris* cv Stringless Green Refugee; 5, healthy *P. vulgaris* cv Bonus; 6, SMV 86/20; 7, PeMoV 88/63; 8, CABMV-SAP 92/749; 9, isolate 93/632; 10, CABMV 87/14; 11, CABMV 90/57; 12, CABMV 93/1510; 13, isolate 90/169; 14, WMV-2 90/106; 15, WMV-Merocco 90/107; 16, PStV 95/399; 17, PVY 92/21; 18, isolate 93/1; 19, healthy *N. benthamiana*; 20, PMV 95/931; 21, healthy *Pisum sativum*; 22 and 23, extraction buffer; 24, positive control (isolate 93/65).

and BCMV 92/750 is unstable in buffer containing PVP (personal experience). As the reaction to viruses other than BCMV and its strains is very weak, this ELISA can be used in future field surveys to detect closely related viruses only. The ELISA is also sensitive enough to detect most field-infected samples.

5.4 CONCLUSION

Purification methods for both viruses were successfully developed. Isolate 93/65 was purified at approximately 2 mg/100 g leaf material from *P. vulgaris* cv Bonus, while isolate 93/1 was purified at approximately 20 mg/100 g leaf material from *N. benthamiana*.

Antisera were successfully prepared against both virus isolates. Both ELISA systems are sensitive and specific enough to be used in future field surveys.

CHAPTER 6

NUCLEIC ACID SEQUENCE DETERMINATION

6.1 INTRODUCTION

Nucleic acid sequence comparisons are probably the most accurate way of identifying a potyvirus. When sequences of the entire genome of potyviruses were compared, it was found that more or less the same picture of virus relationships are obtained, irrespective of the part of the genome that is being compared (Rybicki and Shukla, 1992). Comparison of the partial CP region nucleotide sequences also give a similar pattern to that obtained when the entire CP nucleotide or amino acid sequences are compared. Partial CP sequence comparisons serve to unequivocally identify distinct viruses and their strains (Rybicki and Shukla, 1992; Pappu *et al.*, 1993).

The 3' non-coding region is only conserved among strains of a potyvirus, and little homology is observed between distinct viruses (Shukla *et al.*, 1994).

By comparing the nucleotide sequences of part of the CP region and the 3' non-coding region of unidentified potyviruses to those of known viruses, accurate classification of the virus should be possible.

Sequence data from the existing databases has been used to develop degenerate oligonucleotide primers to conserved areas of the potyviral genome. These primers should be able to amplify all members of the potyvirus group (Pappu *et al.*, 1993; Langeveld *et al.*, 1991). Virus-specific primers that can be used to distinguish closely related viruses or strains of a virus, have also been developed (Bariana *et al.*, 1994; Saiz *et al.*, 1994a).

In this study, we have used group-specific degenerate primers as described by Pappu *et al.*, 1992. One primer is specific for the conserved "WCIEN" region mid-way in the CP, while the other is specific for the poly(A) tail. The nucleic acid sequence data of the PCR product obtained with these primers were used to identify the "unknown" potyviruses.

IC-RT-PCR has been found to be 250 times more sensitive than RT-PCR, 625 times more than hybridisation, and 5 000 times more sensitive than ELISA for the detection of plum pox virus (Wetzel *et al.*, 1992). The immunocapture step serves to concentrate the virus particles and to remove plant substances that would otherwise interfere with the PCR. As no RNA extraction or virus purification is necessary, it saves time and money.

A combination of group-specific antiserum and group-specific primers, should enable one to amplify almost all the members of a virus group. If one wants to amplify only a specific virus using group-specific primers, a virus-specific antiserum will make the PCR more specific.

6.2 MATERIALS AND METHODS

6.2.1 Immunocapture reverse transcription PCR (IC-RT-PCR)

6.2.1.1 Amplification using degenerate primers

The viruses were amplified by IC-RT-PCR using degenerate primers as described by Pappu *et al.*, 1992. One primer was directed at the end of the 3' non-coding region and the other at a conserved block mid-way in the coat protein (WCIEN block). The nucleic acid sequence of the primers was as follows:

5' XTTTTTTTTTTTTTTTTTTTTT 3'
5' TGGTGZATYGANAATGG 3'
where X = A, C, or G
Y = A, C, or T
Z = A, or T
N = A, C, G, or T

The primers were synthesised at the Department of Biochemistry, University of Cape Town, South Africa.

The conditions for the PCR were similar to that described by Langeveld *et al.*, 1991, Pappu *et al.*, 1993, and T. Roussell (personal communication). The optimum concentrations of the enzymes, dNTP mix, primers, and $MgCl_2$, were determined using isolate 93/1. A clone which included the entire coat protein and 3' non-coding regions of CABMV-SAP obtained from Dr Reon Brand (ARC-Infruitec), was used as positive control for the PCR (Brand *et al.*, 1993).

IgG or antiserum was diluted in coating buffer (0.05 M sodium bicarbonate pH 9.6 containing 0.20 g NaN_3) at the optimum conditions as determined for ELISA. Both viruses were trapped with their homologous antiserum or IgG. Use of the potyvirus common epitope Mab was also tested. PCR tubes supplied by Whitehead Scientific (South Africa) were coated with 100 µl of the IgG or serum and incubated at 37°C for 3 hours. The tubes were washed twice with 200 µl of PBS-Tween (0.02 M PBS pH 7.4 + 0.05% Tween-20).

Plant material was macerated at a ratio of 1:10 (w/v) in extraction buffer (0.02 M PBS pH 7.4, 2% PVP, 0.2% ovalbumin) and centrifuged in a microcentrifuge for 10 seconds. 100 µl of the plant extract was added to each tube and incubated at 4°C for 16 to 20 hours. The tubes were washed twice with 200 µl PBS-Tween and briefly centrifuged and all traces of buffer removed.

The PCR/reverse transcriptase buffer (1.76% Triton X-100, 10 mM Tris pH 8.0, 50 mM KCl, 10 mM DTT, 2.5 mM $MgCl_2$), 175 µM of each dNTP, 1 µM of each primer, 18 u HPRI RNAse inhibitor (Amersham International), 60 u M-MLV reverse transcriptase (Life technologies), and 2 u Dynazyme (Finnzymes) was added to each tube. The total volume of the reaction was 100 µl.

The tubes were incubated at 37°C for 45-60 minutes for the reverse transcriptase reaction. The PCR was performed as follows: 94°C for 2 minutes (one cycle); 35 cycles of 94°C for 1 minute, 46°C for 1 minute, 72°C for 2 minutes and one cycle of 72°C for 5 minutes for elongation. The amplification was performed in a JDI thermocycler. The PCR products were run on a 1% agarose gel in TAE buffer for 30-60 minutes at 100 V. These PCR products were used for cloning and sequencing.

6.2.1.2 Determination of the sensitivity of the PCR

Isolate 93/1 was purified from *N. benthamiana* and isolate 93/65 from *P. vulgaris* as described before. Purified virus was diluted from 21 µg to 5 fg in extraction buffer. The viruses were trapped with IgG diluted in coating buffer. The dilutions of the seroreagents used were the same as for ELISA (93/1 IgG diluted 1:100, and 93/65 IgG diluted 1:200). The IC-RT-PCR was performed as described above. The PCR products were analysed with agarose gel electrophoresis as described.

6.2.1.3 Determination of the specificity of the PCR

The specificity of both IC-RT-PCR systems was determined. Closely and distantly related viruses were trapped with antisera against isolates 93/1 and 93/65. Viruses used in the test, were PVY 92/21, BYMV 92/751, BCMV 92/750. PStV 95/399, PStV 91/354, PMV 95/931, CABMV-SAP 92/749; CABMV 93/1510, SMV 96/38, CABMV 94/2097, PeMoV 96/38, PeMoV 94/2097, isolate 93/1, and isolate 93/65. Healthy *N. Benthamiana*, *P. Vulgaris* cv Bonus, and *Pisum sativum* were used as negative controls. A buffer control containing no DNA was also included. The CABMV-SAP 92/749 clone was used as positive control for the PCR. The IC-RT-PCR was performed and the products subjected to agarose gel electrophoresis as described before.

The role of antibodies in the trapping of virus particles, was also tested: virus-infected plant material macerated in extraction buffer was used to coat the PCR tubes directly. The RT-PCR was performed as described before. These amplifications were then compared to amplification of virus trapped with antiserum.

6.2.1.4 Amplification of isolate 93/1 with ClYVV- and BYMV-specific primers

Isolate 93/1 was also amplified by IC-RT-PCR using primers specific for BYMV and ClYVV as described by Bariana *et al.*, 1994. The PCR was adapted to amplify the viruses using the degenerate oligo(dT) primer instead of the communal BYCY primer as described in the article. The sequences of the primers used were:

BYMV	5' GGTTTGGCYAGRTATGCTTTTG 3'
CYVV	5' CATTCCCAGACAGAGACATCAATGCAG 3'
BYCY	5' GAGAATTTAAAGACGGATA 3'
oligo(dT)	5' XTTTTTTTTTTTTTTTTTTTT 3'

Where X = A, C, G
Y = C, T
R = A, G

These primers were synthesised at the Department of Biochemistry, University of Cape Town, South Africa.

6.2.2 Ligation and transformation

The PCR product was prepared for ligation with the Double GeneClean Protocol (Appendix A). The DNA was purified and concentrated with GeneClean (BIO 101) and treated with DNA polymerase I (Amersham) and T4 polynucleotide kinase (Amersham) to make the ends flush. The enzymes were removed and the DNA once more concentrated by purification with GeneClean. The DNA was ligated to an equal amount (w/w) of dephosphorylated SmaI-cut pBluescript KS+ with T4 DNA ligase (Amersham). The ligation was transformed into competent *E. coli* DH5α cells (Appendix A). The cells were mixed with IPTG and X-gal and plated out onto LB-plates containing ampicillin. The cells were grown at 37°C for 18 hours. Preparation of the dephosphorylated vector is described in Appendix A.

In some cases, TA-cloning was performed. The PCR product was ligated without purification into SmaI-cut dT-tailed pBluescript KS+ with T4 DNA ligase (Amersham). The vector was purified according to the alkaline lysis method as described in Appendix A, and cut to completion with SmaI as described before. The plasmid was T-tailed according to the method of Holton and Graham (1990), using Dynazyme.

6.2.3 Selection of positive clones

White colonies were selected and grown in LB-broth for 18 hours at 37°C with shaking. The plasmids were purified using the alkaline lysis miniprep method (Appendix A). The plasmids were run on a 1% agarose gel and compared to intact pBluescript KS+. Plasmids that appeared to be bigger than the control were cut with EcoRI and XbaI (both from Boehringer-Mannheim) as described in Appendix A. Some recombinants were also cut with XbaI and PstI (Boehringer-Mannheim). The digestions were run on a 1% agarose gel and the size of the inserts determined. Plasmids containing the correct inserts were selected for future studies.

6.2.4 DIG cycle sequencing

Four recombinant plasmids of each virus were selected for nucleic acid sequence determination. Plasmids were purified according to the alkaline lysis method as described in Appendix A. The plasmids were subjected to cycle sequencing using a DIG Taq DNA sequencing kit for standard and cycle sequencing (Boehringer-Mannheim). Two sets of DIG-labelled primers were used - the standard M13 forward and reverse primers as supplied in the kit, and custom-made (MWG-Biotech, Germany) SK and KS primers. The DNA was detected on the membrane with the DIG chemiluminescent detection kit (Boehringer Mannheim).

The nucleic acid sequences of the primers used were as follows:

KS-Primer	5'-DIG-TCGAGGTCGACGGTATC-3'
SK-Primer	5'-DIG-CGCTCTAGAACTAGTGGATC-3'
M13 forward primer	5'-DIG-GTAAAACGACGGCCAGT3'
M13 reverse primer	5'-DIG-CAGGAAACAGCTATGAC3'

6.2.5 Sequence analysis

Closely related virus sequences were found with BLAST searches of the Genbank and EMBL databases and their updates (Altschul *et al.*, 1990). Nucleic acid sequence data retrieved from the Genbank and EMBL databases were aligned using DNAMAN (Lynnon Biosoft, 1996) and homology and phylogenetic trees drawn. The nucleic acid sequences were translated with DNAMAN. The amino acid sequences were also aligned to sequences obtained from the Swiss Protein and Genbank databases, and homology and phylogenetic trees drawn using DNAMAN.

6.3 RESULTS AND DISCUSSION

6.3.1. IC-RT-PCR with degenerate potyvirus-specific primers

6.3.1.1 General

Both isolates were successfully amplified with IC-RT-PCR using degenerate potyvirus-specific primers. The type of tube used for this PCR is very important as not all tubes have sufficient protein binding capacities. 0.5 ml polypropylene tubes sold by Whitehead Scientific were found to have the best binding capacity for the antisera (results not shown). No amplification was observed when healthy *P. vulgaris* or *N. benthamiana* was trapped with antiserum, or in the buffer control. When the CABMV-SAP clone was amplified, a 692 bp fragment was obtained. Isolate 93/1 yielded a 640 bp fragment, while isolate 93/65 yielded a 700 bp fragment. The PCR products can be seen in Fig. 6.1. No differences in amplification were observed when trapping with virus-specific IgG or antiserum, or with the potyvirus common epitope Mab (results not shown).

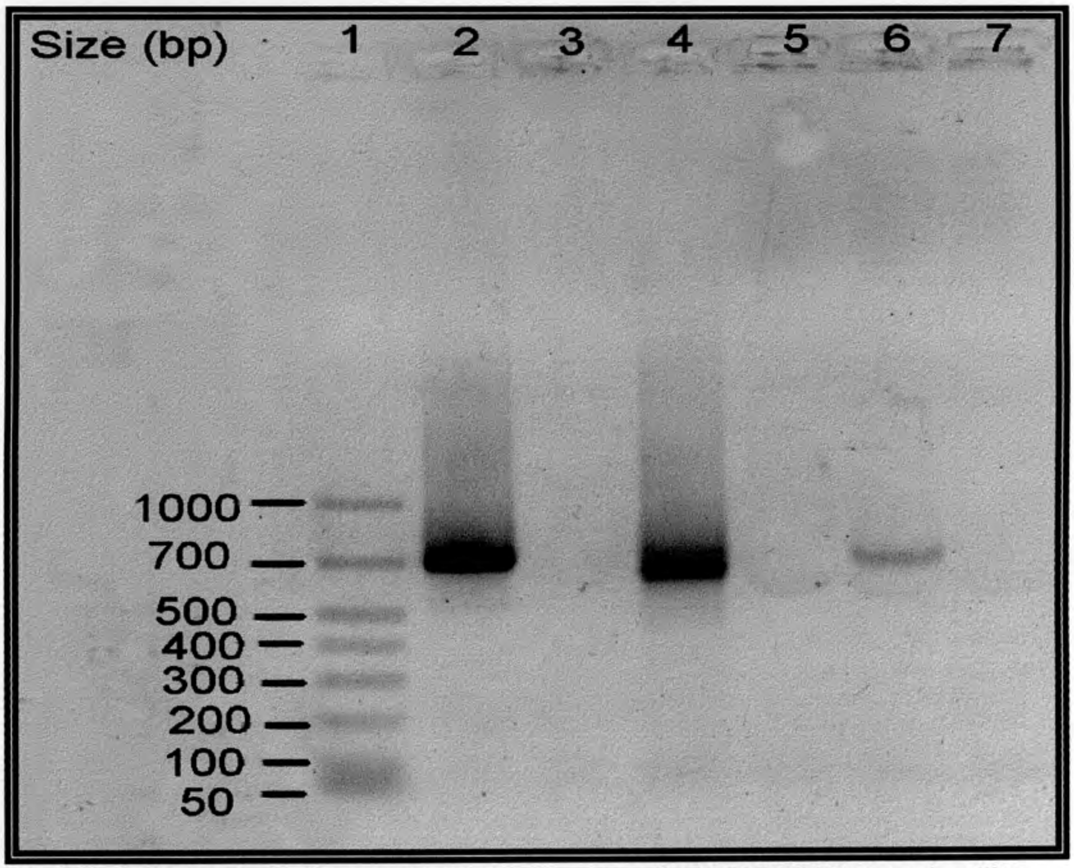


Fig. 6.1 PCR Products. Lane 1, BioMarker Low; lane 2, CABMV-SAP clone (positive control); lane 3, buffer control; lane 4, isolate 93/1; lane 5, healthy *N. benthamiana*; lane 6, isolate 93/65; lane 7, healthy *P. vulgaris*.

6.3.1.2 Sensitivity of the PCR systems

Neither of the PCR systems were as sensitive as expected.

Isolate 93/1 could clearly be detected down to 1.3 ng of virus (Fig 6.2), compared to 2.6 ng in the ELISA. Very faint bands were however still observed for 166 pg of virus.

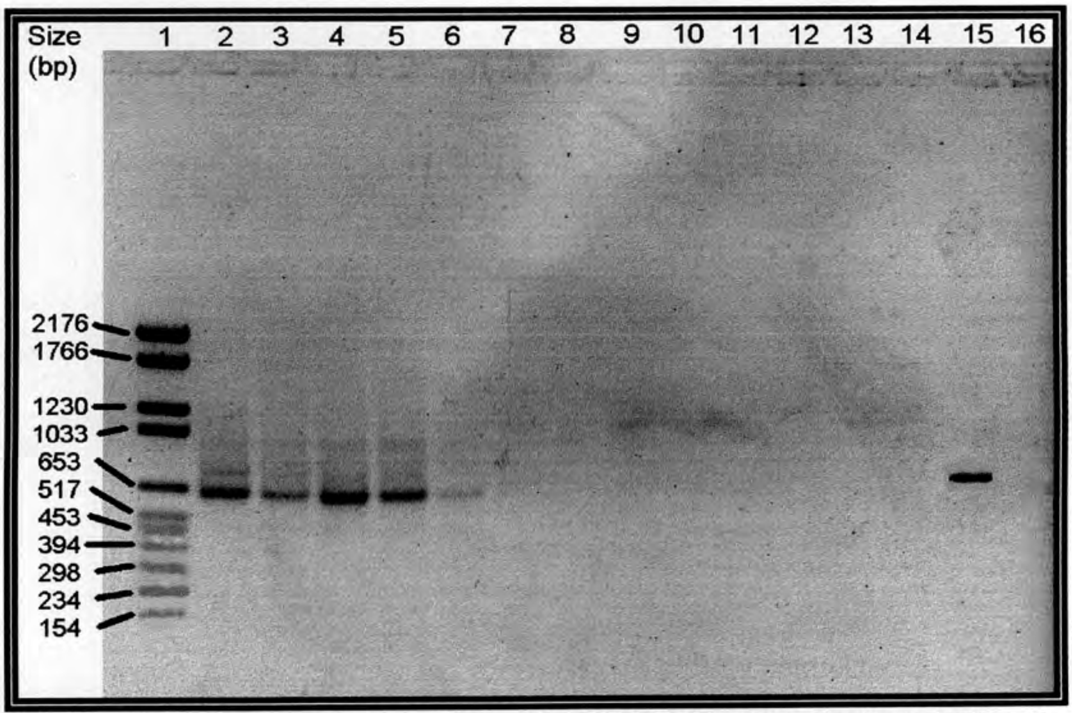


Fig. 6.2 Determination of the sensitivity of the IC-RT-PCR system for the detection of isolate 93/1. Lane 1, marker VI (Boehringer-Mannheim); lane 2, 10.5 µg virus, lane 3, 1.33 µg virus; lane 4, 166 ng; lane 5, 41.6 ng; lane 6, 1.3 ng; lane 7, 164 pg; lane 8, 41 pg; lane 9, 10 pg; lane 10, 1.28 pg; lane 11, 160 fg; lane 12, 40 fg; lane 13, 20 fg; lane 14, 10 fg; lane 15, positive control (CABMV-SAP clone); lane 16, buffer control.

Isolate 93/65 could be detected at 166 ng of virus, which is the same as in the $F(ab')_2$ ELISA. Very faint bands were observed at lower dilutions. These bands were too faint to be regarded as a positive result (Fig 6.3).

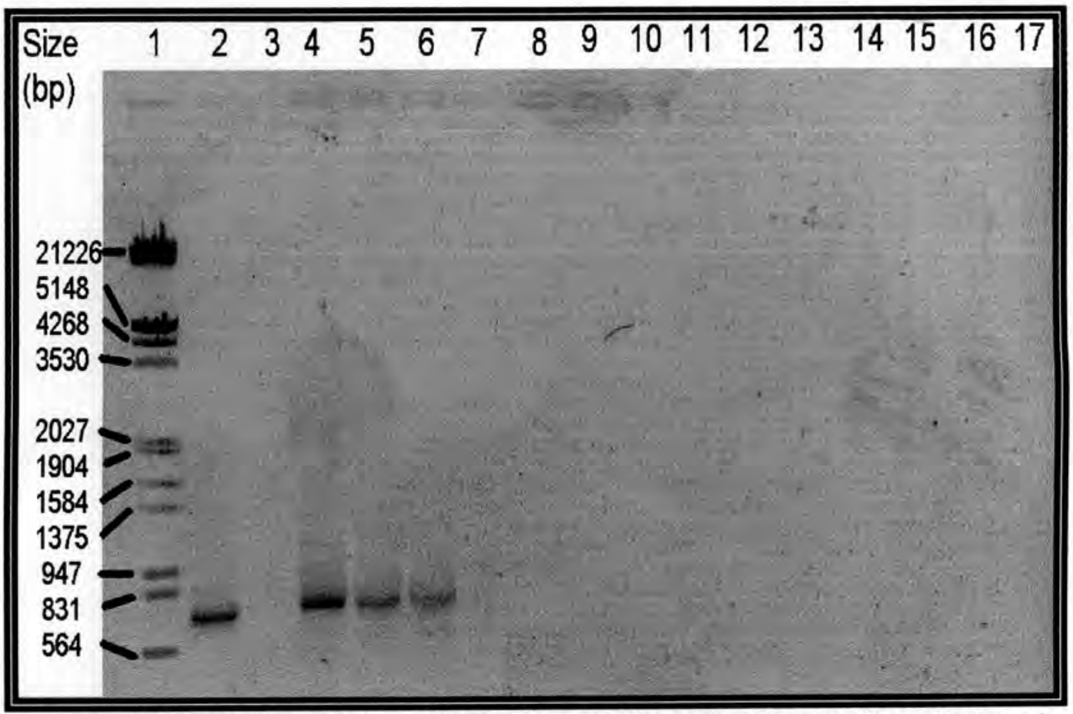


Fig. 6.3 Determination of the sensitivity of the IC-RT-PCR system for the detection of isolate 93/65. Lane 1,Marker III (Boehringer-Mannheim); lane 2, positive control (CABMV-SAP clone); lane 3,buffer control; lane 4, 21 µg; lane 5, 1.33 µg ; lane 6, 166 ng; lane 7, 10 ng; lane 8, 328 pg; lane 9, 41 pg; lane 10, 10 pg; lane 11, 2.56 fg; lane 12, 1.28 pg; lane 13, 640 fg; lane 14, 320 fg; lane 15, 160 fg; lane 16, 80 fg; lane 17, 40 fg.

6.3.1.3 Specificity of the PCR systems

When trapping with IgG directed against isolate 93/1, the following viruses could be detected: PVY 92/21, CABMV-SAP 92/749, CABMV 93/1510, PeMoV 94/2097, isolate 93/65, BYMV 92/751, and isolate 93/1 (Fig 6.4). All these viruses are known to be serologically related to ClYVV, and detection of these viruses with antiserum directed against isolate 93/1 is therefore expected. The ELISA system could however not detect PVY or the CABMV strains. The fact that the ELISA system did not detect PVY 92/21, could be due to the low concentration of the virus in the desiccated tissue that was used. Fresh plant material was used for the PCR. The PCR system was also able to detect CABMV isolates 92/749 and 93/1510 that the ELISA system could not. PCR detection of CABMV-SAP 92/749, CABMV 93/1510 and PeMoV 94/2097 were extremely weak and the bands barely visible. As the PCR is at least twice as sensitive as the ELISA system, the few virus particles trapped by the antiserum when the serological relationship is not very close, can only be detected in RT-PCR and not in ELISA.

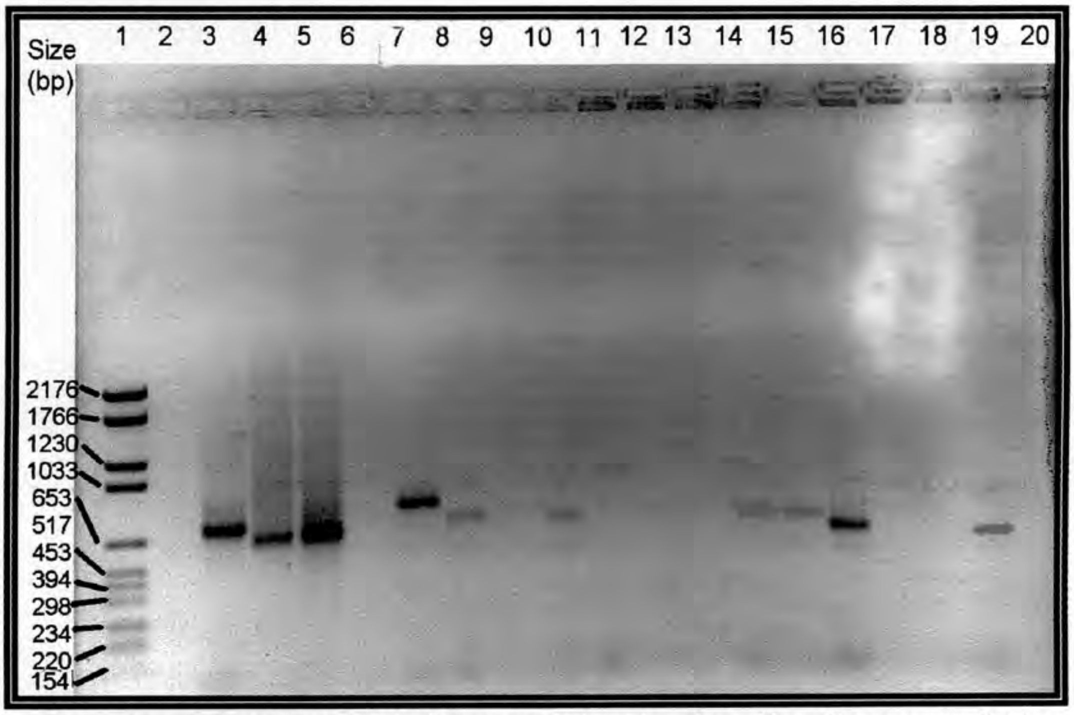


Fig 6.4 Specificity of the IC-RT-PCR system when trapping with antiserum specific for isolate 93/1. All samples, except isolate 93/1 in lane 4, were trapped with 93/1-specific IgG diluted 1: 100 in coating buffer. Lane 1, marker VI (Boehringer Mannheim); lane 2, buffer control; lane 3, positive control (CABMV-SAP clone); lane 4, isolate 93/1 without trapping; lane 5, isolate 93/1; lane 6, PStV 95/399; lane 7, PVY 92/21; lane 8, CABMV-SAP 92/749; lane 9, PStV 91/354; lane 10, CABMV 93/1510; lane 11, PeMoV 96/38; lane 12, PeMoV 94/2097; lane 13, healthy control *N. Benthamiana*; lane 14, isolate 93/65; lane 15, BYMV 92/751; lane 16, BCMV 92/750; lane 17, healthy control *P. Vulgaris* cv Bonus; lane 18, PMV 95/931; lane 19, healthy control *Pisum sativum*.

When trapping with IgG directed against isolate 93/65, more viruses were detected than in the ELISA system. The IC-RT-PCR system could detect isolates 93/1 and 93/65, PVY 92/21, PStV 91/354, BCMV 92/750, and BYMV 92/751 (Fig 6.5). CABMV 93/1510 could also be detected, although the reaction was very weak. The ELISA system could only detect isolate 93/65, PstV 95/399, and had a weak reaction against isolate 93/1. All the viruses detected in the PCR are known to be serologically related to BCMV. Reaction to PStV 95/399 and BCMV 92/750 is as expected, as isolate 93/65 is probably a strain of BCMV. As explained before, the non-detection of BCMV 92/750 by the 93/65-ELISA system, is due to the extraction buffer used in the ELISA. Although serological relatedness between BCMV and PVY has been described, it was not observed in the ELISA. Fresh material was used in the IC-RT-PCR test, while desiccated material was used in the ELISA. The concentration of PVY in the desiccated material could have been too low for detection as not all potyviruses survive desiccation over long periods of time (pers. experience).

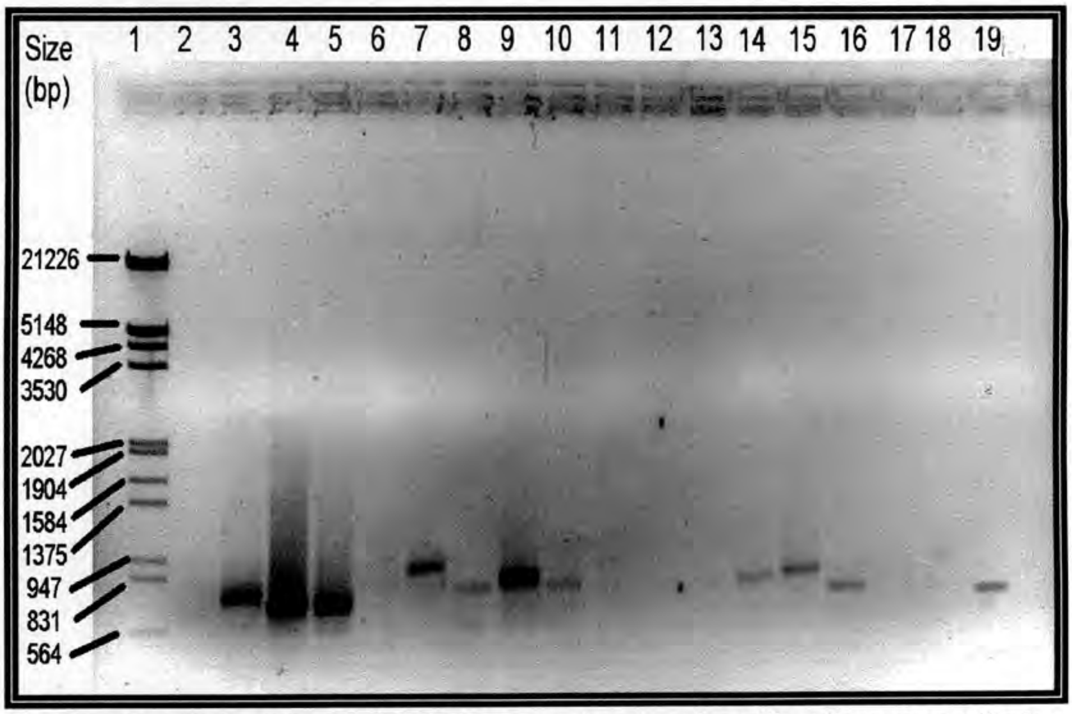


Fig 6.5 Specificity of the IC-RT-PCR system when trapping with antiserum specific for isolate 93/65. Samples were trapped with IgG specific for isolate 93/65 diluted 1:200 in coating buffer. Lane 1, marker III (Boehringer Mannheim); lane 2, buffer control; lane 3, positive control (CABMV-SAP clone); lane 4, isolate 93/1; lane 5, PStV 95/399; lane 6, PVY 92/21; lane 7, CABMV-SAP 92/749; lane 8, PStV 91/354; lane 9, CABMV 93/1510; lane 10, PeMoV 96/38; lane 11, PeMoV 94/2097; lane 12, healthy control *N. Benthamiana*; lane 13, isolate 93/65 (not trapped); lane 14, isolate 93/65; lane 15, BYMV 92/751; lane 16, BCMV 92/750; lane 17, healthy control *P. Vulgaris* cv Bonus; lane 18, PMV 95/931; lane 19, healthy control *Pisum sativum*.

No viruses known to be serologically unrelated to isolates 93/1 and 93/65 were detected in the PCR systems. Both systems were able to detect viruses that are apparently trapped in very low concentrations which the ELISA systems could not. This is due to the higher sensitivity of the PCR.

The different sizes of bands are due to the difference in the 3' untranslated region of the viruses. In Fig. 6.4 it can be seen that PVY 92/21 yielded a larger band than isolate 93/1. The band of isolate 93/1 is again larger than that of BYMV 92/751. This corresponds to the difference in size reported for their 3' untranslated regions: 331 bp for PVY, 177-178 bp for ClYVV, and 169-174 bp for BYMV (Shukla *et al.*, 1994). The bands observed for isolate 93/65 and PStV

91/354 are approximately the same in size. This is also expected as both are strains of BCMV.

Trapping of virus particles with antiserum is not necessary when the virus is present in high concentrations in the infected plants. This is demonstrated by the strong amplification of isolate 93/1 in the absence of trapping antibodies (lane 4, fig. 6.4). If the virus is present in low concentration, amplification of virus added directly to the PCR tube, is not very strong or can be absent (isolate 93/65, lane 13, figs. 6.5 and 6.6). These findings are further illustrated in figs 6.6 and 6.7. Viruses present in low concentrations (PStV 95/399 and PStV 91/354) had to be trapped with IgG directed against a related virus (isolate 93/65), for

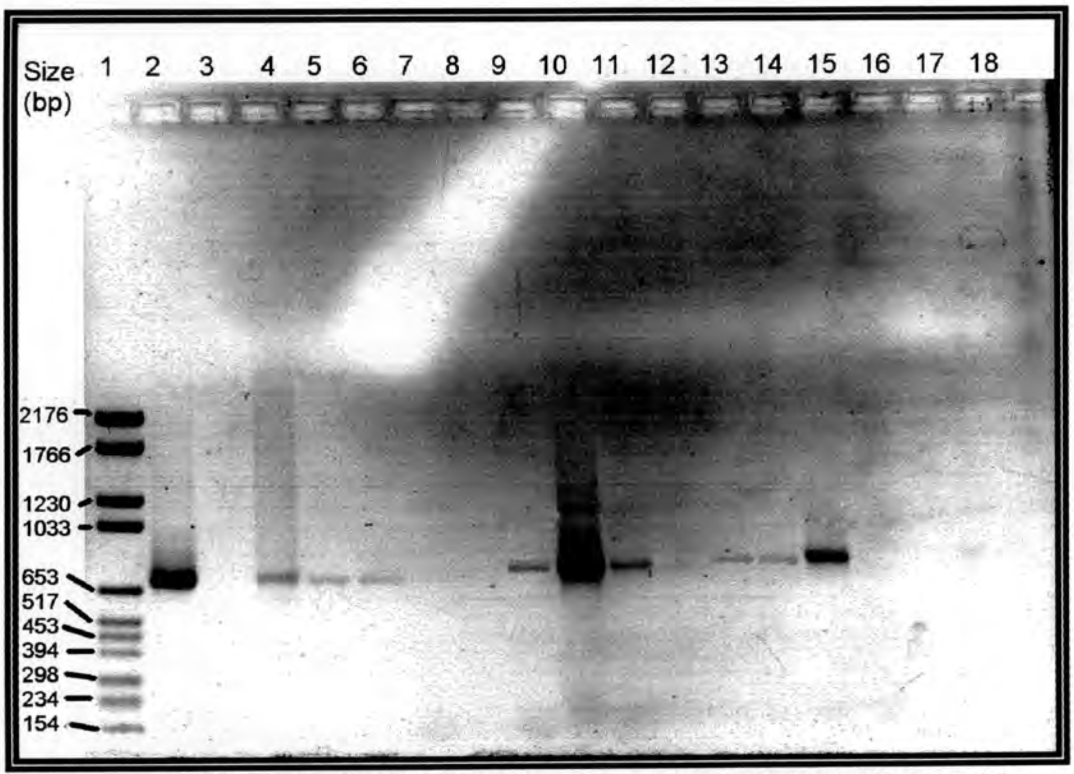


Fig. 6.6 Specificity of the IC-RT-PCR system. Lane 1, molecular weight marker VI (Boehringer Mannheim); lane 2, positive control (CABMV-SAP clone); lane 3, buffer control; lane 4, CABMV 93/1510 not trapped; lane 5, CABMV 93/1510 trapped with 93/1 IgG; lane 6, CABMV 93/1510 trapped with 93/65 IgG; lane 7, PStV 95/399 not trapped; lane 8, PStV 95/399 trapped with 93/1 IgG; lane 9 , PStV 95/399 trapped with 93/65 IgG; lane 10, CABMV-SAP 92/749 not trapped; lane 11, CABMV-SAP 92/749 trapped with 93/1 IgG; lane 12, CABMV-SAP 92/749 trapped with 93/65 IgG; lane 13, isolate 93/65 not trapped; lane 14, isolate 93/65 trapped with 93/1 IgG; lane 15 , isolate 93/65 trapped with 93/65 IgG; lane 16, healthy *N. benthamiana* not trapped; lane 17, *N. benthamiana* trapped with 93/1 IgG; lane 18, *N. benthamiana* trapped with 93/65 IgG.

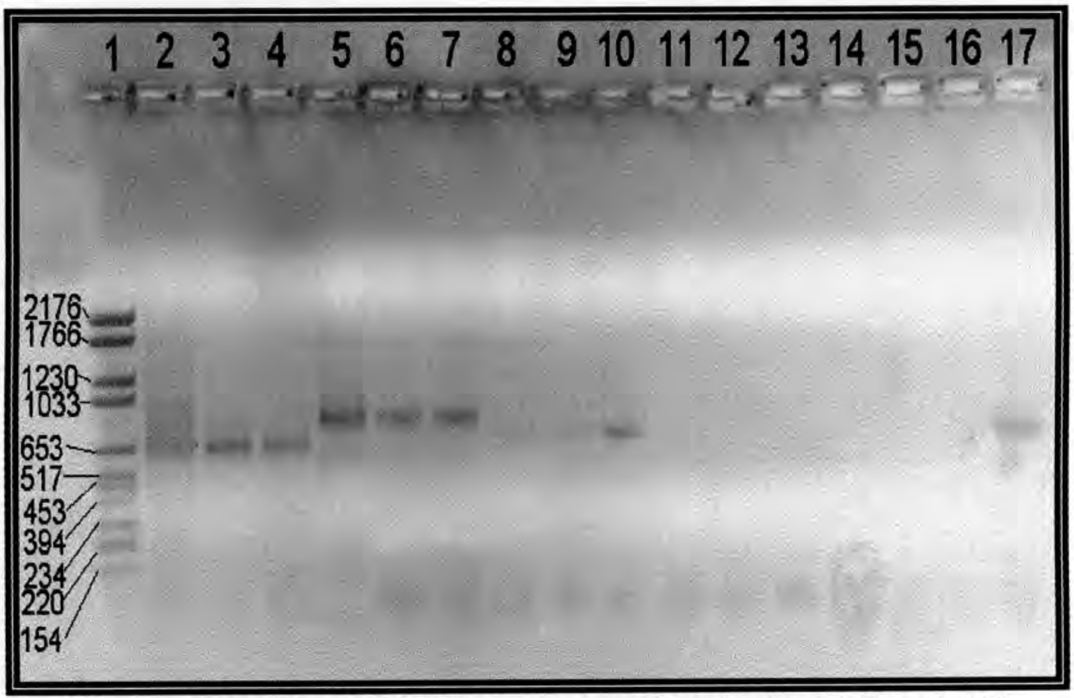


Fig. 6.7 Specificity of the IC-RT-PCR system. Lane 1, molecular weight marker VI (Boehringer Mannheim); lane 2, isolate 93/1 not trapped; lane 3, isolate 93/1 trapped with homologous IgG; lane 4, isolate 93/1 trapped with 93/65 IgG; lane 5, PVY 92/21 not trapped; lane 6, PVY 92/21 trapped with 93/1 IgG; lane 7, PVY 92/21 trapped with 93/65 IgG; lane 8, PStV 91/354 not trapped; lane 9, PStV 91/354 trapped with 93/1 IgG; lane 10, PStV 91/354 trapped with 93/65 IgG; lane 11, PeMoV 96/38 not trapped; lane 12, PeMoV 96/38 trapped with 93/1 IgG; lane 13, PeMoV 96/38 trapped with 93/65 IgG; lane 14, healthy *N. benthamiana* not trapped; lane 15, *N. benthamiana* trapped with 93/1 IgG; lane 16, *N. benthamiana* trapped with 93/65 IgG lane 17, positive control (CABMV-SAP clone).

amplification to occur. Trapping with virus-specific antisera also prevented the amplification of unrelated viruses. This was demonstrated when CABMV 93/1510 was amplified after trapping with IgG against isolates 93/1 and 93/65, or after directly coating the tubes with infected plant material. Amplification only occurred when no antisera was used for trapping. This corresponds to the ELISA results as neither of the systems could detect CABMV 93/1510. Strong amplification of CABMV-SAP 92/749 occurred only when virus material was used to coat the tubes. A very weak PCR product was observed when this virus was trapped with IgG directed against isolate 93/1. This corresponds to the ELISA results where CABMV-SAP 92/749 was only detected in the 93/1 ELISA system. The intensity of the PCR bands corresponded to the relatedness of the virus tested to the IgG used for trapping, as can be seen in the case of isolate 93/65. Stronger DNA bands were observed when the tubes were coated with infected plant material and when virus was trapped with the homologous IgG, than when the virus was trapped with 93/1-IgG. A slight reaction to isolate 93/1 was observed in the 93/65 ELISA system. PeMoV 93/68 was not amplified in this test. The virus occurs in very low concentrations and is not serologically related to isolates 93/1 or 93/65.

These results show that trapping of virus particles with IgG or antiserum makes the RT-PCR system more sensitive and specific.

6.3.1.4 Amplification of isolate 93/1 with BYMV and ClYVV-specific primers

Amplification with using the BYCY primer was not satisfactory (results not shown). Amplifications with the degenerate oligo(dT) primer in combination with the ClYVV- or BYMV-specific primers were satisfactory after the $MgCl_2$ was adjusted to 1.5 mM. Isolate 93/1 was amplified by both sets of primers. BYMV 92/751 was only amplified by the BYMV-specific primer in combination with the degenerate oligo(dT) primer. These results indicate that isolate 93/1 is different from the ClYVV isolates sequenced before (Genbank database) as well as the isolate used in the tests by Bariana *et al.*, 1994. The results of the amplifications can be seen in fig 6.8.

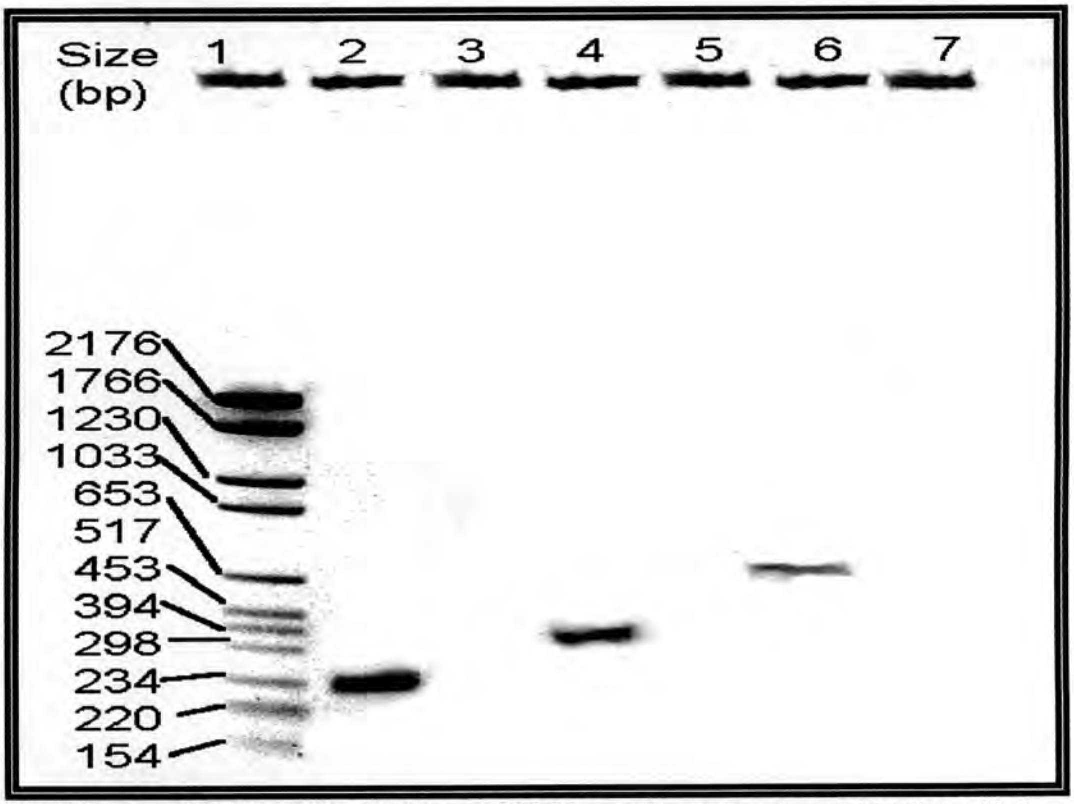


Fig. 6.8 Amplification with BYMV- and ClYVV-specific primers in combination with the degenerate oligo(dT) primer. Lane 1, molecular weight marker VI (Boehringer Mannheim); lane 2, amplification of isolate 93/1 with the ClYVV-specific primer; lane 3, healthy *N. benthamiana*; lane 4, amplification of isolate 93/1 with the BYMV-specific primer; lane 5, healthy *N. benthamiana*; lane 6, amplification of BYMV 92/751 with the BYMV-specific primer; lane 7, healthy *P. vulgaris* cv Bonus.

6.3.2 Ligation and transformation

Ligation of a PCR product prepared according to the Double GeneClean protocol into dephosphorylated vector, was more efficient than TA-cloning. I have subsequently learned that a final elongation step of at least 15 minutes is necessary for the addition of an overhanging A when using Dynazyme (Dynazyme Literature).

Clones 9365.24 and 9365.25 were prepared with TA cloning. All other clones were obtained from ligations into the dephosphorylated vector.

6.3.3 Selection of clones

Clones of isolate 93/65 had inserts of approximately 660 bp. Clones 9365.24, 9365.25, 9365.46, and 9365.48 were selected for sequencing. Fig 6.9 shows an enzyme digestion of various clones of isolate 93/65.

Fig. 6.9 Enzyme digestions of isolate 93/65 PCR product clones. Lanes 1 and 14, molecular marker III (Boehringer-Mannheim); lane 2, uncut pBluescript KS+; lane 3, clone 9365.9; lane 4, clone 9365.18; lane 5, clone 9365.21; lane 6, clone 9365.24; lane 7, 9365.25; lane 8, clone 9365.27; lane 9, clone 9365.39; lane 10, clone 9365.40; lane 11, clone 9365.43; lane 12, clone 9365.46; lane 13, clone 9365.48; lane 14, molecular weight marker III (Boehringer-Mannheim).

Clones of isolate 93/1 had inserts of approximately 660 bp. These inserts also have internal EcoRI, PstI, and XbaI sites. Digestion of the PCR products with XbaI yielded two fragments of approximately 420 bp and 240 bp; PstI digestions yielded fragments of 455 bp and 158 bp; EcoRI digestions yielded 440 bp and 140 bp fragments (Fig 6.10). Clones 931.1, 931.3, 931.44, and 931.50 were selected for sequencing.

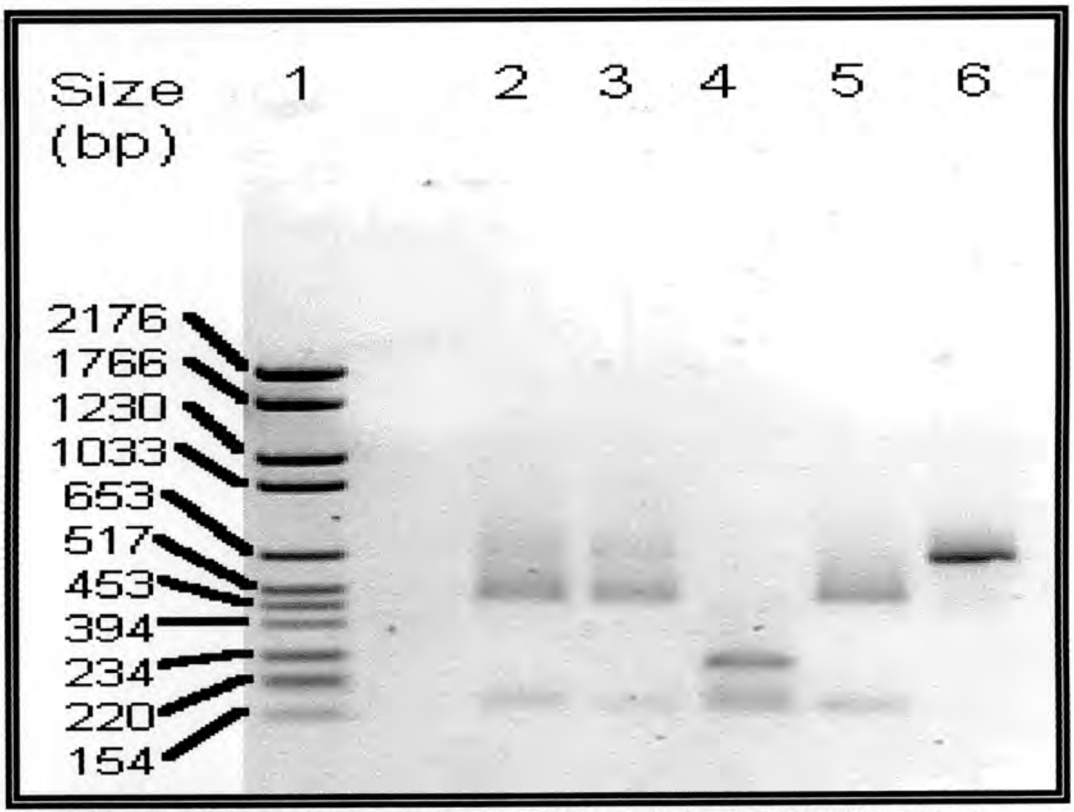


Fig. 6.10 Restriction enzyme digestions of PCR products of isolate 93/1. Lane 1, molecular weight marker VI (Boehringer-Mannheim); lane 2, PstI (Amersham); lane 3, PstI (Boehringer-Mannheim); lane 4, XbaI; lane 5, EcoRI, lane 6, uncut PCR product.

6.3.4 Nucleic acid sequencing

The standard M13 primers were initially used during the sequencing of the clones. As these primers are located far from the SmaI-cloning site, we decided to use the SK and KS primers instead. Overlaps between the reverse and forward primers were only found when using the KS and SK primers. Both isolates had compression areas which could not be overcome by using deaza-ddNTPs instead of normal ddNTPs. The compressions were also not alleviated when the amplification was performed at higher annealing temperatures. Technical assistance in completing the sequencing was therefore obtained from Mrs D. James, Department of Microbiology, UCT. Three clones of each virus were sequenced.

6.3.5 Nucleic acid sequence analysis

Isolate 93/1 showed local identity to ClYVV strains as determined with a BLAST search in Genbank. This corresponds to the serological data. The sequence data as well as the translation product of isolate 93/1 can be seen in fig 6.11.

The 3' non-coding region of isolate 93/1 is 175 nucleotides in length. This falls within the range reported for strains of ClYVV. No AUAUAUA or any other poly-adenylation signals as described for some other potyviruses, were observed in this region. An inverted repeat GAGGT/ACCTC as described for ClYVV-C (Ikegami *et al.*, 1995) is observed. These repeats for part of the consensus sequences described by Bryan *et al.* 1992. The Motif A sequence for isolate 93/1 is AGTGAGGT, and is separated by 3 nucleotides from Motif B.

Isolate 93/65 showed local identity with strains of BCMV. The alignments were however full of gaps. This corresponds to serological data that showed the virus to belong to the BCMV-group of potyviruses. The sequence data and translated protein of isolate 93/65 can be seen in Figure 6.12. The 3' non-coding region of isolate 93/65 is 225 nucleotides long. This is shorter than the 253-256 nucleotides reported by Khan *et al.*, 1993, or the 245 nucleotides reported by Fang *et al.*, 1995. An inverted repeat similar to that described by Bryan *et al.*, is observed in this region. The two motifs contain complementary changes, and are separated by 5 nucleotides. The sequence for Motif A is AGTGTGGT, and CCAC for motif B. A potential polyadenylation signal (TATGT) is observed 101-145 bp upstream from the poly(A) tail, in a similar position as described for BCMV-NL3 (Fang *et al.*, 1995).

```
1       TACATCAGGCGATCTTCAAGGTGAGTGGACAATGATGGATGGAGAGAAACAGGTAACATT
1        T  S  G  D  L  Q  G  E  W  T  M  M  D  G  E  K  Q  V  T  F

61      TCCACTTAAGCCAATTCTTGACTTTGCCAAACCAACATTGAGGCAAATTATGGCCCACTT
21       P  L  K  P  I  L  D  F  A  K  P  T  L  R  Q  I  M  A  H  F

121     TTCTCAGGCAGCCGAATCATACATAGAGTTCAGGAATTCAACAGAGAGGTACATGCCTAG
41       S  Q  A  A  E  S  Y  I  E  F  R  N  S  T  E  R  Y  M  P  R

181     GTATGGGCTGCAAAGAAATCTCACAGACTATGGATTAGCCAGATATGCATTTGACTTCTA
61       Y  G  L  Q  R  N  L  T  D  Y  G  L  A  R  Y  A  F  D  F  Y

241     CAGGTTGACTTCAAAGACACCAGCAAGAGCTCGGGAGGCACATATGCAAATGAAGGCAGC
81       R  L  T  S  K  T  P  A  R  A  R  E  A  H  M  Q  M  K  A  A

301     AGCAATAAGAGGGAAGTCAAATCACATGTTTGGGTTGGATGGCAATGTTGGAACAGACGA
101      A  I  R  G  K  S  N  H  M  F  G  L  D  G  N  V  G  T  D  E

361     GGAGAACACAGAGAGGCACACAGCAAATGATGTTAACAGGAACATGCATCACATTGCTGG
121      E  N  T  E  R  H  T  A  N  D  V  N  R  N  M  H  H  I  A  G

421     AGCACGATTCTAGAGTATCCGTCTTTAAATTCTCCATTTATTTCGAAGTTTTACTATTAT
141      A  R  F

481     AGCACTATGTTGAGTGAGGTTTTACCTCCATTTCATTTTAAGTATAATATGTAATCCATT

541     CTCTTTATTCTGACAGAGTAACTAAGTGAGGTTATACCTCGTTGTGAATCTGATCTTTAT

601     AAACG
```

Fig. 6.11 Translation of the nucleic acid sequence of isolate 93/1 with DNAMAN.

```
1     CACTTCACCGGACGTGAATGGCACATGGGTAATGACGGATGGAGACGAGCAAGTGGAGTA
1       T  S  P  D  V  N  G  T  W  V  M  T  D  G  D  E  Q  V  E  Y

61    CCCGCTCAAGCCAATGGTTGAAAACGCAAAGCCAACACTCCGCCAAATCATGCACCATTT
21      P  L  K  P  M  V  E  N  A  K  P  T  L  R  Q  I  M  H  H  F

121   CTCAGATGCAGCTGAAGCATACATTGAGATGAGAAATTCCGAGAGACCGTACATGCCTAG
41      S  D  A  A  E  A  Y  I  E  M  R  N  S  E  R  P  Y  M  P  R

181   GTACGGTCTACTTAGGAATTTGAGGGATAAAAATCTAGCTCGTTACGCTTTTGATTTCTA
61      Y  G  L  L  R  N  L  R  D  K  N  L  A  R  Y  A  F  D  F  Y

241   TGAAGTGACATCCAAAACATCGGATCGAGCAAGAGAAGCAGTAGCACAGATGAAGGCAGC
81      E  V  T  S  K  T  S  D  R  A  R  E  A  V  A  Q  M  K  A  A

301   AGCCCTCAGCAACGTTAACAGCAAGTTGTTTGGACTTGATGGTAACGTGGCAACAACCAG
101     A  L  S  N  V  N  S  K  L  F  G  L  D  G  N  V  A  T  T  S

361   CGAGAATACTGAAAGGCACACAGCAAGGGACGTTAATCAGAACATGCATACACTTCTTGG
121     E  N  T  E  R  H  T  A  R  D  V  N  Q  N  M  H  T  L  L  G

421   CATGGGCTCCCCGCAGTAAAGATTGGGTCAACTGATCACAGTTAGCATCTCGCGTCGCTG
141     M  G  S  P  Q

481   AATAGTTTCGTATAGTAATCTTTTATGTTCTCTTTAGTTTCAGTGTGGTTTTACCACCTT

541   TGTGTTACTATGTGATAGTGTGGTTGGATCCACCAACATATTGTGAGTACTTTATGTTTA

601   TGAGTAAGCCGGAAGAACCATTGCAATGGCGAGGACATGCAGATGGATTTCATCACGCGT

661   CATGGAGTAGCTACGGCAATGTTTGTTGTTC
```

Fig 6.12 Translation of the nucleic acid sequence of isolate 93/65 with DNAMAN.

Alignment of the nucleic acid sequences of part of the CP and the 3' non-coding regions, showed that isolate 93/1 is closely related, but not identical, to ClYVV strain B. Alignment of the protein sequences showed that isolate 93/1 has a closer relationship to ClYVV isolate 30 than to ClYVV strain B. As the sequence identity as determined in a nucleic acid sequence homology matrix between isolate 93/1 and ClYVV-B, ClYVV-30, and a third strain of ClYVV is 95.4%, 94%, and 93.5% respectively, isolate 93/1 can be considered a new strain of ClYVV.

Protein alignments showed that isolate 93/65 is closely related to BCMV strain NL2, and to BlCMV-W. The nucleic acid alignments showed a closer relationship to BCMV strain US4, with strain NL2 the closest relationship after that. These results do not correspond to the pathogroup determination, where isolate 93/65 was closest to strain NL4. When the relationship between the pathogroup and the nucleic or amino acid sequence data is observed for other strains of BCMV, no consistent patterns are observed. The BCMNV strains do however form a separate group. Isolate 93/65 is not closely related to the two newly described members of the BCMV subgroup, DEMV and AzBMV. The nucleic and amino acid alignments indicate that isolate 93/65 is a new strain of BCMV.

The nucleic acid and amino acid sequence alignments can be seen in Figures 6.13 to 6.16. A detailed list of the nucleic acid and amino acid sequences used in the comparisons, can be seen in Appendix B.

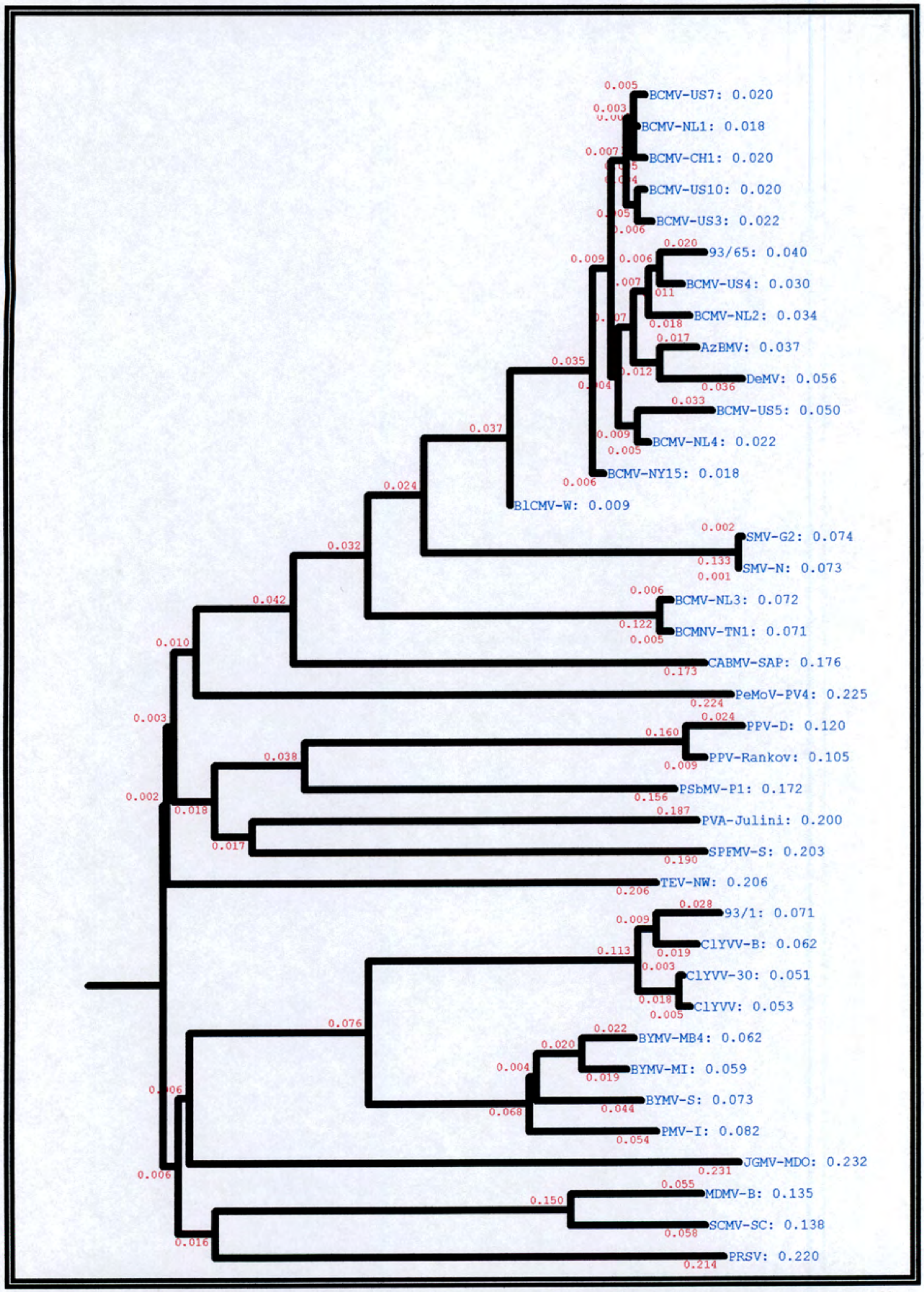


Fig. 6.13 Phylogenetic tree of the nucleic acid sequences of part of the CP gene as well as the 3' non-coding region of several potyviruses, computed from aligned sequences with DNAMAN. Numbers in red indicate branch lengths, while numbers in blue indicate sequence weights.

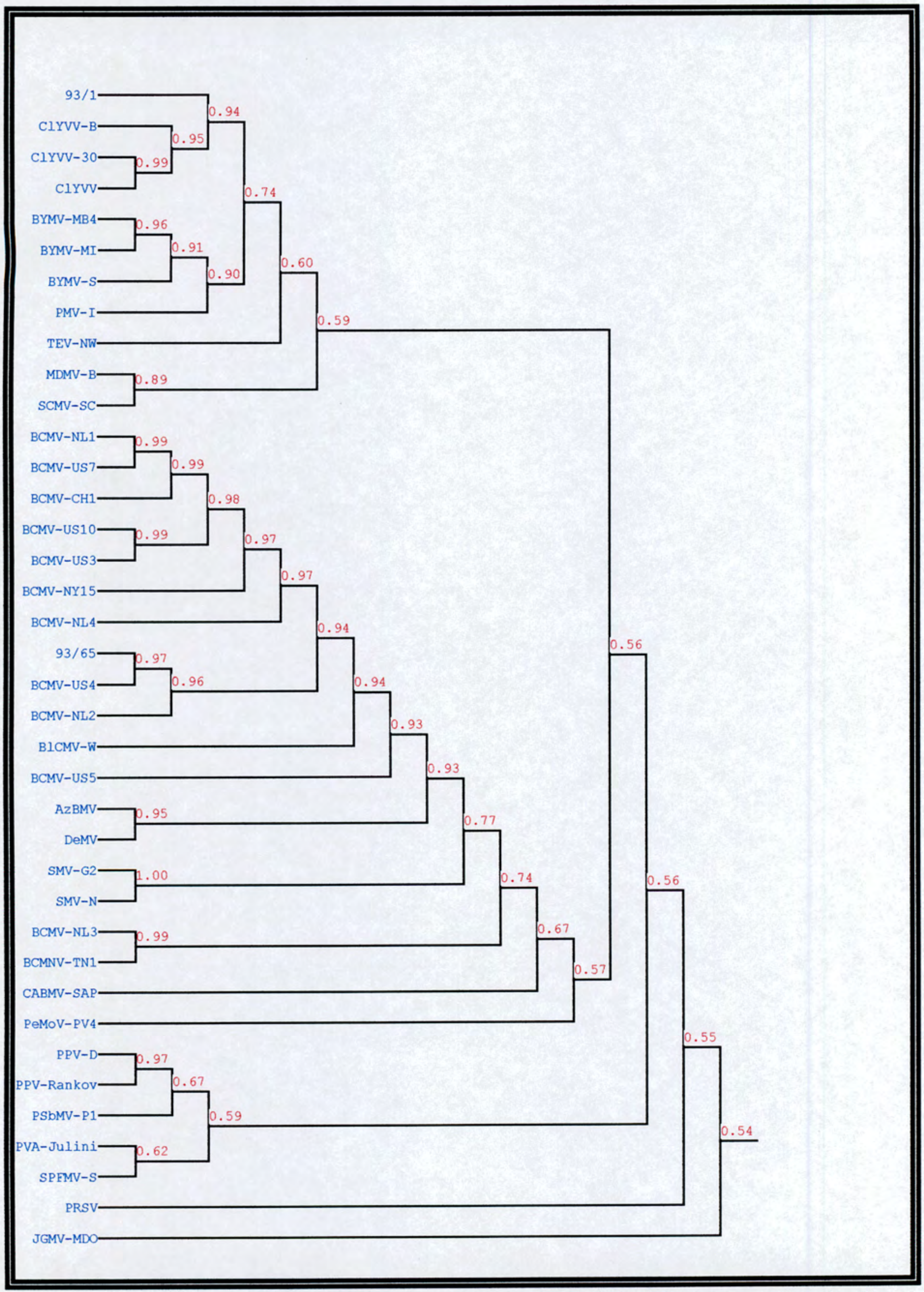


Fig. 6.14 Homology tree of the nucleic acid sequences of part of the CP gene as well as the 3' non-coding region of several potyviruses, computed using DNAMAN from aligned sequences. Numbers in red indicate homology, where 1 equals 100% homology.

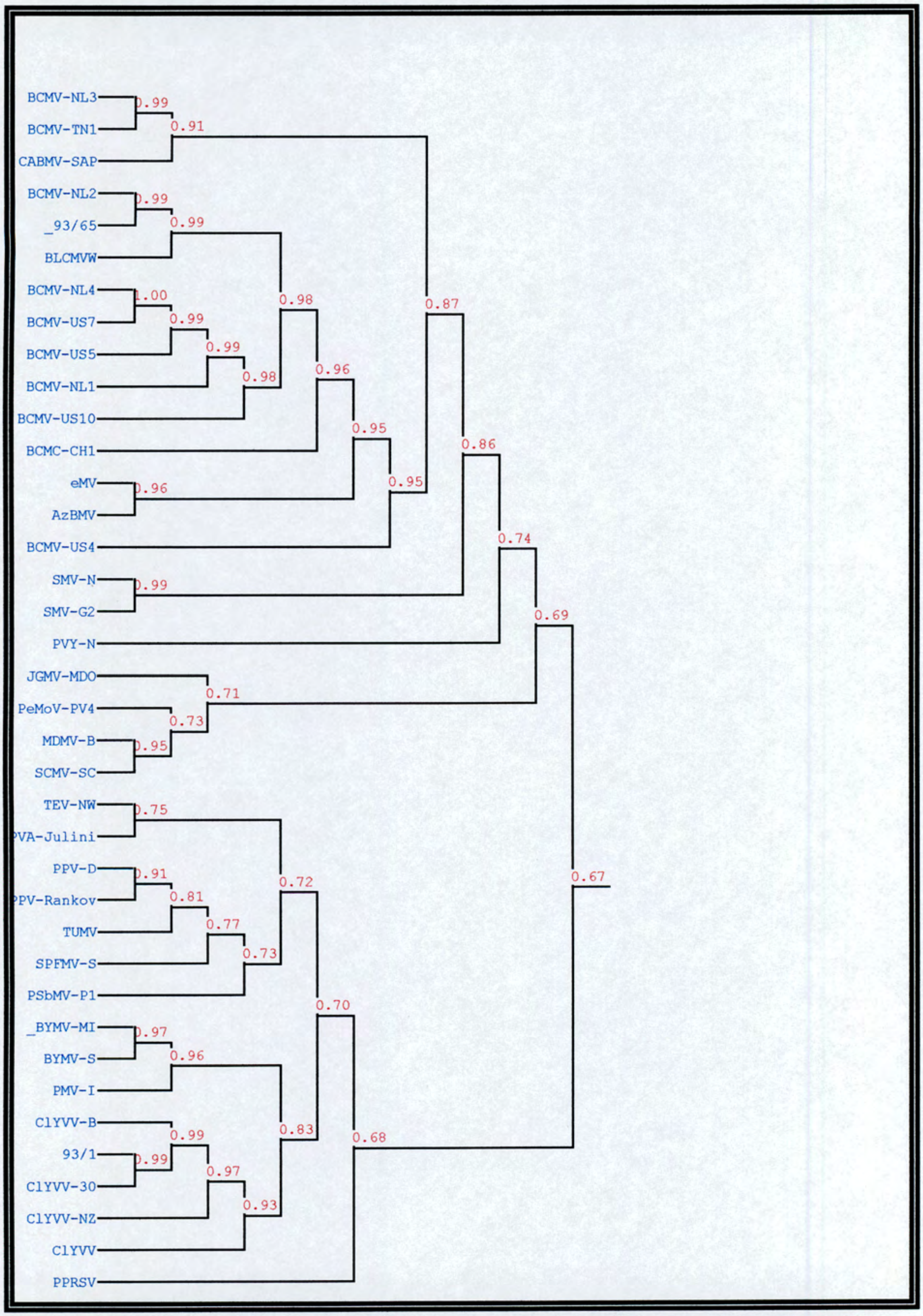


Fig. 6.15 Homology tree drawn with DNAMAN, comparing the partial coat protein amino acid sequences of several potyviruses. Numbers represent homology, with 1 indicating 100% homology.

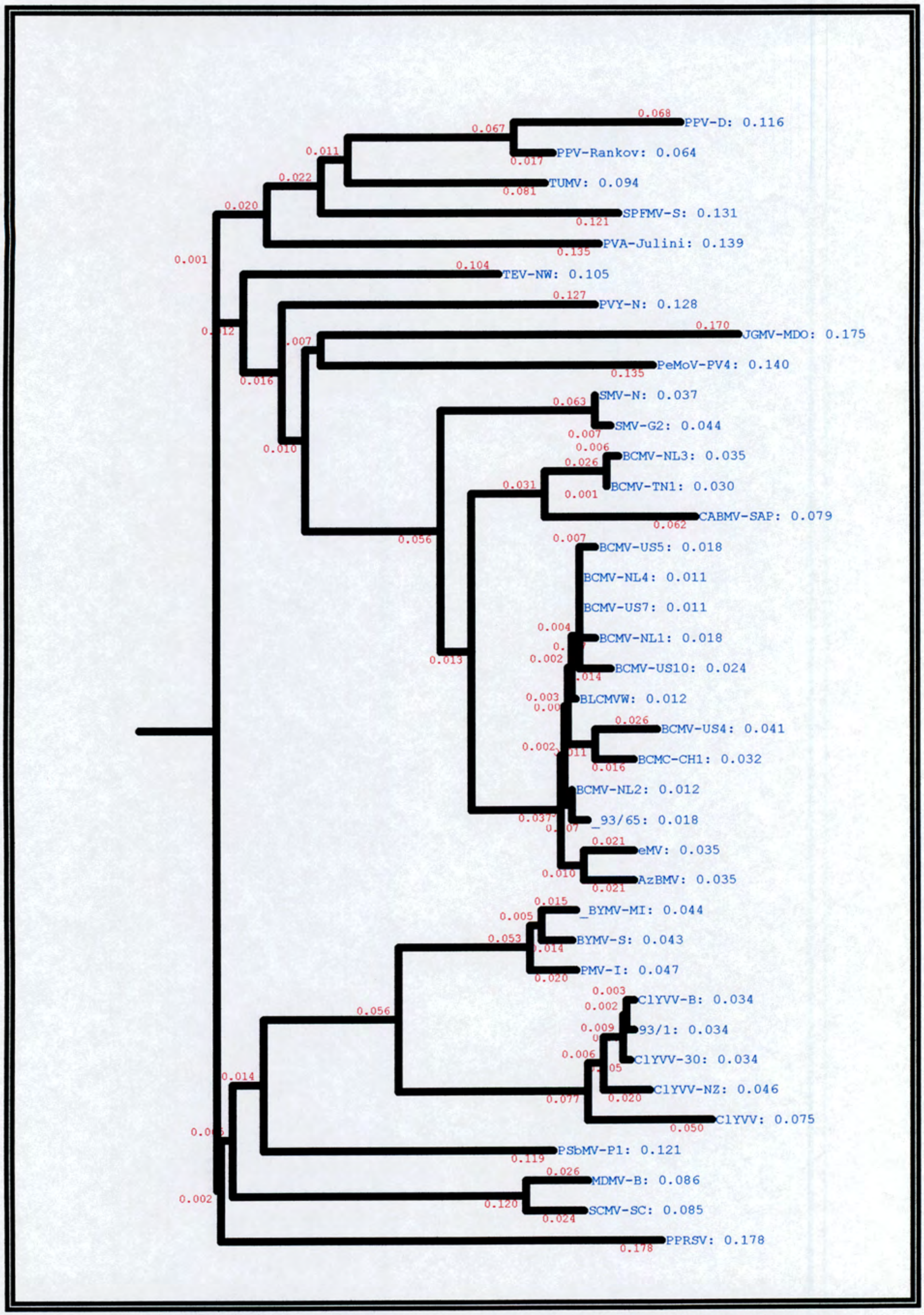


Fig. 6.16 Phylogenetic tree of the amino acid sequences of part of the CP gene of several potyviruses. Numbers in blue indicate sequence weights, and numbers in red indicate branch lengths.

6.3 CONCLUSION

Using nucleic acid sequence data of part of the coat protein gene as well as the 3' non-coding region, isolate 93/1 has been positively identified as a strain of ClYVV, and isolate 93/65 as a strain of BCMV.

Different relationships were observed when the nucleic acid sequences or the translated amino acid sequences were aligned to that of other potyviruses. When the amino acid sequences were compared, isolate 93/65 showed a closer relationship to BCMV-NL2, and 93/1 to ClYVV isolate 30. When the nucleic acid sequences were compared, isolate 93/65 showed a closer relationship to BCMV-US4, with strain NL2 the closest after that. Isolate 93/1 showed a closer relationship to ClYVV strain B when the nucleic acid sequences of different potyviruses were aligned. Both isolate 93/1 and 93/65 can however be considered new strains of ClYVV and BCMV respectively as indicated by their homology with cognate strains of these viruses.

The nucleic acid sequence data therefore confirms the serological data that isolates 93/1 and 93/65 are related, but not identical, to the strains of ClYVV and BCMV used in the tests. It also explains why the host ranges were narrower than expected, and why the CP of isolate 93/1 was slightly larger than reported in the literature for other strains of ClYVV.

Symptomatology, serology, and nucleic acid sequence data all confirmed that isolate 93/1 is a strain of ClYVV and isolate 93/65 a strain of BCMV.

APPENDIX A

STANDARD METHODS

1. Storage of bacterial cultures

Bacterial cell suspensions were grown overnight in LB-broth containing the relevant antibiotic, and stored frozen at -80°C in the presence of 15% glycerol.

2. Preparation of competent cells

Competent *Escherichia coli* DH5α cells were prepared with the $CaCl_2$ method. *E. coli* DH5α cells were grown overnight with shaking at 37°C. One to two ml of the overnight culture was added to 100 ml LB-Broth and incubated at 37°C with shaking. The cells were grown until they reached a value of 0.5 at 550 nm. The cells of 20 ml of the suspension were collected by centrifugation at 5 000 rpm for 5 minutes at 4°C in a Beckman JA 20 rotor. The cell pellet was resuspended in 10 ml of ice-cold 50 mM $CaCl_2$. The cells were centrifuged for 5 minutes at 5 000 rpm, 4°C in a Beckman JA 20 rotor. The cells were resuspended in 1 ml of ice-cold $CaCl_2$ and left on ice for 3 hours or overnight before use.

3. Alkaline lysis miniprep method of purifying plasmids

Plasmids were maintained in *E. coli* DH5α. A culture containing the plasmid was grown overnight at 37°C with shaking in LB-broth containing 100 µg/ml ampicillin. One ml of this culture was used to inoculate 100 ml of LB-broth containing 100 µg/ml ampicillin. The cells were grown overnight at 37°C with shaking. The cells were collected by centrifugation at 10 000 rpm for 5 minutes in a JA 20 rotor. The pellets were resuspended in 8 ml of solution I (50 mM Glucose, 10 mM EDTA, 25 mM Tris-HCl, pH 8.0), and kept on ice for 30 minutes 16 ml of solution II (0.2 N NaOH, 10% SDS) was added, the cells were mixed gently and kept on ice for 5 minutes 12 ml of solution III (3 M sodium acetate pH 4.8) was added and the mixture left on ice for 30 - 60 minutes The mixture was centrifuged for 10 minutes at 15 000 rpm in a JA 20 rotor. The supernatant was transferred to a glass tube (Corex tube) and precipitated with 2 vol 96% ethanol (72 ml) at -20°C for at least 30 minutes The DNA was collected by centrifugation for 10 minutes at 8 000 rpm in a JA 20 rotor. The precipitate was resuspended in 6 ml of TE buffer (10 mM Tris-Cl pH 8.0, 1 mM EDTA). 2.5 ml of 7.5 M ammonium acetate was added and the mixture incubated on ice for at least 30 minutes The RNA precipitate was removed by centrifugation at 8 000 rpm for 10 minutes in a JA 20 rotor. After a final ethanol precipitation, the DNA was collected by centrifugation at 8 000 rpm for 10 minutes and washed with 80% ethanol.The pellet was dried and resuspended in 400 µl 1x TE buffer. Any remaining RNA was removed by RNase digestion and PEG precipitation. 4 µl of a 10mg/ml RNase solution was added to 400 µl of plasmid DNA. The mixture was incubated at 37°C for 60 minutes. 240 µl of PEG 6000 (20% in 2.5 M NaCl) was added and the mixture incubated on ice for 30 minutes. The plasmid was collected by centrifugation for 10 minutes at 8000 rpm in a Sigma microcentrifuge. The pellet was washed three times with 80% ethanol, dried and dissolved in 400 µl TE.

4. Preparation of blunt-ended vector

Plasmid pBluescript KS+ was maintained in *E. coli* DH5α. The plasmid was purified according to the alkaline lysis miniprep method. The SmaI-cut dephosphorylated plasmids were prepared according to the method of Sambrook *et al.* (1989). 10 - 20 µg of the plasmid was digested to completion with a twofold excess of SmaI (Boehringer Mannheim) at 25°C for 60 minutes in buffer A (33 mM Tris-acetate, 66 mM potassium acetate, 10 mM magnesium acetate, 0.5 mM DTT, pH 7.9). The plasmid was purified by phenol:chloroform extraction and ethanol precipitation. The DNA was recovered by centrifugation at 12 000 g for 10 minutes at 4°C in a Sigma 101 M microcentrifuge, and the pellet dissolved in 90 µl TE pH 8.3. 10 µl of 10x calf intestinal alkaline phosphatase (CIAP) dephosphorylation buffer (10 mM $ZnCl_2$, 10 mM $MgCl_2$, 100 mM Tris.Cl pH 8.3) and 1 u CIAP (Promega) per 2 pmol termini was added to the DNA. The reaction was incubated at 37°C for 60 minutes, after which it was stopped by addition of 6 µl SDS and 11 µl EDTA. All trace of the enzyme was removed by performing a phenol:chloroform extraction and ethanol precipitation. The DNA was redissolved in TE buffer pH 7.6 at a concentration of 100 µg/ml and stored in aliquots at -20°C. Test ligations were performed to establish the success of the dephosphorylation.

5. Ligation

PCR products were purified and ligated according to the Double GeneClean protocol as described in the addendum to the GeneClean Kit Instructions (BIO101 Inc.)

After PCR, the reaction mixture was transferred to a new tube, leaving the oil behind. 3 vol of NaI and 15 µl of Glassmilk was added to the PCR product and left on ice for 5 to 60 minutes. The DNA was washed three times with NEW Wash as described in the GeneClean Kit protocol (BIO101 Inc.) and eluted in 50 µl of water. The ends were made flush for blunt end cloning by treating the cleaned product at 37°C for 1 hour with 10 u of DNA polymerase I (Amersham International) and 10 u of T4 polynucleotide kinase (Amersham International) in polymerase buffer (0.05 M Tris pH 7.5, 0.01 M $MgCl_2$, 1 mM DTT, 0.05 mg/ml BSA, 20 µM dNTP's) containing 0.001M ATP. The reaction was stopped by adding 1 µl of 0.5 M EDTA pH 8.0. The DNA was adsorbed to 10 µl of Glassmilk, washed with NEW wash and eluted in 20 µl of water as described in the Kit.

0.5 µg of the PCR product was ligated to 0.5 µg of dephosphorylated vector with 5 u of T4 DNA ligase (Amersham International) in ligation buffer (0.066 M Tris, 0.005 M $MgCl_2$, 1 mM DTT, 1 mM ATP) in a total reaction volume of 10 µl. The mixture was incubated at 22°C for 16 h, before transforming into competent *E. coli* DH5α cells.

6. Transformation

100µl of the competent cells were pipetted into a glass tube (Corex tube) and mixed with 5 - 10 µl of the DNA that was to be used for transformation. The mixture was left on ice for 30 minutes. The cells were given a heat shock for 90 seconds at 42°C, and cooled on ice for 2 minutes. 1 ml L-Broth was added and the mixture incubated with shaking at 37°C for 60 minutes, after which it was mixed with 40 µl of X-gal (2% w/v in N,N-dimethylformamide) and 10 µl of IPTG (2.4% w/v in water) and plated out onto agar plates containing 100 µg ampicillin/ml LB-broth. The plates were incubated at 37°C for 16 hours.

7. Selection of positive clones

Alkaline lysis miniprep plasmid extraction was performed. Selected clones (*i.e.* white colonies) were picked up with toothpicks from the agar plates and were grown overnight in L-broth at 37°C. The cells in 3 ml of the culture were collected by centrifugation at 8 000 rpm for 2 minutes in a Sigma 101M microcentrifuge. The cells were resuspended in 100 µl of solution I (50 mM Glucose, 10 mM EDTA, 25 mM Tris-HCl, pH 8.0). The suspension was incubated at room temperature for 5 minutes and left on ice for one minute. before adding 2 vol. of cold solution II (0.2 N NaOH, 10% SDS). The mixture was left on ice for 5 minutes after being briefly vortexed. 0.5 vol. of solution III (3 M sodium acetate pH 4.8) was added and the mixture left on ice for at least 5 minutes after a brief vortex. After centrifugation for 5 minutes at 12 000 rpm in a Sigma microcentrifuge, the aqueous phase was collected and the plasmids precipitated by addition of 2 vol. of absolute ethanol. The plasmids were collected by centrifugation at 12 000 rpm in a Sigma microcentrifuge for 15 minutes at 4°C after leaving the ethanol mixture at -20°C for 30 minutes. The resulting pellet was dissolved in water.

8. Electrophoresis of plasmids

Plasmids were run on agarose gels in TAE buffer (0.04 M Tris-acetate, 0.02 M EDTA, pH approximately 8.5), and the recombinants compared to the size of standard pBluescript KS+ plasmids. Recombinants larger than the standard plasmids were selected. The sizes of the inserts of the recombinants were determined by digesting the plasmids with XbaI and EcoRI for one hour at 37°C in buffer H (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT). The digestions were run on a 1% agarose gel in TAE buffer (0.04 M Tris-acetate, 0.002 M EDTA, pH approximately 8.5).

9. DIG cycle sequencing

DIG cycle sequencing was performed as per the instructions in the Boehringer Mannheim DIG Sequencing kit. Sequencing was performed on plasmids purified according to the alkaline lysis miniprep method described before. Plasmids were not denatured before making up the annealing mix. The annealing mix consisted of 1 pmol plasmid DNA, 1 pmol primer, 2 µl of the reaction mixture (250 mM Tris-HCl pH 9.0, 50 mM $MgCl_2$), and 3 u Taq DNA polymerase (Boehringer Mannheim, Germany). The total reaction volume was 20 µl.

After mixing the reagents well, 4 µl of the annealing reaction was added to 2 µl of each of the four termination mixtures.

The PCR conditions were as follows:
M13 Forward primer:
    one cycle of 94°C for 5 minutes
    30 cycles of 94°C for 30 sec, 70°C for 60 sec
M13 reverse primer, KS and SK primers:
    one cycle of 94°C for 5 minutes
    30 cycles of 94°C 30 sec, 60°C 30 sec, 70°C 60 sec

The reactions were terminated by adding 2 µl of the formamide buffer (98% deionised formamide, 0.2% bromophenolblue, 0.2% xylene cyanol, 10 mM EDTA). The reactions were be stored at -20°C until used.
The nucleic acid sequence of the custom made primers (MWG-Biotech, Germany) were as follows:
KS-Primer 5'-DIG-TCGAGGTCGACGGTATC-3'
SK-Primer 5'-DIG-CGCTCTAGAACTAGTGGATC-3'

10. Sequencing gel electrophoresis and blotting

The gel was made up as follows:
    21 g Urea
    6 ml 10x TBE pH 8.0 (0.089 M Tris base, 0.089 M boric acid, 0.004 M EDTA)

5 ml Long Ranger concentrate (AT Biochem)
Water to a final volume of 50 ml
25 µl TEMED
250 µl 10% ammonium persulfate

Both glass plates were thoroughly washed before treating one with Gel-slick (AT Biochem) and the other with Bind-Silane (γ-methacryloxypropyltrimethoxysilane, Sigma). One ml of the Gel-slick was poured onto the glass small plate, wiped evenly over the plate and left to dry. The plate was buffed with tissue to ensure as smooth surface. The second plate was wiped with a mixture of 15 µl of bind silane and 375 µl of acetic acid in 12,5 ml of absolute ethanol. The plate was allowed to dry before wiping with ethanol. The larger plate was laid flat on the bench and spacers put along the sides. A strip of filter paper was put along the bottom of the gel, and the small glass plate put on top. The plates were clamped together with the bulldog clamps, and the entire length of the plate as well as the bottom was covered with Scotch tape to make a watertight seal. The gel solution was poured between the plates and the flat side of the shark-tooth comb was inserted at the top of the gel and clamped into position. The gel was polymerised for at least 60 minutes.

The gel was prerun in 0.6x TBE for 60 minutes at 50 W before loading the samples. The samples were denatured immediately before electrophoresis by incubating at 95°C for 3 minutes and cooling on ice. For 2 hour electrophoresis 3.5 µl of each reaction was loaded onto the gel. For longer electrophoresis, the entire sample was loaded.

After electrophoresis, the glass plate treated with Gel-slick was removed and a Nylon membrane (Boehringer-Mannheim, Germany) was layered onto the gel. The membrane was covered with a sheet of Whatman filter paper. The filter paper was covered with a glass plate and charged with weight of approx. 2 kg for at least 20 minutes. The transferred DNA was cross-linked to the membrane for 3 minutes on a UV transilluminator.

11. DIG Detection

DIG detection of the sequences was performed according to the instruction in the Boehringer-Mannheim DIG sequencing kit. The membrane was placed in a plastic bag and rinsed for 1 - 5 minutes in 120 ml of washing buffer (maleic acid buffer containing 0.3% (w/v) Tween 20). The membrane was then incubated overnight at 4°C in 120 ml of blocking buffer (1% w/v blocking reagent in maleic acid buffer). The membrane was then incubated with shaking for 30 minutes at room temperature in 120 ml anti-DIG-alkaline phosphatase conjugate diluted 1:10000 in maleic acid buffer pH 7.5 (0.1 M Maleic acid, 0.15 M NaCl). The membrane was washed twice for 15 minutes in 120 ml of washing buffer, before equilibrating it in detection buffer (0.1 M Tris-HCl pH 9.5 (20°C), 0.1 M NaCl, 50 mM $MgCl_2$). The substrate (CSPD or CDP-Star) was diluted 1:1000 in detection buffer, and the membrane incubated for 5 minutes with shaking in 10 ml of this solution. The membrane was removed from the plastic bag and placed onto a Whatman filter for 1-3 minutes, taking care that the membrane does not dry out. The membrane was placed in a new plastic bag and exposed to X-ray film for 2 h at 37°C when using CSPD or 1 h at room temperature when using CDP-Star.

12. Storage of virus-infected plant material

12.1 Dessication

Two to five leaves were wrapped in tissue paper and placed in a dessicator-vessel containing $CaCl_2$ for three weeks. The leaves were then placed in a polytop bottle over $CaCl_2$, the lids were sealed with wax and the bottles stored at 4°C.

12.2 Freeze-drying

Plant material is macerated in a 1:1 ratio of a freshly prepared solution of 7% (w/v) glucose and 7% (w/v) peptone. The extract was placed in a vial and freeze-dried overnight (Hollings and Lelliott, 1960). The vials were heat-sealed under vacuum and stored at 4°C.

12.3 Storage at -80°C

Leaf material was placed in cryovials and stored at -80°C.

13. ISEM (Immunosorbent electron microscopy)

Grids were coated with antiserum diluted in 0.1 M phosphate buffer pH 7.1 and incubated at 37°C for one hour. The grids were then washed with buffer, placed on the virus extract and incubated at 37°C for four hours. The grids were washed with buffer, placed on antiserum and incubated at 37°C for one hour. The grids were given a final wash and stained with uranyl acetate.

14. Immunisation

Rabbits were immunized with between 0.1 and 1 mg virus per injection. The second immunization was performed two to three weeks after the primary injection, with all other injections at least three to four weeks apart. Freund's complete adjuvant was used for the primary injection, and the incomplete adjuvant for all subsequent injections. All injections were intramuscular. The rabbits were bled at weekly intervals after completion of the immunization schedule. The blood samples were incubated for approximately 2 hours at 37°C before placing it at 4°C for 16 hours. Trapped erythrocytes were removed form the serum by centrifugation for 10 minutes at 8000 rpm in a Beckman JA 20 rotor. The serum was mixed with a equal volume of glycerine and stored at 4°C. The titre of each bleed was determined with F(ab')$_2$ ELISA.

15. Purification of IgG and F(ab')$_2$

IgG was purified from the serum on a CM-Affigel Blue (Biorad) column. A 2 ml aliquot of serum was loaded onto a CM-Affigel Blue column buffered with 0.01 M potassium phosphate buffer. The serum was eluted with the same buffer and the absorbance value at 280 nm of all the fractions determined. Fractions of the IgG with absorbance values higher than 1.4 were pooled and the concentration adjusted to 1 mg/ml with buffer (A_{280nm} = 1.4).

The $F(ab')_2$ fragments were prepared by a modification of the procedure of Campbell *et al.*, 1970. A PD-10 column was equilibrated with 0.07 M sodium acetate buffer pH 4 containing 0.05 M sodium chloride. A 2.5 ml aliquot of the IgG was placed onto the column and eluted with 3.5 ml of the sodium acetate buffer. 3.5 ml of the eluent was collected and treated with 45 µg pepsin (Sigma) per mg of IgG for 18 hours at 37°C. The Fc fragments were removed by dialysis against PBS (0.01 M potassium phosphate buffer pH 7.25 containing 0.15 M sodium chloride and 0.02% sodium azide).

16. $F(ab')_2$ ELISA

$F(ab')_2$ fragments were diluted in coating buffer (0.05 M sodium carbonate pH 9.6) and placed in the central 60 wells of an ELISA plate. The outer wells were filled with water and the plate incubated for 4 hours at 30°C. The plate was then soaked once and washed three times for 3 minutes with PBS-Tween (0.02 M potassium phosphate pH 7.4, 0.15 M NaCl, 2.7 mM KCl, 0.2% sodium azide, 0.05% Tween 20). Plant material was macerated in a 1:10 ratio (w/v) in extraction buffer (PBS-Tween containing 2% PVP and 0.2% ovalbumin) and placed in the wells for 18 hours at 4°C. The plate was soaked once and washed five times for 3 minutes with PBS-Tween. The IgG was diluted in extraction buffer and added to the central 60 wells of the plate. The plate was incubated at 30°C for 4 hours before washing three times for 3 minutes with PBS-Tween. Goat anti rabbit Fc alkaline phosphatase conjugate (GAR-Fc-AP) was diluted in extraction buffer and added to the central 60 wells of the plate. The plate was incubated for 18 hours at 4°C before washing five times for three minutes with PBS-Tween. The enzyme substrate (1 mg/ml ρ-nitrophenyl phosphate in 10% diethanolamine pH 9.8) was added to the wells and the plate incubated at 30°C for 30 minutes before measuring the absorbance at 405 nm with the Multiscan MC spectrophotometer. Results were analyzed with the ELISA-LINK programme and graphs drawn with QuattroPro V1.

17. Titre determination

The titre of all the bleeds were determined with $F(ab')_2$ ELISA as described before, except that antisera was used instead of purified IgG. $F(ab')_2$ fragments for use in this ELISA were prepared from the third bleed, and used at a dilution of 1:100. All antisera were diluted from 1:4 to 1:2048 in extraction buffer.

IgG and $F(ab')_2$ for use in future ELISA were then purified as described before and a $F(ab')_2$ ELISA performed to determine the optimum dilutions.

18. Optimising $F(ab')_2$ ELISA

The checkerboard titration protocol of Voller *et al.*, 1979, was used. $F(ab')_2$ fragments and IgG were diluted 1:100, 1:1000, and 1:10000. Infected plant material was diluted 1:10, 1:100, and 1:1000, while healthy plant material was diluted 1:10 and 1:100. One row was used as buffer control. The ELISA was performed as described before. The optimum dilutions of $F(ab')_2$ and IgG giving absorbance values of at least twice the background values were used in future studies.

19. Indirect ELISA

Plant material was macerated in a ratio of 1:10 w/v in coating buffer, and placed in the central 60 wells of an ELISA plate. The ELISA plate was incubated for 16 hours at 4°C. The plate was washed five times with PBS-Tween. Antiserum or IgG diluted in extraction buffer was incubated at 37°C for four hours, after which the plate was washed three times with PBS-Tween. GAR-Fc-AP conjugate was diluted in extraction buffer and added to the central 60 wells of the plate. The plate was incubated for 18 hours at 4°C before washing five times for three minutes with PBS-Tween. The enzyme substrate (1 mg/ml ρ-nitrophenyl phosphate in 10% diethanolamine pH 9.8) was added to the wells and the plate incubated at 30°C for 30 minutes before measuring the absorbance at 405 nm with the Multiscan MC spectrophotometer. Results were analysed with the ELISA-LINK programme and graphs drawn with QuattroPro V1.

20. Cytopathology

Infected leaf material was thoroughly washed and immersed under a solution of 2.5% glutaraldehyde in 0.1 M sodium phosphate pH 7.2. Sections of 1 x 5 mm were cut from the infected leaf material and left to fix for 18 hours at 4°C. The cuttings were washed thoroughly in 0.1 M sodium phosphate pH 7.2 on a shaker. The material was post-fixed in 1% osmium tetroxide in a fume cupboard for 2 hours at room temperature or 18 hours at 4°C. The material was washed in water and stored overnight in a large volume of water.

Ultrathin sections were cut with a LKB 8800 ultramicrotome fitted with a glass knife. The sections were mounted onto 400 mesh copper grids and stained with uranyl acetate and lead citrate (Reynolds, 1963).

21. Negative staining

Plant material was macerated in a 0.1 M sodium phosphate buffer pH 7.1. All samples were prepared in triplicate as three different stains were used to visualise the virus particles. A 400 mesh copper grid was floated onto a drop of plant extract for 10 minutes. The grid was washed with water before floating it on a drop of stain for 5 minutes. The grid was then given a final wash with water to remove excess stain. Staining was done with 2% uranyl acetate pH 4.3, 2% ammonium molybdate pH 5.3, and 2% potassium phosphotungstate pH 6.0 or 7.0. The grids were examined for the presence of virus particles with a ABT 002A transmission electron microscope.

22. SDS-PAGE

Protein samples were analysed on SDS-PAGE gels consisting of a 12% polyacrylamide resolving gel and a 4% stacking gel, both containing 2.6% bisacrylamide. Samples were mixed in an equal volume of disruption buffer (0.054 M Tris-HCl pH 6.8, 9% (v/v) glycerol, 4.3% (v/v) β-mercaptoethanol, 1.7% (w/v) SDS, and 0.009% bromophenol blue) and boiled for 2 minutes before loading onto the gel. Electrophoresis was for 20 hours at 60 V at 4°C, in a 0.25

M Tris-HCl pH 8.3 buffer containing 0.192 M glycine and 0.1%SDS. The gel was stained overnight in 1% (w/v) Coomassie brilliant blue in 45% (v/v) and 10% (v/v) glacial acetic acid. The gel was destained by repeated changes of 45% (v/v) and 10% (v/v) glacial acetic acid.

APPENDIX B

List of viruses used in nucleic acid and amino acid sequence comparisons.

Virus name	Strain	Database accession number	Used in amino acid sequence comparisons	Used in nucleic acid sequence comparisons
AzBMV		U60100	*	*
BCMNV	TN1	U37076	*	*
BCMV	CH1	L19539	*	*
BCMV	NL1	L15331	*	*
BCMV	NL2	L19472	*	*
BCMV	NL3	U19287	*	*
BCMV	NL4	L21766	*	*
BCMV	NY15	S66252		*
BCMV	US10	U37072	*	*
BCMV	US3	U37073		*
BCMV	US4	U37074	*	*
BCMV	US5	L19474	*	*
BCMV	US7	L19474	*	*
BYMV	MB4	D22819		*
BYMV	MI	X81124	*	*
BYMV	S	S77515	*	*
BICMV-W	W	S66253	*	*
CABMV	SAP	D10053	*	*
CIYVV	B	S77521	*	*
CIYVV	Isolate 30	D86044/D00603	*	*
CIYVV		D00605	*	*
CIYVV	New Zealand (NZ)	229051	*	
DeMV		U23564	*	*
JGMV	Maize dwarf virus O (MDO)	U07217	*	*
MDMV	B	D00949	*	*
PeMoV	PV4	L32959	*	*
PMV	I	S71232	*	*
PPV	D	D00298	*	*
PPV	Rankovik	M21847	*	*
PRSV		S49774/X67672	*	*
PSbMV	DPD1	D10930/D01152	*	*
PVA	Isolate Juliniere	X91968	*	*
SCMV	SC	D00948	*	*
SPFMV	S (severe)	D38543	*	*
SoyMV	G2	S42280	*	*
SoyMV	N	D00507	*	*
TEV	NW (non-wilting)	L38714	*	*
PVY	N	JA0073/PS0281	*	
TUMV	S	S11689/S12507	*	

APPENDIX C

List of viruses used in this study

Virus name	Accession number	Origin	Technique	Condition
BCMV	92/750	PPRI	ELISA, ISEM, IC-RT-PCR	Fresh material
BYMV	92/751	PPRI	ELISA, ISEM, IC-RT-PCR	Fresh material
CABMV-SAP	92/749	R. Brand, SA	ELISA IC-RT-PCR	Freeze-dried material Fresh material
CABMV	87/14	PPRI		
CABMV	90/57	PPRI		
CABMV	93/1510	PPRI	ISEM, IC-RT-PCR	Fresh material
Isolate 90/169	90/169	PPRI	ELISA	Desiccated material
Isolate 93/1	93/1	PPRI	ELISA, ISEM, IC-RT-PCR	Fresh material
Isolate 93/65	93/65	PPRI	ELISA, ISEM, IC-RT-PCR	Fresh material
PeMoV	88/63	PPRI	ELISA IC-RT-PCR	Desiccated material Fresh material
PeMoV	94/2097	PPRI	IC-RT-PCR	Fresh material
PMV	95/931	PPRI	IC-RT-PCR	Fresh material
PStV	91/354	PPRI	ELISA IC-RT-PCR	Desiccated material Fresh material
PStV	95/399	PPRI	ELISA IC-RT-PCR	Desiccated material Fresh material
PVY	92/21	PPRI	ELISA, IC-RT-PCR	Fresh material
SMV	86/20	PPRI	ELISA	Fresh material
SMV	96/38	PPRI	IC-RT-PCR	Fresh material
WMV2	90/106	PPRI	ELISA	Desiccated material
WMV-Merocco	90/107	PPRI	ELISA	Desiccated material

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